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Dietary rumen-protected L-arginine or *N*-carbamylglutamate attenuated fetal hepatic inflammation in undernourished ewes suffering from intrauterine growth restriction

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ABSTRACT

This study aimed to explore whether dietary rumen-protected L-arginine (RP-Arg) or *N*-carbamylglutamate (NCG) supplementation to feed-restricted pregnant ewes counteracts fetal hepatic inflammation and innate immune dysfunction associated with intrauterine growth retardation (IUGR) in ovine fetuses. On d 35 of pregnancy, twin-bearing Hu ewes ($n = 32$) were randomly assigned to 4 treatment groups (8 ewes and 16 fetuses per group) and fed diets containing 100% of the NRC requirements (CON), 50% of the NRC requirements (RES), RES + RP-Arg (20 g/d) (RESA), or RES + NCG (5 g/d) (RESN). At 08:00 on d 110 of gestation, fetal blood and liver tissue samples were collected. The levels of triglyceride, free fatty acid, cholesterol and β-hydroxybutyrate in the fetal blood of RESA and RESN groups were lower ($P < 0.05$) than those of the RES group, but were higher ($P < 0.05$) than those of the CON group. The interleukin (IL)-6 and IL-1 levels in fetal blood and liver tissue as well as the myeloid differentiation primary response 88 (MyD88), transforming growth factor β (TGFβ), and nuclear factor kappa B (NF-κB) mRNA levels in the fetal liver were decreased ($P < 0.05$) by the NCG or RP-Arg supplementation compared to the RES treatment. Similarly, the toll-like receptor (TLR)-4, MyD88, TGFβ, and p-c-Jun N-terminal kinase (JNK) protein levels in the fetal liver were reduced ($P < 0.05$) in the NCG and RP-Arg -supplemented groups compared to the RES group. These results showed that dietary supplementation of RP-Arg or NCG to underfed pregnant ewes could protect against IUGR fetal hepatic inflammation via improving lipid metabolism, down-regulating the TLR-4 and the inflammatory JNK and NF-κB signaling pathways, and decreasing cytokine production in ovine fetal blood and liver tissue.

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1. Introduction

It is well-known that gestational maternal feed restriction is associated with intrauterine growth retardation (IUGR) which increases the predisposition to developmental and metabolic disorders in pre-and postnatal life in ovines (Kelly et al., 2017). The dam responds to gestational feed restriction by the programming of nutrient partitioning to alter the growth rate and functions of the major fetal organs (Prezotto et al., 2016). It has been indicated that IUGR is associated with permanent functional and anatomical adaptations in various organs and tissues in mammals (Maršál, 2018; Chen et al., 2017; Lee et al., 2019). For example, IUGR-induced low birth weight is associated with hypertension, abdominal adiposity, glucose intolerance, dyslipidemia, cardiovascular disease and type 2 diabetes mellitus in adult life (Barker et al., 1989). Further, gestational nutritional deficiency led to permanent impairment in the immunological system in rats (Chandra, 1975).

Sheep models have long been used to study fetal development and physiology due to the following advantages: sheep conceptus are relevant to human fetal physiology and has good tolerance for in utero manipulation (Cavanaugh, 2015). Also, pregnant ewes (particularly those carrying multiple fetuses) display similar symptoms of lipid metabolism disorder, including elevated blood nonesterified fatty acids and β-hydroxybutyrate (BHB) as well as severe fatty liver when they are subjected to feed restriction (Xue et al., 2019). Therefore, a pregnant sheep model can be used to investigate the effect of maternal undernutrition on fetal hepatic inflammation, lipid metabolism and development.

The liver is a vital organ for the differentiation and development of immune precursor cells in the early gestational period (Attreed et al., 2017). The fetal liver shows high sensitivity to various injuries, such as nutritional deficiency, infection and teratogens (Hyatt et al., 2008). Previous studies on ovine fetuses highlighted that IUGR is associated with hepatic dysfunctions including impaired fetal liver growth, nutrient partitioning, insulin sensitivity, stress and antioxidant responses and increased hepatic cell apoptosis (Liu et al., 2018; Thorn et al., 2009). Pregnant dams with restricted feeding experience lipid mobilization which predisposes them to lipid accumulation and inflammation of the fetal liver (Chen et al., 2019). Thus, further research is warranted on the adaptation mechanisms of the fetal liver's immune response to gestational maternal feed restriction and on feeding strategies to alleviate the detrimental effects of IUGR on the fetal liver's immune function.

L-Arginine (Arg), a semi-essential amino acid, has been indicated to attenuate inflammatory responses (Tan et al., 2011). On the other hand, N-carbamylglutamate (NCG) has been recognized as the carbamoyl phosphate synthetase-1 cofactor; the latter can limit flux via Arg synthesis and the urea cycle (Wu et al., 2012). Exogenous Arg supplementation has been suggested to decrease the production of pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-1β induced by LPS-induced inflammation in a rat model (Mohamed et al., 2015). Also, dietary or intravenous administration of Arg has been demonstrated to ameliorate inflammation and immune response in weaned pigs and broiler chickens (Pi et al., 2014; Tan et al., 2014). Similarly, intravenous administration of Arg also attenuated LPS-triggered inflammation and mitigated lactation-induced metabolic stress and inflammation in lactating dairy cows (Zhao et al., 2018; Ding et al., 2020). Furthermore, dietary supplementation of NCG or rumen-protected Arg (RP-Arg) to underfed pregnant ewes from d 35 to 110 of gestation improved the IUGR fetal BW and liver development (Zhang et al., 2016a; Sun et al., 2018). However, little is known about how dietary supplementation of NCG and RP-Arg in nutrient-restricted Hu sheep would affect the fetal hepatic innate

immune system, the toll-like receptors (TLR), the transforming growth factor β (TGFβ), c-Jun N-terminal kinase (JNK) and nuclear factor kappa B (NF-κB) pathways in the circulation and the hepatic tissues of IUGR ovine fetuses.

Therefore, the objectives of the study were to explore the role of NCG or RP-Arg in IUGR fetal liver development and the effects of NCG and RP-Arg on IUGR fetal hepatic inflammation and immune function.

2. Materials and methods

2.1. Animals

All experimental procedures were approved by the Ethics Committee of Yangzhou University (SYXK2013-0057). Forty-eight multiparous Hu ewes with similar body weight (BW = 40.1 ± 1.2 kg), body condition score (BCS = 2.6 ± 0.2: from 0 [thin] to 5 [obesity]; Russel et al., 1969) and age (18.5 ± 0.5 months) were chosen for the study. Hu ewes were housed in an indoor barn at the Jiangyan Experimental Station in Taizhou, Jiangsu, China. The barn was equipped with heating radiators to maintain the average temperature at 15.3 ± 0.88 °C and with automatic lighting control to simulate the natural photoperiod. Following housing, Hu ewes were drenched with ivermectin (0.2 mg/kg) as an anthelmintic and then were subjected to an oestrus synchronization protocol for 12 d using an intravaginal progestogen sponge (30 mg; Pharmp PTY, Herston City, Australia). Two days following the removal of the vaginal device, estrous behavior was detected by 3 vasectomized rams at 08:00 and 16:00 and accordingly ewes were inseminated artificially with fresh semen (d 0 of gestation) and were housed individually in a pen (1.05 m × 1.60 m) for 35 d. On d 35 of gestation, ewes were scanned with ultrasonography (Asonics Microimager 1000 sector scanning instrument; Ausonics, Sydney, Australia) to determine the fetal numbers in each ewe. A total of 32 ewes bearing twin fetuses were selected for this work. The experimental diet (Table 1) was formulated to meet 100% of the nutrient requirements of the estimated National Research Council (NRC, 1985) for pregnant ewes. From d 0 to 35 of gestation, each ewe was given 100% of the nutrient requirements of NRC (1985) once a day at 08:00 and was allowed to drink clean water freely.

2.2. Experiment

On d 35 of gestation, twin-bearing Hu ewes (n = 32) were randomized to 4 treatment groups (each group comprised 8 ewes and 16 fetuses) and fed pelleted diets (Table 1) containing 100% of the NRC (1985) requirements (CON), 50% of the NRC requirements (RES), RES + RP-Arg (20 g/d, Beijing Feeding Feed Science Technology Co., Beijing, China) (RESA), or RES + NCG (5 g/d, the Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China) (RESN) (Zhang et al., 2016a). The RP-Arg used was a 50% Arg product; the NCG used was a 50% NCG product. Therefore, the actual additional amounts RP-Arg and NCG were 10 g/d Arg and 2.5 g/d NCG, respectively. They were mixed into the pelleted mixed diet. The rumen protection of Arg was at least 85%, but the intestinal release of RP-Arg was over 90%, as indicated by previous methods (Chacher et al., 2012). The RP-Arg was extracted from phospholipids and glycerides according to the spray-congealing and spray-drying processes according to Eldem et al. (1991). The Arg dose was determined following previous studies on pregnant ewes receiving Arg supplementation (McCoard et al., 2013; Zhang et al., 2018). The NCG dose was determined based on previous research on pregnant ewes, piglets, and dairy cows (Zhang et al.,

Table 1
Ingredient and nutrient composition of the experimental diets (%, on dry matter basis).

Item	Diet 1 (0 to 90 d of gestation)	Diet 2 (91 to 110 d of gestation)
Ingredients		
Chinese wild rye	50.00	45.00
Corn	35.12	31.32
Soybean meal	12.00	20.00
Dicalcium phosphate	1.67	2.34
Calcium carbonate	0.41	0.54
Salt	0.50	0.50
Premix[1]	0.30	0.30
Total	100	100
Nutrient composition		
DM[2], as fed	90.23	90.36
GE[2], MJ/kg	17.63	18.49
ME[3], MJ/kg	9.23	10.03
CP[2]	9.98	13.59
MP[3]	6.42	8.69
EE[2]	4.21	4.59
NDF[2]	37.12	32.57
ADF[2]	20.98	18.93
Ca[2]	0.57	0.81
P[2]	0.45	0.69

DM = dry matter; GE = gross energy; ME = metabolizable energy; CP = crude protein; MP = metabolizable protein; EE = ether extract; ADF = acid detergent fibre; NDF = neutral detergent fibre.

[1] Provided the following per kilogram of diet: 30,000 IU vitamin A, 10,000 IU vitamin D, 100 mg vitamin E, 90 mg Fe, 12.5 mg Cu, 50 mg Mn, 100 mg Zn, 0.3 mg Se, 0.8 mg I and 0.5 mg Co.

[2] The values were analysed.

[3] The values were estimated according to NRC (1985).

2016b; Zeng et al., 2012; Chacher et al., 2014). The experiment lasted till d 110 of gestation and the BWs of the ewes were measured on an interval of 10 d, and the feed intakes were adjusted accordingly.

2.3. Chemical analyses

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

2.4. Fetal blood and liver sample collection

All ewes were euthanized by the captive bolt gun (Supercash Mark 2; Acceles and Shelvoke) and were killed by exsanguination at 08:00 on d 110 of gestation. Both twins were used for tissue analysis. Blood samples (5 mL) were collected from the fetal umbilical cord; one portion was kept in heparinized tubes whereas another portion was left to coagulate for 40 min and then centrifuged for 15 min at 3,000 × g and finally kept at −80 °C till assayed. The BW of the fetuses were recorded after slaughtering of dams and the weights of their livers were also immediately recorded. Thereafter, hepatic tissues were cleaned with iced PBS, sliced into pieces and then frozen into liquid nitrogen and kept at −80°C until analysis (Chen et al., 2019).

2.5. Biochemical analyses of fetal blood

The plasma total free fatty acid (FFA) was measured by enzyme calorimetry using an assay kit (Waco Chemicals, Waco, TX) (Zhu et al., 2010). The plasma triglyceride and cholesterol levels were analyzed by the Boehringer Mannheim/Hitachi 912 analyzer (Roch Diagnostics, Indianapolis, IN) according to a previous description (Long et al., 2007). The 3-hydroxybutyrate dehydrogenase was used to measure the plasma BHB using spectrophotometry (Wu et al., 1991). The levels of plasma alkaline phosphatase (AKP), albumin (ALB), total protein (TP), alanine transaminase (ALT) and aspartate transaminase (AST) were measured by the Hitachi automatic biochemical analyzer (7600-020, Hitachi Ltd., Tokyo, Japan) using the commercially available AKP (060701, Prodia diagnostics, Germany), ALB (20110622, Kehua Biological Cor. Shanghai, China), TP (20110422, KehuaBiological Cor. Shanghai, China), ALT (050801, Prodia diagnostics, Germany), and AST (070201, Prodia diagnostics, Germany) detection kits, respectively. The globulin (GLB) content was determined as the difference between TP and ALB. The specific commercial ELISA kits (CUSABIO; MEIMIAN) were used to measure the serum amyloid protein A (SAA) and paraoxonase (PON) levels.

2.6. The hepatic lipase, triglycerides, lipoprotein lipase, and total cholesterol of fetal liver

The fetal liver tissue (0.5 g) was rinsed in 0.85% frozen saline, followed by centrifugal homogenization at 1,500 × g for 5 min in a 9-fold volume of 0.85% frozen saline. A 10% liver homogenate was centrifuged at 3,000 × g for 15 min and supernatants were collected and used to determine the concentrations of total cholesterol, triglycerides, hepatic lipase and lipoprotein lipase in fetal liver using spectrophotometry (EMC-61PC-UV, Duisburg, Germany) and commercial kits following the manufacturers' procedures (Diagnostic Product; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) (Yang et al., 2019).

2.7. Cytokine analysis

Commercial kits were used to measure the IL-6 and TNF-α (R&D Systems, Oxford, UK) as well as the IL-1 (BioSource/MED Probe, Camarillo, CA, USA) contents using a BioTek synergy HT microplate reader (BioTek Instruments, Winooski, VT, USA) at 450 nm. The detection limits were 35.0, 10.0, and 7.0 pg/mL for IL-1, IL-6, and TNF-α, respectively. The inter- and intra-assay coefficients of variation were ≤10%. All results were presented as picogram per milliliter of fetal plasma and as nanogram per gram of protein in fetal liver tissue (Zhang et al., 2019).

2.8. Cellularity estimates of fetal liver

A freshly thawed 0.5-g tissue sample was subjected to homogenization in 2 mmol/L EDTA buffer (pH = 7.4), 2.0 mol/L sodium chloride, and 0.05 mol/L Tris aminomethane by a Polytron equipped with a PT-10 s probe (Brinkmann, Westbury, NY, USA). The protein, RNA and DNA contents in the fetal liver homogenate were estimated. The RNA and DNA estimation was done by the orcinol and diphenylamine procedures, respectively (Johnson et al., 1997). The protein content was estimated using Coomassie brilliant blue G with bovine serum albumin (Fraction V; Sigma−Aldrich) as the standard (Johnson et al., 1997; Bradford, 1976). Spectrophotometry (Beckman DU 640; Beckman Coulter Inc., Brea, CA, USA) was used to analyze and assess the prepared samples against the concentration curves of known standards. The DNA content was used as a

parameter to measure hyperplasia. The ratios of RNA to DNA and protein to DNA were utilized as indicators of potential cellular activity and hypertrophy, respectively (Scheaffer et al., 2004).

2.9. Total RNA extraction and real-time qPCR

A Trizol reagent (R1100; Solarbio) was used to isolate the total cellular RNA in the fetal liver samples following the manufacturer's instructions. Spectrometry (A260/A280, NanoDrop 2000; Thermo Scientific) and agarose gel (1%) electrophoresis were used to verify the quality of RNA. A PrimeScript RT reagent Kit was used to generate the first-stranded cDNA in duplicate, along with the gDNA Eraser (RR047A; Takara). Sangon Biotech (Shanghai; China) was responsible for designing and synthesizing primers for reference and target genes (Table 2). Specifically, β-actin was a stable housekeeping gene and selected to be the reference gene in this study. Real-time qPCR was carried out 3 times for amplifying β-actin and target genes through 2 × SYBR Green I PCR mix (Solarbio, SR1110). The Roche Light Cycler 480II Sequence Detection System was utilized to carry out specific reactions. Notably, the specific parameters of thermal cycling are shown below, for 10 min at 95 °C to activate Hot start DNA polymerase, followed by 40 amplification cycles for 20 s at 95 °C as well as for 1 min at 60 °C. The $\triangle$cycle threshold ($\triangle$Ct) approach was used to determine the relative level of mRNA (Livak and Schmittgen, 2001). Briefly, the $\triangle$Ct value represented the difference in Ct between reference and target genes ($\triangle$Ct = Ct$_{target}$ − Ct$_{reference}$). The $\triangle\triangle$Ct value for each sample was calculated by taking away the mean $\triangle$Ct in a specific control/restriction group. Moreover, the relative mRNA level was calculated according to R = $2^{-\triangle\triangle Ct}$.

2.10. Western blot analysis

A commercial kit (Beyotime Biotechnology, Jiangsu, China) was used to extract the total protein in fetal liver tissues following the manufacturer's protocols. The bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) was adopted to detect protein levels. In this study, the primary antibodies included anti-p IκBα and anti-IκBα (both are 1:1,000, CST), anti-TLR4 (1:1,000, Abcam), pcJun (Ser73) (1:1,000, SIGMA), anti-SAPK/JNK, anti-pcJun (Ser63), anti-pIKKβ, IKKβ and anti-TGFβ (all are 1:1,000, Cell Signaling), anti-MyD88 (1:2,000, SIGMA), anti-pSAPK/JNK (Thr183/Tyr185) and anti-cJun (both are 1:2,000, Cell Signaling), anti-pp65 and anti-p65 (both are 1:300, Cell Signaling), anti-TNF-α (1:300, Santa Cruz Biotechnology) and anti-β-actin (1:1,500, Santa Cruz Biotechnology). In brief, total protein (50 μg) was mixed with the loading buffer (150 mmol/L Tris−HCl [pH 6.8], 20% glycerol, 2 mmol/L 2-mercaptoethanol, and 0.004% [wt/vol] bromophenol blue), followed by a 5-min denaturation at 100 °C. The protein contents of the lysates were separated using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred into the polyvinylidene fluoride (PVDF) membranes. A 5% skimmed milk supplemented in Tris-buffered saline (TBST) was used to block the membranes for 1 h under ambient temperature, followed by overnight incubation with primary antibodies in TBST with 5% bovine serum albumin at 4 °C. The membranes were washed with TBST 3 times, followed by further 1 h incubation with anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) (1:5,000, Antgene Biotech) under ambient temperature. The immunoreactive bands were washed and observed under the ECL western blotting detection system (Fujifilm, Tokyo, Japan). The Image J software (Wayne Rasband, Maryland, USA) was used for the densitometric detection of the band intensity. The band density among different blots was normalized according to the density of a reference sample and the β-actin content.

2.11. Statistical analyses

The fetal data obtained on d 110 of gestation was tested by one-way analysis of variance (ANOVA) using the PROC GLM procedure of SAS (version 9.2). The fetal gender was incorporated in the initial model but revealed no statistical significance ($P > 0.05$), hence was eliminated from the final model, which only contained maternal nutritional treatments. Duncan's multiple range test was used to detect differences among treatments. Values are presented as least

Table 2
Primer sequences used in the real-time PCR[1].

Gene	Sequences (5′-3′)	Product size, bp	References
CD11b	F: GTCATTGGGGTGGGAGATG	80	Yan et al., (2011)
	R: TCAGCAGGGGGCTTAGATG		
CD14	F: CTCAGCGTGCTTGATCTCAG	98	Yan et al., (2011)
	R: AAGGGATTTCCGTCCAGAGT		
CD68	F: CAGGGGACAGGGAATGACT	144	Yan et al., (2011)
	R: CCAAGTGGTGGTTCTGTGG		
MyD88	F: ATGGTGGTGGTTGTCTCTGAC	133	Jiang et al., (2017)
	R: GGAACTCTTTCTTCATTGGCTTGT		
TLR-2	F: CAAGAGGAAGCCCAGGAAG	112	Yan et al., (2010)
	R: TGGACCATGAGGTTCTCCA		
TLR-4	F: TGCTGGCTGCAAAAAGTATG	139	Yan et al., (2010)
	R: CCCTGTAGTGAAGGCAGAGC		
TGFβ	F: AAAAGAACTGCTGTGTTCGTCA	149	Wang et al., (2012)
	R: GACCTTGCTGTACTGTGTGTCC		
IL-6	F: AGGAAAAAGATGGATGCTTCCA	122	Herdrich et al., (2010)
	R: GACCAGCAGTGGTTTTGATCAA		
IL-1β	F: CGTCTTCCTGGGACGTTTTAG	97	Yang et al., (2016)
	R: CTGCGTATGGCTTCTTTAGGG		
NF-κB	F: ATACGTCGGCCGTGTCTAT	125	Zhang et al., (2019)
	R: GGAACTGTGATCCGTGTAG		
TNF-α	F: ACACCATGAGCACCAAAAGC	109	Puttabyatappa et al., (2019)
	R: AGGCACAAGCAACTTCTGGA		
β-actin	F: CACCGCAAATGCTTCTAGGC	96	Dong et al., (2020)
	R: ACGAGGCCAATCTCATCTCG		

CD = cluster of differentiation; MyD88 = myeloid differentiation factor 88; TGFβ = Transforming growth factor β; TLR = toll-like receptor; IL = interleukin; TNF-α = tumor necrosis factor α; NF-κB = nuclear factor kappa B (p65); F = forward; R = reverse.

squares means ± SEM. A *P*-value of ≤0.05 stood for statistical significance.

3. Results

3.1. The dry matter intake (DMI) of ewes, maternal and fetal body weights, and liver weights

On d 110 of pregnancy, the DMI of ewes and maternal BW in the CON group were greater (*P* < 0.05) than those of the RES, RESN and RESA groups (Appendix Table 1). The fetal BW and liver weight were lower (*P* < 0.05) in the RES group compared to the CON group. Relative to the RES group, fetal BW and liver weight were greater (*P* < 0.05) in the RESN group. Dietary NCG or RP-Arg supplementation reduced (*P* < 0.05) the ratio of liver weight to fetal BW in the RES group. These variables have also been presented in our previous publication (Zhang et al., 2016).

3.2. Fetal plasma biochemical indices

The TP, GLB, ALT and AKP concentrations in the fetal plasma of animals in the RES group were lower relative to those in the CON group (*P* < 0.05) (Table 3). Compared with the RES group, dietary NCG or RP-Arg supplementation increased the GLB, TP and ALT contents in fetal plasma (*P* < 0.05). The AST, ALB:GLB ratio, AST:ALT ratio, and SAA content in fetal blood were higher (*P* < 0.05) in the RES group compared to those in the CON group. Dietary NCG or RP-Arg supplementation decreased the contents of AST:ALT ratio, AST, ALB:GLB ratio, and SAA in the fetal plasma of the RES group (*P* < 0.05). The PON and ALB contents of fetal blood did not differ (*P* > 0.05) among treatment groups.

3.3. Fetal liver cellularity estimates

There was no difference in the DNA content in fetal liver tissues among treatments (*P* > 0.05) (Table 4). The protein and RNA content, the ratios of protein to DNA and RNA to DNA of fetal liver in the RES group were lower (*P* < 0.05) relative to those in the CON group, but were higher (*P* < 0.05) in fetuses born to ewes treated with RP-Arg or NCG.

Table 3
Effects of dietary *N*-carbamylglutamate (NCG) and rumen-protected L-arginine (RP-Arg) supplementation on indices of the fetal blood in underfed Hu ewes on d 110 of gestation[1].

Item	CON[2]	RES[3]	RESA[4]	RESN[5]	SEM	*P*-value
TP, g/L	42.38[a]	29.78[c]	34.57[b]	35.09[b]	1.13	0.009
ALB, g/L	22.09	21.89	21.97	22.01	0.78	0.301
GLB, g/L	9.38[a]	6.75[c]	8.01[b]	8.35[b]	0.45	0.013
ALB:GLB ratio	2.36[c]	3.24[a]	2.74[b]	2.65[b]	0.17	0.007
AST, IU/L	22.67[b]	32.38[a]	23.09[b]	22.97[b]	1.55	0.019
ALT, IU/L	3.99[a]	2.07[c]	3.02[b]	3.91[a]	0.12	0.006
AST:ALT ratio	5.68[c]	15.64[a]	7.65[b]	5.88[c]	1.01	0.007
AKP, IU/L	137.65[a]	108.87[b]	110.34[b]	109.78[b]	7.09	0.021
PON, IU/L	165.23	160.29	163.63	161.97	8.03	0.126
SAA, g/mL	1179.07[c]	2087.89[a]	1609.36[b]	1648.85[b]	113.87	0.008

TP = total protein; ALB = albumin; GLB = globulin; AST = aspartate transaminase; ALT = alanine transaminase; AKP = alkaline phosphatase; PON = paraoxonase; SAA = serum amyloid protein A.
[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* = 16 per group).
[2] CON: ewes fed 100% of NRC (1985) recommendations for pregnancy.
[3] RES: ewes fed 50% of NRC (1985) recommendations for pregnancy.
[4] RESA: RES ewes supplemented RP-Arg at 20 g per day.
[5] RESN: RES ewes supplemented NCG at 5 g per day.

Table 4
Effects of dietary *N*-carbamylglutamate (NCG) and rumen-protected L-arginine (RP-Arg) supplementation on fetal liver estimates of cellularity in underfed Hu ewes on d 110 of gestation[1].

Item	CON[2]	RES[3]	RESA[4]	RESN[5]	SEM	*P*-value
DNA, mg/g	11.46	11.53	11.48	11.41	1.03	0.221
RNA, mg/g	14.97[a]	8.59[c]	11.62[b]	11.96[b]	1.38	0.009
RNA:DNA ratio	1.31[a]	0.74[c]	1.01[b]	1.05[b]	0.19	0.016
Protein, mg/g	137.69[a]	87.68[c]	104.26[b]	110.27[b]	8.97	0.008
Protein:DNA ratio	12.02[a]	7.61[c]	9.08[b]	9.67[b]	1.03	0.021

[a, b, c] With in a row, means without a common superscript differ *P* < 0.05.
[1] Data are means and pooled SEM (*n* = 16 per group).
[2] CON: ewes fed 100% of NRC (1985) recommendations for pregnancy.
[3] RES: ewes fed 50% of NRC (1985) recommendations for pregnancy.
[4] RESA: RES ewes supplemented RP-Arg at 20 g per day.
[5] RESN: RES ewes supplemented NCG at 5 g per day.

3.4. Total free fatty acids, cholesterol, triglyceride, and hydroxybutyrate contents in fetal plasma

The fetal plasma FFA, cholesterol, triglyceride, and BHB levels in the RES group were higher (*P* < 0.05) compared to those in the CON group (Table 5). The fetal plasma FFA, cholesterol, triglyceride, and BHB levels in RESA and RESN groups (*P* < 0.05) were lower relative to those in the RES group.

3.5. The lipoprotein lipase, total cholesterol, and triglycerides in fetal liver

The contents of hepatic lipase, lipoprotein lipase, total cholesterol and triglycerides in the fetal liver in the RES group were lower (*P* < 0.05) than those of the CON group (Table 6). The triglycerides, total cholesterol, and lipoprotein lipase contents were higher (*P* < 0.05) in the fetal liver in RESA or RESN group relative to those in the RES group.

3.6. The cytokine levels in fetal plasma and liver

The IL-6 and IL-1 levels in fetal plasma and liver tissue in the RES group were higher than those in the CON group (*P* < 0.05) (Table 7). Compared to the RES group, dietary NCG or RP-Arg supplementation decreased the IL-6 and IL-1 levels in fetal plasma and liver tissue (*P* < 0.05) and decreased the TNF-α content in the fetal liver tissue (*P* < 0.05).

3.7. The mRNA abundance of genes in fetal liver

The mRNA levels of cluster of differentiation 11 b (*CD11bI*), *CD14*, *CD68*, *NF-κB*, *TLR-4*, *MyD88*, *TGFβ*, *IL-1β*, *IL-6*, and *TNF-α* in the fetal

Table 5
Effects of dietary *N*-carbamylglutamate (NCG) and rumen-protected L-arginine (RP-Arg) supplementation on total free fatty acid (FFA), triglyceride, cholesterol, and β-hydroxybutyrate (BHB) content of fetal plasma in underfed Hu ewes on d 110 of gestation[1].

Item	CON[2]	RES[3]	RESA[4]	RESN[5]	SEM	*P*-value
FFA, mmol/L	0.41[c]	0.63[a]	0.52[b]	0.54[b]	0.04	0.006
Cholesterol, mg/dL	28.69[c]	36.73[a]	32.06[b]	28.83[c]	1.78	0.012
Triglyceride, mg/dL	32.15[c]	43.64[a]	37.27[b]	36.61[b]	1.39	0.009
BHB, mmol/L	0.14[c]	0.31[a]	0.25[b]	0.21[b]	0.03	0.007

[a, b, c] With in a row, means without a common superscript differ *P* < 0.05.
[1] Data are means and pooled SEM (*n* = 16 per group).
[2] CON: ewes fed 100% of NRC (1985) recommendations for pregnancy.
[3] RES: ewes fed 50% of NRC (1985) recommendations for pregnancy.
[4] RESA: RES ewes supplemented RP-Arg at 20 g per day.
[5] RESN: RES ewes supplemented NCG at 5 g per day.

Table 6
Effects of dietary *N*-carbamylglutamate (NCG) and rumen-protected L-arginine (RP-Arg) supplementation on triglycerides, total cholesterol, lipoprotein lipase, and hepatic lipase of the fetal liver in underfed Hu ewes on d 110 of gestation[1].

Item	CON[2]	RES[3]	RESA[4]	RESN[5]	SEM	*P*-value
Triglycerides, mmol/g	1.24[a]	0.88[c]	1.09[b]	1.21[a]	0.06	0.006
Total cholesterol, mmol/g	0.48[a]	0.29[c]	0.39[b]	0.37[b]	0.02	0.005
Lipoprotein lipase, U/mg	0.71[a]	0.42[b]	0.67[a]	0.69[a]	0.06	0.014
Hepatic lipase, U/mg	0.69[a]	0.51[b]	0.61[ab]	0.59[ab]	0.05	0.009

[a, b, c] With in a row, means without a common superscript differ $P < 0.05$.
[1] Data are means and pooled SEM ($n = 16$ per group).
[2] CON: ewes fed 100% of NRC (1985) recommendations for pregnancy.
[3] RES: ewes fed 50% of NRC (1985) recommendations for pregnancy.
[4] RESA: RES ewes supplemented RP-Arg at 20 g per day.
[5] RESN: RES ewes supplemented NCG at 5 g per day.

Table 7
Effects of dietary *N*-carbamylglutamate (NCG) and rumen-protected L-arginine (RP-Arg) supplementation on cytokine concentrations in the fetal plasma and fetal liver in underfed Hu ewes on d 110 of gestation[1].

Item	CON[2]	RES[3]	RESA[4]	RESN[5]	SEM	*P*-value
Plasma						
TNF-α, pg/mL	138.72	130.65	132.34	137.28	13.25	0.219
IL-1, pg/mL	149.23[c]	231.28[a]	194.58[b]	192.89[b]	11.41	0.006
IL-6, pg/mL	107.26[c]	175.68[a]	142.62[b]	144.21[b]	10.58	0.006
Liver						
TNF-α, ng/g protein	9.12[c]	16.28[a]	12.89[b]	13.01[b]	0.93	0.007
IL-1, ng/g protein	17.29[c]	28.09[a]	22.09[b]	21.88[b]	1.78	0.011
IL-6, ng/g protein	12.78[c]	23.13[a]	17.73[b]	17.09[b]	1.14	0.008

IL = interleukin; TNF-α = tumor necrosis factor α.
[a, b, c] With in a row, means without a common superscript differ $P < 0.05$.
[1] Data are means and pooled SEM ($n = 16$ per group).
[2] CON: ewes fed 100% of NRC (1985) recommendations for pregnancy.
[3] RES: ewes fed 50% of NRC (1985) recommendations for pregnancy.
[4] RESA: RES ewes supplemented RP-Arg at 20 g per day.
[5] RESN: RES ewes supplemented NCG at 5 g per day.

Fig. 1. Effects of dietary *N*-carbamylglutamate (NCG) and rumen-protected L-arginine (RP-Arg) supplementation on the mRNA abundance of genes of the fetal liver in underfed Hu ewes on d 110 of gestation. Values are means with standard errors represented by vertical bars ($n = 16$ per group). CON: ewes fed 100% of NRC (1985) recommendations for pregnancy; RES: ewes fed 50% of NRC (1985) recommendations for pregnancy; RESA: RES ewes supplemented RP-Arg at 20 g per day; RESN: RES ewes supplemented NCG at 5 g per day. [a, b, c] Bars without a common letter differ ($P < 0.05$). CD = Cluster of differentiation; MyD88 = myeloid differentiation factor 88; TLR = toll-like receptor; TGFβ = Transforming growth factor β; IL = interleukin; NF-κB = nuclear factor kappa B (p65); TNF-α = tumor necrosis factor α.

liver in the RES group were higher ($P < 0.05$) than those in the CON group (Fig. 1). Dietary NCG or RP-Arg supplementation decreased ($P < 0.05$) the above-mentioned genes in the fetal liver relative to those in the RES group. There was no difference ($P > 0.05$) in the *TLR*-2 mRNA level in the fetal liver among treatments.

3.8. Protein levels in fetal liver

Compared with the CON group, the TNF-α, TLR-4, MyD88, p65, phospho (p)-p65, TGFβ, p-IκBα, p-IKKβ, p-c-Jun (Ser63), p-JNK, and p-c-Jun (Ser73) protein levels were higher in the fetal liver in the RES group ($P < 0.05$) (Fig. 2. A to I). Compared with RES, NCG or RP-Arg treatment declined the TNF-α, TLR-4, MyD88, p65, TGFβ, p-IκBα, p-c-Jun (Ser73) and p-JNK protein levels in fetal liver ($P < 0.05$). The protein levels of Jun, IκBα, IKKβ, and JNK were not changed among treatments ($P > 0.05$). Compared to the RES group, NCG or RP-Arg treatment did not decrease ($P > 0.05$) p-c-Jun (Ser63) and p-p65 protein levels in fetal liver.

4. Discussion

It has been reported that IUGR primarily results from exposure to chronic hypoxia and malnutrition due to uteroplacental insufficiency, in which the impairment of uteroplacental blood flow curtails the supply of oxygen and macro-and micro-nutrients from the mother to the fetus (Zhang et al., 2021). Under such conditions, a hemodynamic adaptation occurs with the blood flow redistribution, benefiting cerebral blood flow at the expense of fetal liver circulation. This adaptation is favorable to fetal survival under an
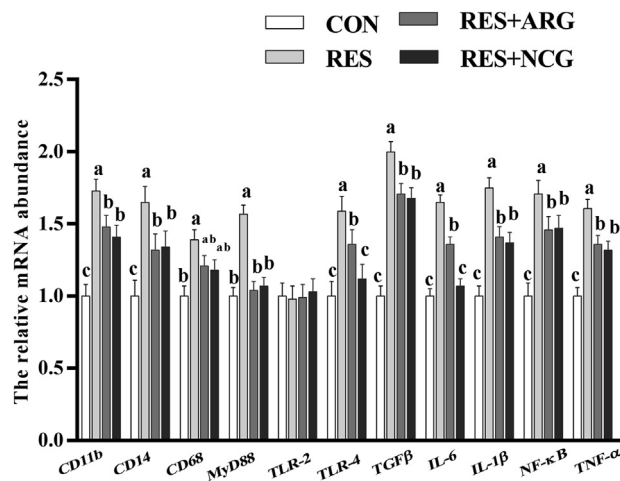
adverse intrauterine environment, but it inevitably impairs the liver function and compromises fetal growth (Cianfarani et al., 2012). In our study, gestational feed restriction suppressed liver development and decreased the plasma contents of AKP and increased the acute phase protein SAA in the ovine fetus. Our findings are consistent with previous research which suggested that undernutrition of late pregnant ewes led to fibrosis, dysfunction and antioxidant imbalance in the fetal liver (Liu et al., 2018). Generally, AKP exerts a vital role during skeleton development and liver metabolism. Besides, the positive acute-phase protein SAA is suggested to modulate the host immune response (Gruys et al., 2005). The fetal liver is a primary organ for hematopoiesis in the process of embryonic development, which has additional metabolic effects during the perinatal or late gestational periods (Kamiya et al., 1999). Tests for liver function have been recognized as effective tools to evaluate liver dysfunction, such as tests for albumin, bilirubin, AKP, AST and ALT (Ogunkeye and Roluga, 2006). These tests express various liver functions, such as protein synthesis, integrity and the formation of the hepatic cell (transaminases) as well as free bile flow (AKP and bilirubin) (Limdi and Hyde, 2003). The results in the current work (Table 3) suggested that dietary NCG or RP-Arg supplementation improved fetal liver functions in the ovine IUGR model, leading to an increase in hepatocellular integrity. The liver has multiple important physiological functions during embryonic development and after birth, including hematopoiesis, metabolism and immunity (Chen et al., 2019). The liver produces proteins, lipids and carbohydrates, and is involved in their metabolism (Seifer and Englard, 1994). Besides, the liver synthesizes insulin-like growth factor I (IGF-I), IGF-II, IGF binding proteins and other growth factors (Sjögren et al., 1999). Therefore, improved liver function can further promote fetal growth.

The DNA content was selected to be the hyperplasia indicator, whereas the RNA to DNA and protein to DNA ratios were selected to be the protein synthesis activity and hypertrophy indicators, respectively (Scheaffer et al., 2004). It was shown that the liver tissue of fetuses born to the control ewes had a greater cell size, as
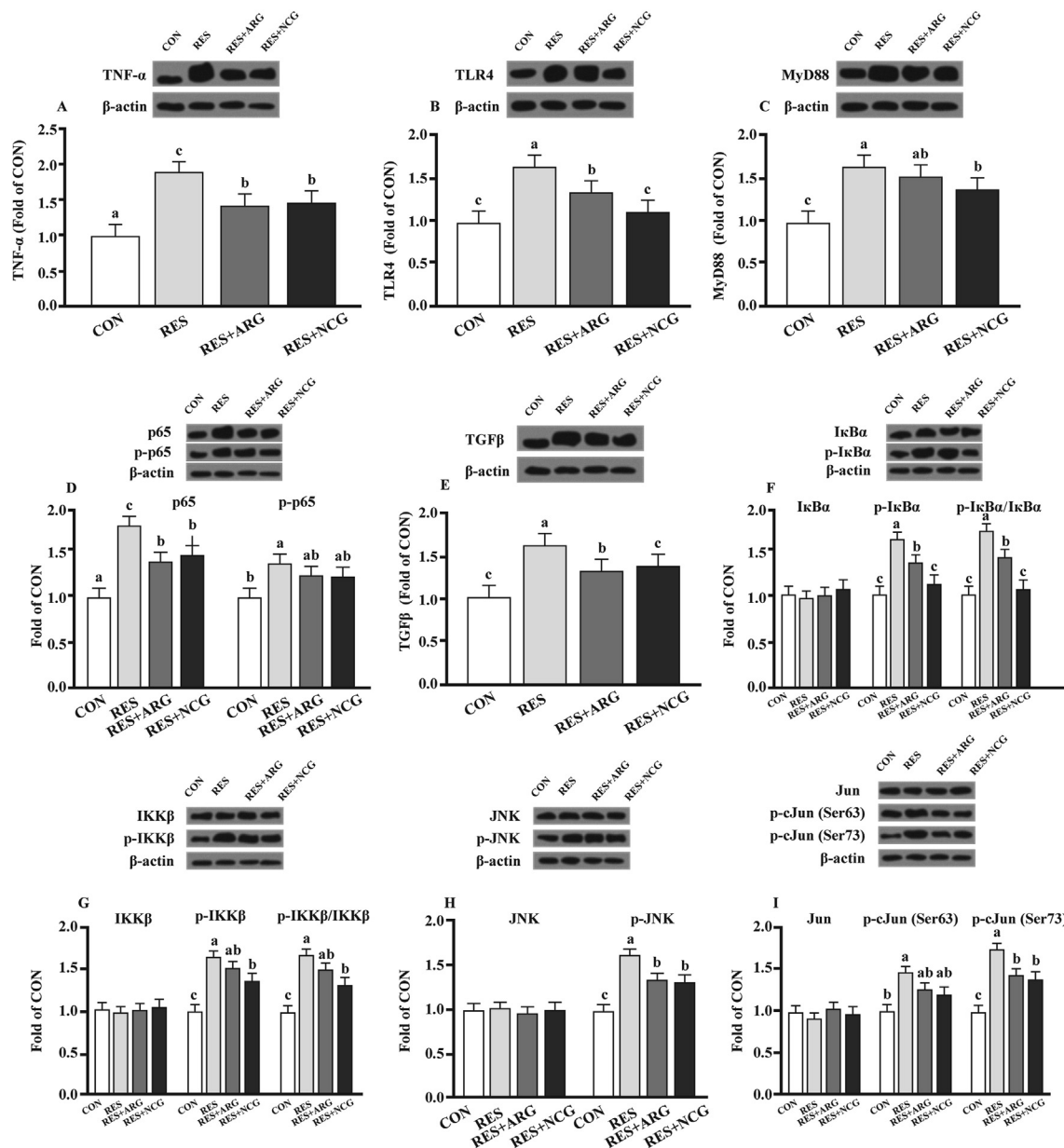
Fig. 2. Effects of dietary *N*-carbamylglutamate (NCG) and rumen-protected L-arginine (RP-Arg) supplementation on the immune function related protein expression of the fetal liver in underfed Hu ewes on d 110 of gestation. (A) TNF-α; (B) TLR4; (C) MyD88; (D) p65 and p-p65; (E) TGFβ; (F) IκBα and p-IκBα; (G) IKKβ and p-IKKβ; (H) JNK and p-JNK; (I) Jun, p-c-Jun (Ser63), and p-c-Jun (Ser73) were determined. The protein expression value is calculated as the densitometry unit of selected protein divided by the densitometry unit of β-actin detected by Western blotting. Values are means with standard errors represented by vertical bars (n = 16 per group). CON: ewes fed 100% of NRC (1985) recommendations for pregnancy; RES: ewes fed 50% of NRC (1985) recommendations for pregnancy; RESA: RES ewes supplemented RP-Arg at 20 g per day; RESN: RES ewes supplemented NCG at 5 g per day. [a, b, c] Columns without a common letter differ ($P < 0.05$). TNF-α = tumor necrosis factor α; TLR = toll-like receptor; NF-κB = nuclear factor kappa B (p65); MyD88 = myeloid differentiation factor 88; TGFβ = Transforming growth factor β; IκB = inhibitor of NF-κB; IKK = IκB kinase; JNK = c-Jun N-terminal kinase.

indicated by a greater protein: DNA ratio, compared to those born to underfed ewes (Gao et al., 2009), which confirms our hypothesis. In our study, both protein level and the protein: DNA ratio was increased in the liver of fetuses born to ewes receiving RP-Arg or NCG supplementation compared to in those born to the RES ewes. Thus, the NCG- or RP-Arg-induced increase in the hepatic cell size might be attributed to the increased protein concentration and the protein to DNA ratio.

Lipids are very important for the metabolism and physiological function of animals' growth and development, especially for fetuses, which play a very important role in embryonic development (Woollett, 2001). Fetal lipid metabolism can be summarized as

follows: fatty acids are transferred to the fetus through the placenta, released into the fetal blood and taken up by the fetal liver, esterified and released back into the fetal circulation as triglycerides. In this study, we reported that FFA, cholesterol, BHB and triglycerides were elevated in the plasma of fetuses born to the RES ewes compared to those born to the CON ones. This change might be due to the enhanced placental uptake of fatty acids, which could have resulted from higher lipid content in maternal circulation (due to feeding restriction which predisposes to lipid mobilization) and/or higher efficiency of cross-placental fatty acid transport (Jones et al., 2017). Our findings suggested that FFA, cholesterol, triglycerides, and BHB in fetal plasma were up-regulated in the IURG

group in comparison to the CON group. However, the greater accumulation of FFA and BHB in the blood triggers a series of metabolic diseases in animals (Smith et al., 1997), which may affect the development of the fetal liver and the health of postnatal lambs. In the present study, total cholesterol, triglycerides, hepatic lipase and lipoprotein lipase were lower in the fetal liver of the RES group compared to the CON group, which also showed that the capacity of fat synthesis and storage is reduced in the fetal liver. NCG or RP-Arg treatment decreased the fetal plasma levels of BHB, total FFA, cholesterol, and triglyceride, and increased the levels of lipoprotein lipase, total cholesterol and triglycerides in the fetal liver, indicating that NCG or RP-Arg administration was beneficial for lipid metabolism in the IUGR ovine fetus.

Ewes experiencing a nutrient deficiency have no nutrient reserve due to the gradually lost body condition (Russel et al., 1969), and thus the levels of FFA in circulation are elevated as the gestation advanced. The FFA mobilization potentially results in inflammation in the underfed ewes, since the continuously increased FFA levels can trigger the innate immune system via the TLR (Li et al., 2020), which represent the transmembrane receptors with specific responses to danger-related molecular patterns or the pathogens. In mammals, TLR play roles as the pattern-recognition receptors, which exert vital parts in recognizing the microbial components (Akira et al., 2001). TLR4 is the lipopolysaccharide (LPS) receptor, while TLR2 is the lipoprotein receptor within the cellular membranes in bacteria (Reyna et al., 2008). When LPS binds to TLR4 and its co-receptor, CD14 (cluster of differentiation 14), the adaptor protein MyD88 (myeloid differentiation factor-88) is recruited to the TLR4 receptor, leading to autophosphorylation of IL-1R-associated kinase (IRAK) and activation of NF-κB signaling (Reyna et al., 2008). FFA are the TLR4 ligands, which link the TLR4 with inflammation. In this study, the TLR4 in the fetal liver was increased in the RES group in comparison with the CON group (Schaeffler et al., 2009). Additionally, the increased FFA levels were also found in fetal blood, which was primarily produced by maternal FFA that traveled to the maternal placenta, incorporated into the entire fetal circulation, and transported to the fetal placenta (Jones et al., 2017). The elevated activation of TLR4 and the FFA levels potentially activates the inflammatory signaling. The JNK and NF-κB signaling pathways in the fetal liver were up-regulated in the RES group, which is supported by the above mechanism. Equils et al. (2005) found that a 50% restriction of daily feed intake for 10 gestational d (from d 11 to 21 of gestation) improved the mRNA levels of TNF-α and IL-1β in rat offspring, which have previously been shown to modulate TLR gene expression (Equils et al., 2005; Alvesrosa et al., 2010). Maternal feed intake restriction affected liver protein synthesis in early fetal goats, and up-regulated the proteins and genes participating in the MyD88-dependent signal transduction pathways, enhancing the target cytokine levels (Chen et al., 2019). These findings conform to our results which reported that maternal undernutrition activated the inflammation signal transduction pathways in the fetal liver. Additionally, macrophage marker expression including CD68, CD4 and CD11b were enhanced in the fetal liver of the RES group, which conformed to the upregulation of inflammation signal transduction pathways.

Amino acids (AA) are required for the synthesis of a variety of specific proteins (including cytokines and antibodies) and regulate key metabolic pathways of the immune response to infectious pathogens (Li et al., 2007). In ovine, a 50% nutritional restriction between d 28 and 78 of gestation reduced concentrations of total α-amino acids (particularly serine, arginine-family amino acids, and branched-chain AA, BCAA) in the fetal plasma (Kwon et al., 2004). Maternal RP-Arg or NCG supplementation to nutrient-restricted
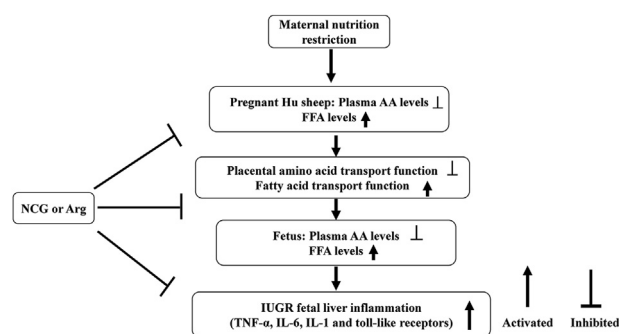

Fig. 3. The possible mechanism of L-arginine (Arg) or N-carbamylglutamate (NCG) on the regulation of the liver inflammation in intrauterine growth retardation (IUGR) Hu fetus. AA = amino acids; FFA = free fatty acid; IL = interleukin; TNF-α = tumor necrosis factor α.

ewes has been shown to improve the concentrations of AA (particularly arginine-family AA and BCAA) and polyamines in maternal and fetal plasma and fetal allantoic and amniotic fluids (Zhang et al., 2016a). In the present study, the results suggested that NCG or RP-Arg treatment resulted in lowering the p-c-Jun (Ser73), TNF-α, p-JNK, TLR-4, MyD88, p65, p-IκBα, and p-IKKβprotein levels in fetal liver, at least in part, by increasing AA concentrations and decreasing FFA, cholesterol, triglycerides, and BHB levels in fetal plasma, which showed that NCG or RP-Arg possesses potentially protective effects in IUGR fetal hepatic inflammatory responses in undernourished ewes (Fig. 3). TGFβ modulates naive T-cell differentiation by promoting the differentiation of T-helper 17 and inducing fibrogenesis (Monteleone et al., 2008). In our results, the mRNA and protein levels of TGFβ were up-regulated in the fetal liver of the RES group, which combined with the up-regulated IL6, further caused inflammation. Moreover, our findings indicated that NCG or RP-Arg treatment resulted in a lowering of the TGFβ levels of the fetal liver in the RES group. These findings demonstrated that NCG or RP-Arg decreased fetal hepatic inflammation by decreasing the TGFβ expression.

5. Conclusion

Our findings showed that the dietary RP-Arg or NCG supplementation could protect against the IUGR fetal hepatic inflammation in under-nourished pregnant ewes via improving fetal lipid metabolism, down-regulating TLR4 and the inflammatory JNK and NF-κB signaling pathways and reducing the cytokine production.

Author contributions

Hao Zhang and **Hongrong Wang** designed the research; **Ying Zhang** and **Yi Ma** conducted the research; **Yi Ma** and **Hao Zhang** analyzed the data; **Hao Zhang** wrote the paper; **Mabrouk Elsabagh** and **Mengzhi Wang** revised the manuscript; **Hao Zhang, Mengzhi Wang** and **Hongrong Wang** had primary responsibility for the final content. All authors read and approved the final manuscript.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgments

Appendix

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