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Original Research Article

Dietary rumen-protected L-arginine or N-carbamylglutamate enhances placental amino acid transport and suppresses angiogenesis and steroid anabolism in underfed pregnant ewes

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ABSTRACT

This study aimed to investigate the effects of dietary supplementation of underfed Hu ewes from d 35 to 110 of gestation with either rumen-protected L-arginine (RP-Arg) or N-carbamylglutamate (NCG) on placental amino acid (AA) transport, angiogenic gene expression, and steroid anabolism. On d 35 of gestation, 32 Hu ewes carrying twin fetuses were randomly divided into four treatment groups, each consisting of eight ewes, and were fed the following diets: A diet providing 100% of NRC's nutrient requirements for pregnant ewes (CON); A diet providing 50% of NRC's nutrient requirements for pregnant ewes (RES); RES diet plus 5 g/d NCG (RES + NCG); or RES diet plus 20 g/d RP-Arg (RES + ARG). On the d 110 of pregnancy, blood samples were taken from the mother, and samples were collected from type A cotyledons (COT; the fetal portions of the placenta). The levels of 17β-estradiol and progesterone in the maternal serum and both the capillary area density (CAD) and capillary surface density (CSD) in type A COT were decreased in response to Arg or NCG supplementation when compared to the RES group. The concentrations of arginine, leucine, putrescine and spermidine in type A COT were higher ($P < 0.05$) in the RES + ARG or RES + NCG group than in the RES group. The mRNA expression levels of inducible nitric oxide synthase (*iNOS*) and solute carrier family 15, member 1 (*SLC15A1*) were increased ($P < 0.05$) while those of progesterone receptor (*PGR*) and fibroblast growth factor 2 (*FGF2*) were decreased in type A COT by supplementation with either NCG or RP-Arg compared to the RES group. The results suggest that providing underfed pregnant ewes from d 35 to 110 of gestation with a diet supplemented with NCG or RP-Arg improves placental AA transport, and reduces the expression of angiogenic growth factor genes and steroid anabolism, leading to better fetal development.

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1. Introduction

Intrauterine growth restriction (IUGR) is a condition in which a fetus does not grow at the normal rate during pregnancy, leading to potentially negative outcomes such as low birth weight, increased risk of death, chronic health conditions and poor performance in adulthood (Gonzalez-Bulnes et al., 2016; Wu et al., 2006). It has

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been indicated that IUGR infants who survive are at an increased risk of developing a variety of health complications including respiratory, neurological, cardiovascular, and intestinal diseases (Gluckman and Hanson, 2006). Several factors can contribute to IUGR, including maternal health conditions, placental problems, genetic disorders, poor nutrition, infection, or environmental toxins; these factors can limit the availability of nutrients to the fetus, leading to changes in placental development and ultimately resulting in IUGR (Wu et al., 2006).

Adequate maternal nutrition is essential for the proper growth and development of the placenta, which is crucial for optimal fetal nutrition and growth. Proper placental growth and vascularity ensure efficient nutrient transport from the mother to the fetus, which can help counteract the development of IUGR (Regnault, 2003). The placental membrane acts as a barrier, separating maternal and fetal blood and allowing nutrient exchange between the mother and the developing fetus via diffusion channels, concentration gradients, and protein transporters. These mechanisms are crucial for the proper growth and development of the fetus, ensuring that it receives the necessary nutrients (Brett et al., 2014). The formation of new blood vessels within the placenta, also known as placental angiogenesis, plays a crucial role in the transport of nutrients from the mother to the developing fetus, and its improvement facilitates fetal development (Grazul-Bilska et al., 2010; Rogers et al., 2009). Steroid hormones play a crucial role in regulating both placental angiogenesis and development in mammals, and placental steroidogenesis is essential for maintaining a healthy pregnancy (Reynolds et al., 2018; Song et al., 2020). On the other hand, providing certain functional nutrients during gestation can improve the placental transport of nutrients to the fetus, which may have positive effects on fetal growth and development. Research suggests that supplying methionine to late-gestating dairy cows is associated with higher birth weight in their newborns (Batistel et al., 2017). This effect is thought to be due to the enhanced placental transport of amino acids (AA) and glucose as indicated by the greater mRNA expression of genes involved in the neural transport of amino acids and glucose as well as genes involved in the mammalian target of rapamycin (mTOR) signalling pathways (Batistel et al., 2017).

L-arginine (Arg), nitric oxide (NO) and polyamine precursors have been shown to promote angiogenesis in the placenta (Raghavan and Dikshit, 2004), increase blood flow from the fetus to the placenta (Wu et al., 2022) and have antioxidant properties during gestation (Morris, 2009). These functional nutrients are essential for proper placental function and fetal development. N-carbamylglutamate (NCG), the structural analogue of N-acetylglutamate, plays an important role in the production of endogenous Arg (Gessler et al., 2010). In our previous research, we found that providing dietary supplements of NCG or rumen-protected Arg (RP-Arg) to underfed pregnant Hu ewes from d 35 to 110 of gestation led to an increase in placental growth and fetal body weight (Zhang et al., 2016a, 2016b). However, the specific mechanisms behind these effects are not yet fully understood. In addition, supplementation of NCG to dairy cows during the last d 28 of pregnancy led to an increase in the body weight of newborn calves and upregulation of mRNA expressions of genes involved in placental angiogenesis, AA transport, and mTOR signalling pathways (Gu et al., 2021). However, it is yet to be determined how the dietary supplementation of NCG or RP-Arg influences placental AA transport function, angiogenesis growth factors, and steroid anabolism to enhance fetal body weight in underfed ewes.

With this in mind, the purpose of this study was to investigate the role of NCG or RP-Arg in preventing the risks of underfeeding-induced IUGR of fetal lambs and to understand the effects of these supplements on placental AA transport function, angiogenesis growth factor gene expression, and steroid catabolism in underfed Hu pregnant ewes from d 35 to 110 of gestation. The current study builds upon our previous research, which examined the impact of dairy supplementation of RP-Arg or NCG on fetal weight and placental morphology in underfed ewes during early-to-mid gestation (Zhang et al., 2016a, 2016b). By investigating the effects of these nutrient interventions on placental function, this study aims to gain a deeper understanding of how they may prevent underfeeding-induced IUGR. The findings of this study will be a valuable addition to the existing literature in this field.

2. Materials and methods

2.1. Animal ethics statements

The study protocols were approved by the Ethics Committee of Yangzhou University (SYXK2013-0057).

2.2. Animals

Forty-eight multiparous Hu sheep ewes similar in age (18.5 ± 0.5 months), BW (40.1 ± 1.2 kg) and body condition score (BCS = 2.6 ± 0.2; range, 0 to 5, indicating thin to obese; Russel et al., 1969) were initially selected in this study. Each Hu ewe was individually housed in the indoor barn of Jiangyan Experimental Station, Taizhou, Jiangsu, China. Details of feeding management and breeding have been described previously (Zhang et al., 2016a, 2016b). In brief, Hu ewes were subjected to a 12 d oestrus synchronisation protocol and then were artificially inseminated with fresh semen collected from Hu sheep rams. Following insemination (d 0 of gestation) until d 35 of gestation, Hu ewes were individually housed in separate pens (each of 1.05 m × 1.60 m). On d 35 of gestation, ewes were scanned for twin pregnancy diagnosis using ultrasonography (Asonics Microimager 1000 sector scanning instrument; Ausonics, Sydney, Australia). Thirty-two ewes were diagnosed to be twin-bearing out of the initial 48 ewes and were used in this study. An experimental diet (Supplementary Table S1) was prepared to meet the 100% nutritional requirement for all ewes, as established by the National Research Council (NRC, 2007).

2.3. Experimental procedure

On d 35 of gestation, ewes were randomly assigned into four treatment groups ($n = 8$) and fed one of the following diets until d 110 of gestation: a diet providing 100% of NRC's (NRC, 2007) nutrient requirements for pregnant ewes (CON); a diet providing 50% of NRC's (NRC, 2007) nutrient requirements for pregnant ewes (RES); RES diet plus 5 g/d NCG (RES + NCG); or RES diet plus 20 g/d RP-Arg (RES + ARG). The NCG purity was 97% (Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China). Using Eldem's method (Eldem et al., 1991), RP-Arg was prepared from phospholipids and glycerides through spray-drying and spray-congealing (Beijing Feeding Feed Science Technology Co., Beijing, China). The protection of RP-Arg in the rumen was ≥85%, and the release of RP-Arg in the intestine was ≥90%, which were evaluated based on previously described methods (Chacher et al., 2012; Hervás et al., 2000). The effective doses of RP-Arg and NCG (blended with a pellet diet) were 10 and 2.5 g/d, respectively, according to our previous work (Zhang et al., 2016a, 2016b). Half of the overall diet (determined for meeting 100% NRC requirements) was offered to ewes to achieve a 50% of nutrient requirements set by NRC (2007) (restricted nutrition). During the experiment, the ewes' BW was measured at 10 d intervals, and the feed intake was adjusted according to the change in BW as stated in our previous work (Zhang et al., 2016b). The DMI, maternal BW, and fetal BW in

Hu ewes on d 110 of gestation were recorded (Supplementary Table S2).

2.4. Chemical analyses

Feed samples were analyzed for crude protein (CP), dry matter (DM), calcium (Ca), phosphorus (P) and ether extract (EE) (methods 990.02, 930.15, 968.08, 965.17, and 920.39, respectively, AOAC, 1990). The acid detergent fiber (ADF) and natural detergent fiber (NDF) concentrations were quantified according to Van Soest et al. (1991). The gross energy (GE) in dietary ingredients and feces was measured using a bomb calorimeter (C200; IKA Works Inc., Staufen, Germany). The GE in the urine sample was determined according to Deng et al. (2014).

2.5. Maternal serum and type A placentome sampling

On the morning of the last day of the experiment (on d 110 of gestation), blood samples (10 mL) were collected from the jugular vein of ewes using a 20 gauge × 3.8 cm blood collection needles (Vacutainer; Becton, Dickinson and Company) into sterile, anticoagulant-free vacuum tubes (Vacutainer; Becton, Dickinson and Company, Suzhou, Jiangsu, China), immediately centrifuged at 3,000 × *g* at 4 °C for 15 min and sera were separated and preserved at −80 °C for later analyses.

Immediately after blood sampling (at 08:00), ewes were euthanized using a captive bolt gun (Supercash Mark 2; Acceles and Shelvoke) and exsanguinated and the weights of all fetuses were measured and recorded as previously explained (Zhang et al., 2016b). From all the ewes carrying twins in all the groups, the placentomes were taken from the placenta, cleaned, identified by type, weighed separately and the total weight was calculated. The placentomes were identified as type A according to the standards previously established (Vonnahme et al., 2006). We selected type A placentomes (n = 8, each for all the groups) to investigate any changes that may have occurred in placentae exposed to the dietary interventions used in this study. One type A placentome from each ewe was used in the present study. The average diameter/weight and the total weight of type A placentomes were measured as stated in our previous work (Zhang et al., 2016a). Samples of cotyledons (COT, the fetal portion of the placenta) were collected from several type A placentomes of similar size within a 10 cm radius of the umbilical attachment as previously described (Song et al., 2020; Zhu et al., 2009), and stored in liquid nitrogen at −80 °C for subsequent analyses.

2.6. Determination of serum concentrations of progesterone (P4) and 17β-estradiol (E2)

The P4 concentration was measured by the specific solid-phase radioimmunoassay (RIA) using corresponding reagents and methods, as recommended for use with the Diagnostic Products Corp (DPC) Coat-A-Count (Diagnostic Corporation, Los Angeles, CA, USA). The assay sensitivity was 0.1 nmol/L (0.031 ng/mL) and the inter- and intra-assay coefficient of variation (CV) values were 8.5% and 4.9%, respectively. The E2 concentration was determined using double-antibody RIA with no prior extraction by using relevant reagents and methods, as recommended by DPC (Diagnostic Corporation, Los Angeles, CA, USA). The assay sensitivity was 5.18 pmol/L (1.4 pg/mL) and the determined inter- and intra-assay CV values were 4.9% and 3.5%, respectively (Parraguez et al., 2013). Each sample was analyzed twice for P4 and E2.

2.7. Assay of NO, insulin and insulin-like growth factor 1 (IGF-1) concentrations and NO synthase (NOS) activity in type A COT tissues

The insulin and IGF-1 concentrations were measured using ovine-specific enzyme-linked immunosorbent assay microplate kits (Mercodia, Guangzhou, China). The NO and NOS levels were measured using the respective kits (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) and following the previously described protocols (Liu et al., 2009; Zhang et al., 2020). The protein concentration was measured using the bicinchoninic acid protein assay kit (BCA) (Pierce, Rockford, IL, USA). The NO content was determined as micromole per gram (μmol/g) of protein. Additionally, total NOS (tNOS) and inducible NOS (iNOS) activities were measured using a NOS activity detection kit. Subsequently, the tNOS activity was subtracted from the iNOS activity to determine the constitutive NOS (cNOS) activity. The tNOS and iNOS activities were calculated as units per gram (U/g) of protein. Furthermore, the optical density was measured at 450 nm. The inter-assay CV was set at < 15% (Guo et al., 2019).

2.8. Determination of cellular proliferation and vascularity parameters in type A COT tissues

The 5 μm paraffin-embedded type A placentome sections were rehydrated with ethanol and subjected to 10 min antigen retrieval using 0.05% Tween 20 (pH 9.0), 1 mmol/L ethylenediaminetetraacetic acid and 50 mmol/L glycine at 120 °C. Further, a primary antibody against CD31 (ab28364, Abcam, Cambridge, UK) was used to treat the sections, and all blood vessels (maternal/fetal) were identified by immunofluorescent labelling with CF633 goat anti-rabbit immunoglobin (Ig) G secondary antibodies (20122, Biotium, Hayward, CA, USA). The trophoblast layer was stained with 20 μg/mL FITC-labelled BS1 lectin (FL-1101; Vector Laboratories, Burlingame CA, USA), and proliferating cells were stained with an anti-Ki67 mouse monoclonal antibody (VP-k452, Vector Laboratories), as described previously (Redmer et al., 2013). To determine the overall number of nuclei, 4′,6-diamidino-2-phenylindole (P36931, Life Technologies) was used to counter-stain the cell nuclei. Proliferation was expressed based on the proportion of Ki67-stained nuclei. The placental vascularity was determined as reported previously (Borowicz et al., 2007). Cell proliferation and vascularity were determined using an average of four images from each section of the type A placentome in each ewe. In addition, anti-CD31 immunofluorescence (IF) was used to detect vascularity. The COT compartments were identified using specific fetal trophoblast lectin staining followed by individual circumscription. Further, the number (as unit/mm^2), area (mm^2) and perimeter (mm) of capillaries were determined to calculate the following vascular indexes (within COT): capillary number density (CND, capillary number/tissue area); capillary area density (CAD, capillary area/tissue area); area per capillary (APC, capillary area/capillary number), capillary surface density (CSD, capillary perimeter/tissue area) (Carr et al., 2016).

2.9. Analysis of AA concentrations, putrescine, spermidine and spermine in type A COT tissues

The AA profiles in type A COT tissues were determined using reverse-phase high-performance liquid chromatography (HPLC, HP1100; Agilent), using norleucine as the internal standard, as reported previously (Bidlingmeyer et al., 1984; Wu and Knabe 1995). Additionally, HPLC (involving pre-column derivatisation using *N*-acetyl-L-cysteine and *o*-phthaldialdehyde) was used to analyse the

polyamines (putrescine, spermine, and spermidine) concentrations in type A COT tissues (Dai et al., 2014). Briefly, the grounded placental powders (25 mg) were added to a 1.5-mL Eppendorf tube containing pre-chilled 1.5 mmol/L $HClO_4$ (100 μL) and neutralised using 2 mmol/L K_2CO_3 (50 μL). After the sample was centrifuged at 15,000 × *g* for 10 min at 4 °C, HPLC was conducted to analyse the polyamine concentrations in the supernatant (50 μL) (Liu et al., 2019).

2.10. Quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

Some angiogenic factors associated with placental vascularity were identified using RT-qPCR. Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) was used to extract cotyledonary RNA from the type-A placentome, and the Agilent 2100 Bioanalyzer (Agilent Technologies) was used to measure the total RNA content through capillary electrophoresis. Nanodrop was adopted for RNA quantification, with the 260/280 ratio ≥1.8. cDNA was prepared from total RNA (1,000 ng) using a SuperScript VILO kit according to the manufacturer's instructions (Life Technologies). Five reference genes, including β-actin, ribosomal protein L19 (RPL19), cyclophilin, 18S rRNA and GAPDH, were analysed by the GeNorm programme (Vandesompele et al., 2002) to determine the suitable reference gene to express accurately the relative gene expression of the selected markers. The overall stability of the tested reference genes was measured by calculating the gene expression stability (M-value). 18S rRNA, RPL19 and β-actin across nutritional treatments had the best stability which were suitable references for the relative gene expression of placental development, steroidogenesis, AA transport and Arg–NO pathway, respectively. The mRNA levels of the placental development-related genes were measured using RT-qPCR as recommended previously (Redmer et al., 2012), and multiplex reactions were conducted upon the addition of 18S mRNA in each well as an internal standard (Redmer et al., 2012; Vonnahme et al., 2008). The gene levels were measured based on the gene-to 18S RNA ratio of each gene of interest. The relative expression of steroidogenesis-related genes was determined using the $2^{-\Delta\Delta CT}$ method, which was followed by normalisation to the expression of RPL19, the internal control gene (Rak et al., 2017; Song et al., 2020). The target gene expression was determined for AA transport and Arg–NO pathway compared with β-actin by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The target gene mRNA level was determined based on the control. The primer sequences used to amplify the target genes and the GenBank accession numbers are presented in Supplementary Table S3.

2.11. Western blot (WB) analysis

A specific kit (Beyotime Biotechnology, Jiangsu, China) was used to extract the total protein from type A COT tissues, which was further homogenised following a previously described protocol (Zhang et al., 2018). The protein concentration was determined using a BCA protein detection kit (Pierce, Rockford, IL, USA). The extracted protein (30 mg) was then denatured, separated by 12% sodium dodecyl-sulphate polyacrylamide gel electrophoresis (Bio-Rad, Richmond, CA, USA) and transferred to 0.45 mm polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Subsequently, the membranes were incubated with 5% bovine serum albumin (wt/vol) for 1 h to block non-specific binding. The membranes were further incubated overnight at 4 °C using the primary antibodies and washed with Tris-buffered saline/Tween 20. The following primary antibodies were used for WB assay: alanine-serine-cysteine AA transporter 2 (ASCT2) (1:500, A6981, ABclonal Technology, Wuhan, China); β-actin (1:1,000, AC006, ABclonal Technology, Wuhan, China); peptide transporter 1 (PEPT1) (1:500, bs-10588R, Bioss, Beijing, China); cationic AA transporter, y + system, member 1 (CAT1, 1:500, A14784, ABclonal Technology, Wuhan, China); progesterone receptor (PGR) (1:1000, AF7737, Beyotime Biotechnology, Shanghai, China); fibroblast growth factor 2 (FGF2) (1:500, bs-0217R, Bioss, Beijing, China); epithelial NOS (eNOS) (1:1,000, NB300-500, Novus, CO, USA). Furthermore, goat anti-rabbit IgG (H + L) secondary antibody (1:5,000; AS014, ABclonal Technology, Wuhan, China) was used to treat the membranes for 1 h at the ambient temperature, following which protein signals were visualized using an electro-chemiluminescence WB assay system (Fujifilm, Tokyo, Japan). Image J (National Institute of Health, Bethesda, MD, USA) was used to calculate the chemiluminescence intensities of protein blots against β-actin.

2.12. Statistical analysis

A one-way analysis of variance (ANOVA) was applied to analyze the data collected at d 110 of gestation, using the GLM procedure of SAS (version 9.2). Although fetal sex was initially included in the analysis, it was found to have no significant impact ($P > 0.05$) and was subsequently removed from the final model, which only took into account maternal nutritional treatments. To compare the results of different treatment groups, Duncan's multiple range test was used. The data is presented as the mean ± standard error of the mean (SEM) and a P value of ≤0.05 was considered statistically significant.

3. Results

3.1. The P4 and E2 concentrations in maternal sera

The P4 ($P = 0.01$) and E2 ($P = 0.02$) concentrations were greater in the sera of RES ewes than those in the sera from the CON ones (Table 1). However, dietary RP-Arg or NCG supplementation mitigated the RES-induced alterations in maternal serum P4 ($P = 0.01$) and E2 ($P = 0.02$) concentrations.

3.2. Insulin, IGF-1 and NO concentrations and NOS activity in type A COT tissues

The cotyledonal insulin, IGF-1 and NO levels and the tNOS, iNOS and cNOS activities were decreased (all $P < 0.05$) in RES ewes compared to the corresponding values obtained from CON ewes (Table 2). Compared to ewes from the RES group, dietary administration of NCG or RP-Arg resulted in a significant increase (15.8% to 72.9%) in insulin/IGF-1/NO contents and tNOS/iNOS/cNOS activities (all $P < 0.05$) in ewes from the RES + NCG or RES + ARG group.

3.3. Cellular proliferation and vascularity parameters in type A COT tissues

When compared to ewes from the CON group, ewes from the RES group had higher levels (52.1% to 77.9%) of CAD ($P = 0.01$), APC ($P = 0.01$), and CSD ($P = 0.01$) in type A COT tissue (Table 3). Compared to ewes from the RES group, those treated with RP-Arg or NCG had lower levels (15.9% to 25.2%) of CAD, APC, and CSD in type A COT tissue (all $P < 0.05$). Cellular proliferation ($P = 0.13$) and CND ($P = 0.12$) in type A COT tissues did not change in response to the treatments.

Table 1
Effects of dietary supplementation of N-carbamylglutamate (NCG) or rumen-protected L-arginine (RP-Arg) on serum concentrations of P4 and E2 in underfed Hu ewes on d 110 of gestation[1,2].

Item	CON	RES	RES + ARG	RES + NCG	SEM	*P*-value
P4, ng/mL	12.03[c]	18.57[a]	15.24[b]	15.15[b]	1.871	0.01
E2, ng/mL	13.18[c]	20.49[a]	16.47[b]	16.21[b]	2.213	0.02

E2 = 17β-estradiol; P4 = progesterone.
[a, b, c] Means in a row with superscripts without a common letter differ ($P < 0.05$).
[1] Data are means and pooled SEM ($n = 8$/group for ewes, $n = 16$/group for the fetus).
[2] CON = ewes fed 100% of NRC nutrient recommendations for pregnancy; RES = ewes fed 50% of NRC nutrient recommendations for pregnancy; RES + ARG = RES ewes supplemented with 20 g/d RP-Arg; RES + NCG = RES ewes supplemented with 5 g/d NCG.

3.4. Concentrations of AA in the type A COT tissues

Except for that of aspartate/alanine, the AA contents in type A COT tissues in the RES ewes were decreased (all $P < 0.05$) compared to those in the CON ones (Table 4). By including RP-Arg or NCG in the RES diet, the drop in the concentrations of AA (cysteine, histidine, arginine, lysine, leucine, isoleucine, tryptophan, citrulline, glutamate, ornithine, glutamine, and proline) in type A COT tissues caused by RES was counteracted by 15.2% to 61.3% (all $P < 0.05$).

3.5. Polyamine concentrations in type A COT tissues

The concentrations of putrescine ($P = 0.01$), spermidine ($P = 0.01$), spermine ($P = 0.01$) and total polyamines ($P = 0.02$) in type A COT tissues were reduced by 35.2% to 50.3% in the RES ewes than in the CON ones (Table 5). However, these metabolites were increased by 25.9% to 60.3% (all $P < 0.05$) in the RES + ARG or RES + NCG group compared to those in the RES group, whereas they were decreased by 13.2% to 24.3% (all $P < 0.05$) compared to those in the CON group, except for spermine in RES + NCG group.

3.6. mRNA expression in the type A COT tissues

The mRNA expression of AA transport-related genes [solute carrier family 7 (AA transporter light chain, y^+ L system), member 7 (*SLC7A7*), solute carrier family 7 (*CAT1*), member 1 (*SLC7A1*), solute carrier family 15 (*PEPT1*), member 1 (*SLC15A1*), solute carrier family 1 (*ASCT2*), member 5 (*SLC1A5*)] and Arg–NO pathway-related genes (*eNOS* and *iNOS*) in type A COT tissues decreased by 37.9% to 61.3% (all $P < 0.05$) in the RES group than in the CON group (Table 6). The mRNA expression of the aforementioned genes in type A COT tissue increased by 41.2% to 98.2% (all $P < 0.05$) in the NCG or RP-Arg-supplemented groups compared to that in the RES group. The mRNA expression of steroidogenesis-related genes [aromatase (*CYP19*), 17 alpha-hydroxylase (*CYP17*), estrogen 1(*ESR1*), estrogen 2 (*ESR2*) and *PGR*] and placental development-related genes [*FGF2*, hypoxia-inducible factor 1 (*HIF1A*), alpha subunit (basic helix-loop-helix transcription factor), tyrosine endothelial kinase (*TEK*), neuropilin 2 (*NRP2*) and *FGFR2*] in type A COT tissue increased by 56.3% to 80.1% (all $P < 0.05$) in the RES group than in the CON group. The expression of the aforementioned genes in type A COT tissues declined by 13.5% to 33.8% after dietary RP-Arg or NCG supplementation (all $P < 0.05$) compared to that in the RES group. No significant difference was observed in the gene expression of solute carrier family 1 (neuronal/epithelial high-affinity glutamate transporter, system X_{AG}), member 1 (*SLC1A1*) ($P = 0.10$) and *NRP1* ($P = 0.12$) in response to the treatment.

3.7. Protein expression in type A COT tissues

Compared to that in the CON ewes, CAT1 ($P = 0.01$), ASCT2 ($P = 0.02$), PEPT1 ($P = 0.01$) and eNOS ($P = 0.03$) protein expression

Table 2
Effects of dietary supplementation of N-carbamylglutamate (NCG) or rumen-protected L-arginine (RP-Arg) on insulin, IGF-1 and NO contents and the activity of NOS in type A cotyledonary (COT) tissues of underfed Hu ewes on d 110 of gestation[1,2].

Item	CON	RES	RES + ARG	RES + NCG	SEM	*P*-value
Insulin, pmol/g protein	4.78[a]	1.76[c]	2.91[b]	3.04[b]	0.182	0.01
IGF-1, pmol/g protein	168.37[a]	108.92[c]	131.36[b]	136.83[b]	9.893	0.01
NO, μmol/g protein	2.98[a]	1.57[c]	1.82[b]	1.92[b]	0.558	0.00
tNOS, U/g protein	487[a]	325[c]	412[b]	416[b]	16.2	0.02
iNOS, U/g protein	302[a]	208[c]	258[b]	269[b]	11.3	0.01
cNOS, U/g protein	185[a]	117[c]	154[b]	147[b]	8.2	0.01

IGF-1 = insulin-like growth factor 1; NO = nitric oxide; NOS = nitric oxide synthase; tNOS = total nitric oxide synthase; iNOS = inducible nitric oxide synthase; cNOS = constitutive nitric oxide synthase.
[a, b, c] Means in a row with superscripts without a common letter differ ($P < 0.05$).
[1] Data are means and pooled SEM ($n = 8$/group for ewes, $n = 16$/group for the fetus).
[2] CON = ewes fed 100% of NRC nutrient recommendations for pregnancy; RES = ewes fed 50% of NRC nutrient recommendations for pregnancy; RES + ARG = RES ewes supplemented with 20 g/d RP-Arg; RES + NCG = RES ewes supplemented with 5 g/d NCG.

Table 3
Effects of dietary supplementation of N-carbamylglutamate (NCG) or rumen-protected L-arginine (RP-Arg) on cellular proliferation and vascularity parameters in type A cotyledonary (COT) tissues of underfed Hu ewes on d 110 of gestation[1,2].

Item	CON	RES	RES + ARG	RES + NCG	SEM	*P*-value
Cellular proliferation, %	3.47	3.13	3.26	3.39	0.284	0.13
CAD, %	13.57[c]	20.67[a]	16.34[b]	16.09[b]	1.032	0.01
CND, $\times 10^6$	894	865	873	890	34.2	0.12
APC, μm^2	126[c]	205[a]	166[b]	171[b]	11.8	0.01
CSD, $\mu m/\mu m^2$	5.23[c]	9.29[a]	6.97[b]	7.04[b]	0.587	0.01

CAD = capillary area density; CND = capillary no. density; APC = area per capillary; CSD = capillary surface density.
[a, b, c] Means in a row with superscripts without a common letter differ ($P < 0.05$).
[1] Data are means and pooled SEM ($n = 8$/group for ewes, $n = 16$/group for the fetus).
[2] CON = ewes fed 100% of NRC nutrient recommendations for pregnancy; RES = ewes fed 50% of NRC nutrient recommendations for pregnancy; RES + ARG = RES ewes supplemented with 20 g/d RP-Arg; RES + NCG = RES ewes supplemented with 5 g/d NCG.

Table 4
Effects of dietary supplementation of N-carbamylglutamate (NCG) or rumen-protected L-arginine (RP-Arg) on concentrations of AA (nmol/g wet weight) in type A cotyledonary (COT) tissues of underfed Hu ewes on d 110 of gestation[1,2].

Item	CON	RES	RES + ARG	RES + NCG	SEM	*P*-value
Essential amino acid						
Arginine	1612[a]	1007[c]	1297[b]	1309[b]	17.8	0.01
Cysteine	784[a]	319[c]	509[b]	497[b]	9.6	0.02
Histidine	197[a]	123[c]	159[b]	161[b]	7.9	0.02
Isoleucine	268[a]	171[c]	213[b]	209[b]	9.7	0.01
Leucine	367[a]	262[c]	304[b]	353[a]	13.8	0.03
Lysine	637[a]	462[c]	562[b]	571[b]	10.8	0.01
Methionine	106[a]	75[b]	91[ab]	88[ab]	6.5	0.01
Phenylalanine	198[a]	101[b]	99[b]	110[b]	4.3	0.01
Threonine	304[a]	241[b]	276[ab]	271[ab]	6.6	0.01
Tryptophan	125[a]	66[c]	89[b]	94[b]	5.2	0.03
Valine	448[a]	332[b]	349[b]	342[b]	9.2	0.01
Nonessential amino acid						
Alanine	4775	4894	4432	4581	189.3	0.23
Aspartate	513	499	509	503	12.9	0.33
Glutamate	2811[a]	1725[c]	2219[b]	2158[b]	89.7	0.01
Glutamine	1257[a]	685[c]	935[b]	1159[a]	28.1	0.01
Citrulline	478[a]	301[c]	389[b]	402[b]	9.5	0.01
Ornithine	101[a]	65[c]	82[b]	97[a]	5.8	0.01
Proline	3421[a]	2011[c]	2678[b]	2606[b]	91.2	0.02

[a, b, c] Means in a row with superscripts without a common letter differ ($P < 0.05$).
[1] Data are means and pooled SEM ($n = 8$/group for ewes, $n = 16$/group for the fetus).
[2] CON = ewes fed 100% of NRC nutrient recommendations for pregnancy; RES = ewes fed 50% of NRC nutrient recommendations for pregnancy; RES + ARG = RES ewes supplemented with 20 g/d RP-Arg; RES + NCG = RES ewes supplemented with 5 g/d NCG.

Table 5
Effects of dietary supplementation of N-carbamylglutamate (NCG) or rumen-protected L-arginine (RP-Arg) on polyamine concentrations (nmol/g wet weight) in type A cotyledonary (COT) tissues of underfed Hu ewes on d 110 of gestation[1,2].

Item	CON	RES	RES + ARG	RES + NCG	SEM	*P*-value
Putrescine	6.48[a]	3.81[c]	4.89[b]	5.03[b]	0.221	0.01
Spermidine	324.37[a]	203.62[c]	276.13[b]	283.32[b]	15.253	0.01
Spermine	124.78[a]	63.28[c]	90.38[b]	111.23[a]	6.871	0.01
Total	455.63[a]	270.71[c]	371.42[b]	399.58[b]	17.412	0.02

[a, b, c] Means in a row with superscripts without a common letter differ ($P < 0.05$).
[1] Data are means and pooled SEM ($n = 8$/group for ewes, $n = 16$/group for the fetus).
[2] CON = ewes fed 100% of NRC nutrient recommendations for pregnancy; RES = ewes fed 50% of NRC nutrient recommendations for pregnancy; RES + ARG = RES ewes supplemented with 20 g/d RP-Arg; RES + NCG = RES ewes supplemented with 5 g/d NCG.

in type A COT tissues declined by 50.3% to 65.4% but that of PGR ($P = 0.01$) and FGF2 ($P = 0.03$) increased by 62.3% to 71.8% in RES ewes (Fig. 1; Fig. 2). In contrast, compared to that in the RES ewes, NCG or RP-Arg supplementation induced an increase of 23.5% to 86.1% in CAT1, ASCT2, PEPT1 and eNOS protein expression and a decrease of 14.1% to 40.2% in PGR and FGF2 protein expression in the type A COT tissues (all $P < 0.05$).

4. Discussion

The findings of this study confirmed an increase in the COT vascular indexes (CAD, APC and CSD) in underfed ewes compared to those in control ewes. Such findings suggest that an adaptation in placental vascularity occurred in response to the maternal nutritional status and the fetal demand for nutrients from early to mid-gestation. The increase in COT vascularity, which may be a natural compensatory process, was first observed on d 90 of gestation in overfed ewes compared to that in control ewes, however, placental vascularity was not affected by nutrition plane after d 130 of gestation (Redmer et al., 2009). In contrast to the previous study by Redmer et al. (2009), where only CND showed significant differences, our study found significant differences in CSD, APC, and CAD, but not in CND. The inability to detect differences in CND at d 110 of gestation may be owing to the increased variability when CND showed a 10-fold increase from d 50 to 140 (Carr et al., 2016; Reynolds et al., 2010). Despite this, our results indicated that pregnant ewes receiving 100% of their nutrient requirements had the highest level of COT vascularity, indicating a significant vascular compensation during pregnancy that help maintain fetal growth, as previously reported by Carr et al. (2016). Based on our findings, it can be deduced that the group with RES diet did not achieve optimal vascular adaptation, leading to a delayed onset of IUGR. Furthermore, our findings demonstrated that RP-Arg/NCG supplementation to underfed ewes suppressed the vascular adaptive response in type A COT tissues compared to that observed in RES ewes.

The placenta controls the transport of nutrients to the fetus to ensure proper fetal growth and development. Abnormalities or perturbations in the placenta can lead to placental dysfunction, which can negatively impact fetal growth and development, potentially resulting in conditions such as IUGR where the fetus does not grow at a normal rate (Sibley et al., 2005). Placental blood flow ensures that the nutrients reach the fetus in adequate amounts, and the levels or activities of specific transporters, such as those for AA and glucose, represent the rate-limiting step during nutrient delivery (Jones et al., 2007). Maternal dietary protein deprivation has been reported to reduce placental AA transporter expression, which precedes fetal growth suppression in rats, highlighting the importance of maternal nutrition in ensuring proper placental function and fetal growth and development

Table 6
Effects of dietary supplementation of N-carbamylglutamate (NCG) or rumen-protected L-arginine (RP-Arg) on the mRNA abundance of genes in type A cotyledonary (COT) tissues of underfed Hu ewes on d 110 of gestation[1,2].

Item	CON	RES	RES + ARG	RES + NCG	SEM	*P*-value
Amino acid transport-related genes						
SLC7A1	1.00[a]	0.39[c]	0.67[b]	0.74[b]	0.062	0.00
SLC1A1	1.00	0.89	0.96	0.93	0.088	0.10
SLC7A7	1.00[a]	0.56[c]	0.79[b]	0.81[b]	0.112	0.01
SLC1A5	1.00[a]	0.62[b]	0.87[a]	0.92[a]	0.083	0.01
SLC15A1	1.00[a]	0.42[c]	0.79[b]	0.76[b]	0.058	0.01
Arg–NO pathway-related genes						
iNOS	1.00[a]	0.50[c]	0.72[b]	0.80[b]	0.087	0.01
eNOS	1.00[a]	0.46[c]	0.70[b]	0.91[a]	0.102	0.02
Steroidogenic-related genes						
CYP17	1.00[c]	1.72[a]	1.40[b]	1.38[b]	0.124	0.02
CYP19	1.00[c]	1.67[a]	1.35[b]	1.31[b]	0.087	0.01
ESR1	1.00[c]	1.62[a]	1.36[b]	1.44[b]	0.058	0.01
ESR2	1.00[c]	1.69[a]	1.39[b]	1.31[b]	0.132	0.02
PGR	1.00[c]	1.57[a]	1.32[b]	1.08[c]	0.104	0.02
Placental angiogenesis factor-related genes						
FGF2	1.00[c]	1.79[a]	1.42[b]	1.39[b]	0.052	0.02
HIF1A	1.00[c]	1.64[a]	1.32[b]	1.10[c]	0.108	0.01
TEK	1.00[c]	1.61[a]	1.29[b]	1.31[b]	0.138	0.01
NRP1	1.00	0.98	1.10	0.96	0.123	0.12
NRP2	1.00[c]	1.71[a]	1.44[b]	1.35[b]	0.159	0.01
FGFR2	1.00[c]	1.62[a]	1.35[b]	1.33[b]	0.093	0.01

CYP19 = aromatase; *CYP17* = 17 alpha hydroxylase; *ESR* = estrogen; *eNOS* = epithelial NO synthase; *FGF2* = fibroblast growth factor 2; *FGFR2* = fibroblast growth factor receptor 2; *HIF1A* = hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor); *iNOS* = inducible NO synthase; *NRP* = neuropilin; *PGR* = progesterone receptor; *SLC1A1* = solute carrier family 1 (neuronal/epithelial high-affinity glutamate transporter, system X_{AG}), member 1; *SLC7A1* = solute carrier family 7 (cationic amino acid transporter, y^+ system), member 1; *SLC1A5* = solute carrier family 1 (alanine-serine-cysteine amino acid transporter 2), member 5; *SLC7A7* = solute carrier family 7 (amino acid transporter light chain, y^+ L system), member 7; *SLC15A1* = solute carrier family 15, (peptide transporter 1) member 1; *TEK* = tyrosine endothelial kinase.

[a, b, c] Means in a row with superscripts without a common letter differ ($P < 0.05$).

[1] Data are means and pooled SEM ($n = 8$/group for ewes, $n = 16$/group for the fetus).

[2] CON = ewes fed 100% of NRC nutrient recommendations for pregnancy; RES = ewes fed 50% of NRC nutrient recommendations for pregnancy; RES + ARG = RES ewes supplemented with 20 g/d RP-Arg; RES + NCG = RES ewes supplemented with 5 g/d NCG.

(Jansson et al., 2006). Similarly, in our study on underfed ewes carrying IUGR fetuses, we observed that the placentas of these ewes showed lower levels of transporters for cationic and neutral AA. Additionally, supplementing the maternal diet with RP-Arg or NCG increased the expression of placental AA transporters in ewes carrying IUGR fetuses. This suggests that there is a mechanism that can ameliorate the negative effects of IUGR on nutrient absorption by increasing the expression of these transporters. For investigating the relationship between the changes in placental AA concentrations and absorption, the mRNA and protein expression of AA transporters, including CAT1, PEPT1 and ASCT2 (encoded by *SLC7A1*, *SLC15A1* and *SLC1A5*, respectively), was analysed. Based on our results, the mRNA expression levels of *SLC7A1*, *SLC1A5* and *SLC15A1* were higher in the placentas of IUGR fetuses from underfed ewes that were supplemented with RP-Arg or NCG compared to those from RES ewes. Dietary supplementation with RP-Arg or NCG also

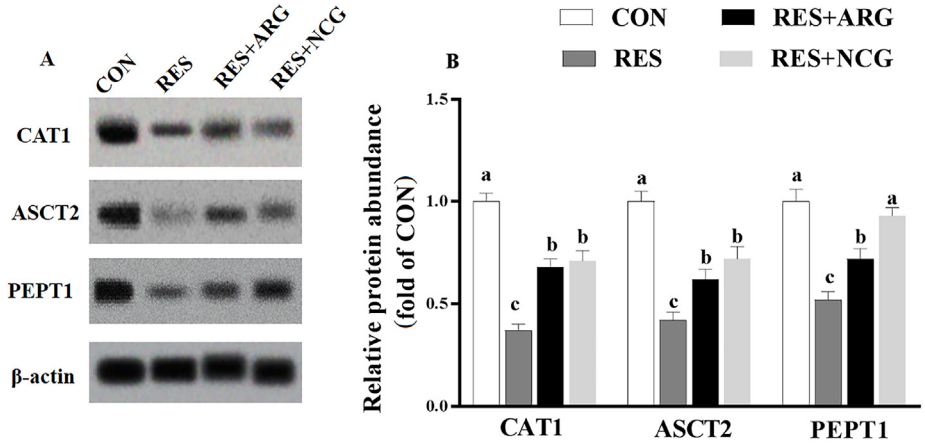

Fig. 1. Effects of dietary N-carbamylglutamate (NCG) or rumen-protected L-arginine (RP-Arg) supplementation on the amino acid and peptide transporters-related protein expression in type A cotyledons (COT) tissues of underfed Hu ewes on d 110 of gestation. *SLC7A1*, *SLC1A5*, and *SLC15A1* were responsible for encoding CAT1, ASCT2, and PEPT1, respectively. (A) Typical charts showing the Western blotting analysis. (B) Densitometric data were standardized using β-actin as a reference and presented as a relative change in the values. Results are expressed as means ± SEM ($n = 8$/group for ewes, $n = 16$/group for the fetus). Means labelled with different letters indicate statistical significance ($P < 0.05$). ASCT2 = alanine-serine-cysteine amino acid transporter 2; CON = ewes fed with 100% of nutrient requirements recommended by NRC (2007); CAT1 = cationic amino acid transporter, y + system, member 1; PEPT1 = peptide transporter 1; RES = 50% of nutrient requirements recommended by NRC (2007); RES + ARG = RES ewes supplemented with 20 g/d RP-Arg; RES + NCG = RES ewes supplemented with 5 g/d NCG.

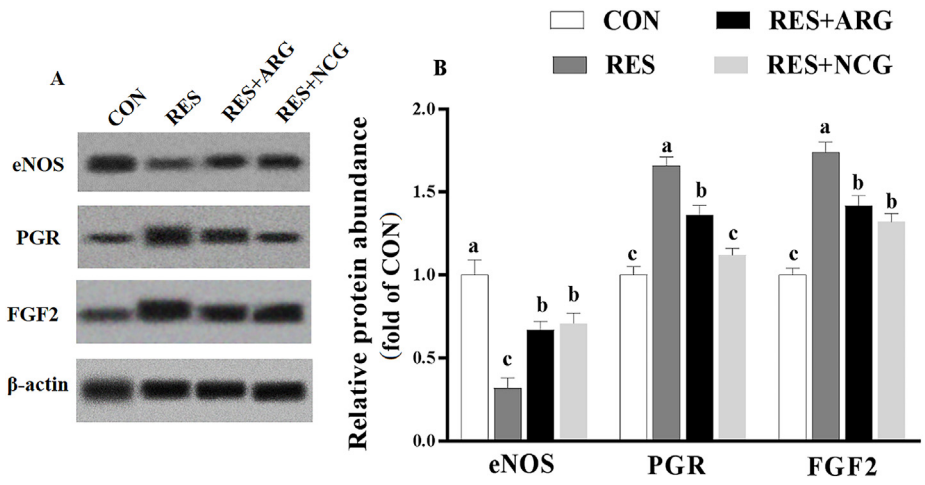

Fig. 2. Effects of dietary N-carbamylglutamate (NCG) or rumen-protected L-arginine (RP-Arg) supplementation on the eNOS, PGR and FGF2 protein expression in type A cotyledons (COT) tissues of underfed Hu ewes on d 110 of gestation. (A) Typical charts showing the Western blotting analysis. (B) Densitometric data were standardized using β-actin as a reference and presented as a relative change in the values. Results are expressed as means ± SEM (n = 8/group for ewes, n = 16/group for the fetus). Means labelled with different letters indicate statistical significance ($P < 0.05$). CON = ewes fed with 100% of nutrient requirements recommended by NRC (2007); eNOS = epithelial NO synthase; FGF2 = fibroblast growth factor 2; PGR = progesterone receptor; RES = 50% of nutrient requirements recommended by NRC (2007); RES + ARG = RES ewes supplemented with 20 g/d RP-Arg; RES + NCG = RES ewes supplemented with 5 g/d NCG.

increased the protein expression of other transporters like CAT1, PEPT1 and ASCT2 in the placentas of IUGR fetuses from underfed ewes. This increase in protein expression aligns with the observed changes in AA contents in the placentas of these IUGR fetuses, indicating that supplementing with RP-Arg or NCG can enhance nutrient transport and support fetal growth and development in IUGR pregnancies by upregulating the expression of these transporters.

It is interesting to note that many AA, as well as their metabolites, have important roles in regulating placental vasodilation and angiogenesis. The catabolism of Arg results in the production of NO, which plays a vital role in regulating blood flow to the placenta and the fetus and promoting the formation of new blood vessels in the placenta, both of which are crucial for proper fetal growth and development (Bird et al., 2003). Besides, Arg, glutamate, and proline are metabolized in the body to form ornithine, which is a precursor for the production of polyamines (Wu et al., 2008). Polyamines, a class of organic compounds, play important roles in various physiological processes such as cell growth and differentiation, gene expression, angiogenesis, and stress response (Kwon et al., 2003). It has been reported that inhibiting the production of polyamines in rats and mice leads to impaired growth of the placenta and causes IUGR (Lopez-Garcia et al., 2008). Our findings further confirmed that selective nutrients play important roles in optimal fetal development, either directly or by promoting placental growth. The levels of the AA (proline, arginine, ornithine, and glutamate) and the polyamines (spermidine, spermine, and putrescine) were elevated in the type A COT of IUGR fetuses from underfed ewes supplemented with RP-Arg or NCG compared to those from RES ewes. Additionally, our study found that the placental mRNA expression of the AA transporter responsible for transporting proline, Arg, ornithine and glutamate was increased in underfed ewes that received supplementations of NCG and RP-Arg.

FGF2 is thought to be a key factor in regulating endothelial cell growth and blood vessel formation (Bai et al., 2018). Besides, the FGF2-induced activation of endometrial FGFR2 plays an important role in improving fertility and uterine development, as has been reported in swine (Lim et al., 2017). Our findings indicated that the FGF2 protein expression was increased in the type A COT of IUGR fetuses from underfed ewes, but was remarkably decreased by dietary RP-Arg or NCG supplementation. Additionally, the ovine vascularity parameters in type A COT tissue exhibited consistent FGF2 mRNA and protein expression. As a result, the underfeeding-induced adverse energy balance promoted placental angiogenic growth factor expression among RES ewes, which was reduced by dietary RP-Arg/NCG supplementation. Therefore, the varying expression of different angiogenic growth factors is a potentially important factor related to the alteration of vascularity parameters in ovine COT tissues.

Hypoxia-inducible factor 1a (HIF-1A) may be involved in the induction of angiogenesis by oxidative stress (Kim and Byzova, 2014). As reported in research on the human placenta, HIF-1A promotes the proliferation of arterial endothelial cells by increasing the FGF2/VEGF levels (Wang et al., 2009). Furthermore, our study demonstrated that RES ewes showed a significant increase in *HIF-1A* mRNA expression, which was decreased markedly upon RP-Arg/NCG supplementation. Accordingly, RP-Arg/NCG supplementation and underfeeding affect oxidation-antioxidation in ovine COT tissues, thus altering the placental angiogenic growth factor expression.

In the last trimester, the P4/E2 concentration in malnourished ewes increased compared to that in controls (Lemley et al., 2014), which is consistent with our results. The dietary supplementation of 0.8% Arg remarkably decreased the P4 contents in pregnant gilts (Bazer et al., 2014). According to our results, the maternal P4/E2 concentrations also declined in RES ewes supplemented by dietary NCG or RP-Arg compared to that in RES ewes. The decline in the P4 and E2 concentrations in circulation was associated with reduced placental steroid synthesis or increased placental and liver catabolism (Sangsritavong et al., 2002), since the elevated activity of NO and NOS, Arg catabolic products, increased the placental and hepatic blood flow and promoted steroid catabolism. However, contrary to our findings, Arg supplementation did not affect the maternal progesterone contents in malnourished sheep . A such discrepancy might be associated with the duration of fetal nutrient deprivation and litter size as well as the timing, dose and duration of Arg supplementation.

The placenta functions as the steroidogenic organ that connects the mother and the foetus as well as the target of steroid action (Miller and Auchus, 2011). Placental dysfunction is related to alterations in the levels of steroidogenic enzymes in humans (Hogg et al., 2013). According to our findings, malnutrition elevated PGR

mRNA expression in the COT tissues of pregnant Hu ewes. This result is consistent with findings from previous research on breast cancer tumours, suggesting that PGR expression shows a weak inverse relationship with energy intake (Lagiou et al., 2011). The change in ESR1, ESR2 and PGR levels in response to malnutrition may have resulted in P4 and E2 secretion disorders and disturbed placental function. In our study, RP-Arg or NCG supplementation compensated for the effects of malnutrition on ESR1, ESR2 and PGR levels in pregnant ewes. Thus, RP-Arg or NCG modulates placental activities via steroid hormones and their corresponding receptors. As previously suggested, Arg-NO enhances estradiol/progesterone production in luteinised granular cells in goats (Guo et al., 2019). The expression of steroidogenic enzyme-related genes was inhibited by RP-Arg or NCG in bovine granular cells (Feng et al., 2018). Therefore, RP-Arg or NCG plays important roles during steroidogenesis, and the Arg-NO pathway possibly regulates the ESR1, ESR2 and PGR levels.

5. Conclusion

In conclusion, our study revealed that dietary RP-Arg or NCG supplementation increases IUGR fetal weight by improving placental AA transport, angiogenic growth factor expression and steroid catabolism in underfed ewes. Additionally, dietary RP-Arg or NCG supplementation is a potential treatment for IUGR-related ovine placental dysfunctions.

Author contributions

Hao Zhang, **Hongrong Wang**, and **Mengzhi Wang** designed the research; **Hao Zhang**, **Yi Zheng**, **Xia Zha**, and **Bei Zhang** conducted the research; **Mengzhi Wang**, **Xiaoyun Liu**, and **Hao Zhang** analyzed the data; **Hao Zhang**, **Mabrouk Elsabagh**, and **Yi Ma** wrote the paper; **Hao Zhang**, **Mengzhi Wang**, and **Guihua Shu** had primary responsibility for the final content. All authors read and approved the final manuscript.

Declaration of competing interest

We confirm that the manuscript has not been published elsewhere and is not under consideration by other journals. All authors have approved the manuscript and agree with submission to Animal Nutrition. The authors have no conflicts of interest to declare.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.08.005.

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