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Original Research Article

Dietary *N*-carbamylglutamate and L-arginine supplementation improves redox status and suppresses apoptosis in the colon of intrauterine growth-retarded suckling lambs

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ABSTRACT

Previous studies have revealed that dietary *N*-carbamylglutamate (NCG) or L-arginine (Arg) improves small intestinal integrity and immune function in suckling Hu lambs that have experienced intrauterine growth retardation (IUGR). Whether these nutrients alter redox status and apoptosis in the colon of IUGR lambs is still unknown. This study, therefore, aimed at investigating whether dietary supplementation of Arg or NCG alters colonic redox status, apoptosis and endoplasmic reticulum (ER) stress and the underlying mechanism of these alterations in IUGR suckling Hu lambs. Forty-eight 7-d old Hu lambs, including 12 with normal birth weight (4.25 ± 0.14 kg) and 36 with IUGR (3.01 ± 0.12 kg), were assigned to 4 treatment groups (n = 12 each; 6 males and 6 females) for 3 weeks. The treatment groups were control (CON), IUGR, IUGR + Arg and IUGR + NCG. Relative to IUGR lambs, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) content, as well as proliferation index, were higher ($P < 0.05$) whereas reactive oxygen species (ROS), malondialdehyde (MDA) levels and apoptotic cell numbers were lower ($P < 0.05$) in colonic tissue for both IUGR + Arg and NCG lambs. Both mRNA and protein levels of C/EBP homologous protein 10 (CHOP10), B-cell lymphoma/leukaemia 2 (Bcl-2) -associated X protein (Bax), apoptosis antigen 1 (Fas), activating transcription factor 6 (ATF6), caspase 3, and glucose-regulated protein 78 (GRP78) were lower ($P < 0.05$) while glutathione peroxidase 1 (GPx1), Bcl-2 and catalase (CAT) levels were higher ($P < 0.05$) in colonic tissue for IUGR + Arg and IUGR + NCG lambs compared with IUGR lambs. Based on our results, dietary NCG or Arg supplementation can improve colonic redox status and suppress apoptosis via death receptor-dependent, mitochondrial and ER stress pathways in IUGR suckling lambs.

1. Introduction

Proper maternal nutrition and placental sufficiency are crucial for optimal fetal development and growth. Maternal malnutrition during pregnancy and/or placental dysfunction predispose the fetus to a developmental defect known as intrauterine growth retardation (IUGR) i.e., birth weight below the 10th percentile of

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the same gestational age (Desir-Vigne et al., 2018). It has been indicated that IUGR may be associated with the development of gastrointestinal diseases such as irritable bowel syndrome and inflammatory bowel disease (IBD) (Khalili et al., 2013). Also, it has been demonstrated that the permeability of the colon is enhanced by IUGR, which compromises cell growth and differentiation and may cause cell death (Fanca-Berthon et al., 2009; Le Dréan et al., 2014). Colonocytes isolated from IUGR adult rats revealed that the expression of the endoplasmic reticulum (ER) and mitochondrial genes (especially the ER stress markers) was altered in response to a high-lipid diet (Segain et al., 2015), suggesting a sustained effect of IUGR at adulthood. An association of ER stress with the pathophysiology of IUGR was demonstrated in various animal models of IUGR, with enduring effects in a few adult organs (Vo and Hardy, 2012).

As a folding and synthesizing site for membrane and secretory proteins, the ER is considerably vulnerable to oxidative stress which lowers its protein-folding capacity (Szegezdi et al., 2006). With the onset of ER stress, unfolded or misfolded proteins are deposited within the ER lumen causing cellular dysfunction and, in prolonged ER stress, apoptosis (Urra et al., 2013; Wang et al., 2019). To counteract the harmful consequences of ER stress, organisms have evolved diverse mechanisms. There are 3 ER transmembrane receptors involved in mediating these mechanisms and they are collectively known as the unfolded protein response (UPR): the inositol-requiring enzyme 1 (IRE1), the PKR-like ER kinase (PERK) and the activating transcription factor 6 (ATF6) (Walter and Ron, 2011). The UPR work could reduce unfolded protein deposition and recover ER functionality (Schroder and Kaufman, 2005).

Oxidative stress plays a complex pathophysiological role in IUGR-related intestinal damage and is regarded as an inducer of the development and progression of intestinal dysfunction (Ozsurekci and Aykac, 2016). With the onset of birth, fetuses experience an abrupt shift from a low-oxygen intrauterine environment (low free radical levels) to an oxygen-rich extrauterine environment, which leads to substantial production of reactive oxygen species (ROS) and evokes oxidative stress in various organs including the intestine (Yin et al., 2013). The foregoing process occurs when the mechanisms for intestinal antioxidant capacity remain underdeveloped, especially among IUGR newborns, such that the overproduction of ROS and consequent oxidative injury cannot be resolved (Yin et al., 2013). Hence, one effective strategy for counteracting IUGR-related dysfunction may be enhancing the intestinal antioxidant systems through the supply of antioxidants.

Currently, great attention is being paid to investigating the role of L-arginine (Arg) on gut mucosal physiology. *N*-carbamylglutamate (NCG), a metabolically-stable analogue of *N*-acetylglutamate synthase (NAG), promotes endogenous production of Arg via activating intestinal carbamylphosphate synthase-1 and pyrroline-5-carboxylate synthase (Li et al., 2022). The role of Arg in facilitating mitochondrial function and biogenesis via the nitric oxide (NO) axis is well-known, where NO is a major signalling molecule that participates in intracellular redox regulation (Yin et al., 2014). Despite a growing body of knowledge on the role of Arg in preventing the development of various metabolic disorders in neonates, little information is available on how Arg and NCG affect colonic redox status, apoptosis and ER stress in IUGR suckling lambs.

Thus, the current work aimed to study how dietary Arg or NCG supplementation affects colonic redox status, apoptosis and ER stress in IUGR suckling Hu lambs. We hypothesized that dietary Arg or NCG supplementation improves colonic redox status and suppresses apoptosis. Accordingly, the data theoretically support the use of Arg or NCG as functional dietary or formula constituents for IUGR neonates.

2. Materials and methods

All experimental procedures were carried out following animal protection laws as well as the Guide for the Care and Use of Laboratory Animals issued by the Ethics Committee of Yangzhou University (SXXY 2015-0054).

2.1. Milk replacer (MR) diets

The nutrient composition of the MR diets is reported in Appendix Table 1 (Zhang et al., 2020). Isoenergetic and isonitrogenous MR diets were designed for all 4 treatments based on ovine feeding standards (NRC, 2007). Nitrogen content in diets was adjusted by L-alanine (Ala). *N*-carbamoylglutamate (purity of 97%) was purchased from Sigma-Aldrich (MO, USA) whereas Arg and Ala were products of Ajinomoto (Beijing, China).

2.2. Chemical analyses of MR

The MR samples were analyzed for ash, dry matter (DM), crude protein (CP), ether extract (EE), calcium (Ca) and total phosphorus (TP) (methods 942.05, 930.15, 990.02, 920.39, 968.08, and 965.17, respectively, AOAC 1990). Gross energy (GE) was measured using a bomb calorimeter (C200; IKA Works Inc., Staufen, Germany). The amino acid (AA) profile was measured by reverse-phase HPLC (HP1100; Agilent) using norleucine as the internal standard according to published methods (Bidlingmeyer et al., 1984).

2.3. Animal treatments

Suckling lambs with a birth weight of at least 1.5 standard deviations (SD) below the average were defined as IUGR and those with birth weight within 0.5 SD were classed as normal birth weight (NBW) (Zhang et al., 2020). At 7 d old, 48 newborn Hu lambs, of which 36 were IUGR (3.01 ± 0.12 kg) and 12 NBW (4.25 ± 0.14 kg) were selected out of 432 twin lambs born to Hu sheep at the Jiangyan Experimental Station (Taizhou, Jiangsu, China). The lambs were weaned at 7 d of age, and based on initial BW were then randomly divided into 4 treatment groups ($n = 12$ per group with 3 replicates each, 2 males and 2 females): control (CON, lambs with NBW of 4.25 ± 0.14 kg and fed MR), IUGR (IUGR lambs with BW of 3.01 ± 0.12 kg and fed MR), IUGR + Arg (IUGR lambs with BW of 2.99 ± 0.13 kg and fed MR plus 1% Arg) and IUGR + NCG (IUGR lambs with BW of 3.03 ± 0.11 kg and fed MR plus 0.1%NCG) (Zhang et al., 2020). The dietary doses of Arg and NCG (1 and 0.1% of DM, respectively) were adopted from previous studies in mice (Cao et al., 2016) and pigs (Yang et al., 2013).

Lambs were housed in a 4 m × 1 m indoor pen in each replicate from d 7 to 28 after birth. Lambs had free access to clean fresh water and were fed MR (as dry matter) at a rate of 10% of their live weight and the daily amount was adjusted at 10-d intervals. Lambs were fed the MR diets separately at 07:00, 13:00 and 19:00 each day over a 21-d period. Before feeding, 40 °C water was used to dissolve the MR and other additives to give solutions with a DM content of 16.67%, which were then bottled and fed to lambs by skilled farm staff. This way, confounding variables like daily handling and management were eliminated. Daily MR intake was measured for each lamb; later, DM level within MR was multiplied by average daily MR intake to calculate average daily DM intake (ADMI). The lambs were weighed for initial (d 7 of age) and final (d 28 of age) BW. The results of growth performance have been reported in our previous study (Zhang et al., 2020).

2.4. Sample collection

At the end of the experiment, lambs were euthanized by intravenous injection of sodium pentobarbital (15 mg/kg BW). The entire small intestine was harvested and the colon was carefully separated as previously described (Zhang et al., 2018). Then, each colon segment was cleaned, rinsed with saline and subsequently arranged on an ice cold surface. The colonic mucosa was gently scraped with a glass slide and the scraped contents were put in Eppendorf tubes then snap-frozen in liquid nitrogen before being preserved at −80 °C until analysis. The colon subsamples were fixed in 0.1 mol/L paraformaldehyde (pH 7.4) followed by paraffin embedding.

2.5. Colonic levels of insulin, insulin-like growth factor 1 (IGF-1), protein, nitric oxide (NO), and nitric oxide synthase (NOS)

Ovine-specific ELISA microplate kits (Mercodia, Guangzhou, China) were used to analyze IGF-1 and insulin content. In addition, commercially available kits (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) were used to analyze NOS and NO contents following specific protocols (Liu et al., 2009). Protein content was determined using the bicinchoninic acid (BCA) protein detection kit (Pierce, Rockford, IL, USA).

2.6. Intestinal mitochondrial isolation and mitochondrion ROS production measurement

Mitochondria were isolated from fresh colon samples using mitochondrial isolation kits (Beyotime Institute of Biotechnology, China) and following previously described procedures (Cao et al., 2018, 2019). The colonic mucosa was homogenized in MSH buffer (10 mmol/L HEPES, pH 7.5, containing 200 mmol/L mannitol, 70 mmol/L sucrose, 1.0 mmol/L egtazic acid and 2.0 mg/mL serum albumin) followed by centrifuging the homogenate at 1,000 × *g* for 10 min at 4 °C. The supernatant was collected and further centrifuged at 3,500 × *g* for 10 min at 4 °C to harvest the mitochondrial pellet (Pintaa et al., 2014).

Mitochondrion ROS production measurements were performed after treating the intestinal mitochondria with 2′,7′-dichlorohydro fluorescein diacetate (2 μmol/L), and the samples were subjected to a 20-min incubation at 24 °C, followed by a microplate reader-assisted measurement of fluorescence intensity as previously reported (Pipatpiboon et al., 2012; Cao et al., 2018).

2.7. Analysis of antioxidant status in colonic tissue

Approximately 300 mg of colonic mucosal sample (frozen) was rinsed in saline (1:9, wt/vol), and homogenized with a fast benchtop homogenizer (Tekmar, OH, USA). The mucosal homogenate was centrifuged at 15,000 × *g* for 10 min at 4 °C. Then, the supernatant was obtained and used for determining superoxide dismutase (SOD), reduced glutathione (GSH), hydrogen peroxide (H_2O_2), glutathione peroxidase (GSH-Px), glutathione reductase (GR), oxidized glutathione (GSSG), total antioxidant capacity (T-AOC), protein carbonyl and malondialdehyde (MDA) using colourimetric kits (Nanjing Jiancheng Bioengineering Institute). For inter-sample contrasts, the data for each sample were normalized to the protein concentration.

2.8. Caspase 3, caspase 8 and caspase 9 activities and mitochondrial cytochrome C assay in the colon

Caspase 3 (KGA203), caspase 8 (KGA303) and caspase 9 (KGA403) were assayed using colourimetric kits (Keygen Biotech, Nanjing, China). Approximately 0.1 g of colonic tissue was homogenized and lysed in cold buffer (50 mL), and then the cellular lysates were centrifuged at 10,000×*g* for 5 min at 4 °C. Subsequently, supernatant aliquots (50 mL) were collected for analysis as reported previously (Liu et al., 2017).

Colonic homogenate (10%) was centrifuged (at 2,000 × *g*) for 10 min, and then supernatants were centrifuged (at 10,000 × *g*) for 15 min. After resuspending the pellets obtained, lysis proceeded with 1.5 mL of cold buffer to assess cytochrome C colourimetrically using a WFJ 2100 Nanodrop (UNIC Instrument, Shanghai, China). A calibration curve was established with the use of bovine cytochrome C as previously described (Liu et al., 2017).

2.9. Colonic DNA content and proliferation index

Following homogenization of 0.5 g of the frozen colon in a pH 7.4 buffer (20 mL) comprising Na_3PO_4 (0.05 mol/L), NaCl (2.0 mol/L) and EDTA (0.002 mol/L), the supernatants were harvested for DNA level determination (in duplicate) with the Hoechst 33,258 (1 μg/mL; Sigma-Aldrich, B2338) using the DNA Type I derived from bovine liver as a reference (Sambrook and Russell, 2001).

Fresh portions of the colon were washed with pH 7.4 PBS, crushed, and then filtered through a 300 mesh (stainless steel). Propidium iodide (PI) staining buffer involving propidium iodide (0.5%), TritonX-100 (0.25%) and Rnase (10 mg/mL; 4ABIO, Beijing, China) was utilized for examining the colonic proliferation index. CellQuest software (Becton Dickinson) was used for data analysis. The proliferation index was recorded as the proportions of S, G2 and M phase cells in varying cellular cycle phases (Liu et al., 2017).

2.10. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of the colon in histologic sections

After deparaffinization, 4 mm thick colonic sections were subjected to a 40-min treatment with proteinase K (50 mg/mL; Keygen Biotech, Nanjing, China) at 37 °C. After rinsing in PBS for 3 times, the DNA fragments in the apoptotic cells were used for the TUNEL assay according to the manufacturer's protocols (KGA7032, KeyGEN Biotech, Nanjing, China). The initial step involved incubation of sections with terminal deoxynucleotidyl transferase (TdT) solution, which was a working-strength mixture of digoxigenin-deoxyuridine triphosphate (dUTP) and TdT stirred for 60 min at 37 °C. Next, slides were subjected to rinsing in PBS for 3 times, incubated using streptavidin-horseradish peroxidase (HRP) for 30 min at 37 °C, and another rinsing in PBS for 3 times. After identifying TUNEL positive cells using diaminobenzidine, they were stained again with hematoxylin. In every case, both positive and negative controls were included. Standard microscopy was employed for the observation of each specimen, and the overall counts of TUNEL-positive cells were recorded in 10 random HPF (high power fields) under 400× magnification by 2 blinded observers. Data are presented in terms of TUNEL-positive cell counts per field (Liu et al., 2017).

2.11. Total RNA extraction and qRT-PCR assay

Total RNA was separated from colonic mucosa using RNAiso Plus Reagent (TaKaRa Biotechnology, Dalian, China). The NanoDrop 1000 (Thermo Fischer Scientific, Waltham, USA) was utilized to assess the mass and concentration of isolated RNA and the integrity of RNA was evaluated via agarose gel electrophoresis (2.0%). Next, an RT Master Mix kit (PrimeScript) from TaKaRa Biotechnology was used for generating complementary DNA. A Premix Ex Taq kit (TB Green) from the same company was used to assay β-actin and mRNA expression levels, which was accomplished by qRT-PCR

Table 1
Effect of dietary Arg or NCG supplementation on protein, insulin, IGF-1 and NO levels and activity of NOS in the colon of IUGR suckling lambs.[1]

Item	Groups[2]				SEM	*P*-value
	CON	IUGR	IUGR + Arg	IUGR + NCG		
Protein, mg/g wet wt	28.2[a]	15.9[c]	22.4[b]	21.1[b]	2.11	0.008
Insulin, pmol/g protein	4.99[a]	3.04[c]	4.01[b]	3.93[b]	0.212	0.023
IGF-1, pmol/g protein	200[a]	132[c]	159[b]	163[b]	9.9	0.007
NO, μmol/g protein	2.89[a]	1.14[c]	1.92[b]	2.03[b]	0.361	0.014
tNOS, U/g protein	699[a]	513[c]	602[b]	607[b]	19.3	0.039
iNOS, U/g protein	402[a]	323[c]	371[b]	368[b]	11.8	0.009
cNOS, U/g protein	297[a]	190[c]	231[b]	239[b]	6.9	0.010

Arg = L-arginine; NCG = *N*-carbamylglutamate; IGF-1 = insulin-like growth factor 1; NO = nitric oxide; NOS = NO synthase; IUGR = intrauterine growth retardation; iNOS = inducible NOS; tNOS = total NOS; cNOS = constitutive NOS.
[a,b,c] Within a row, mean values without a common letter differ significantly ($P < 0.05$).
[1] Mean values with SEM ($n = 12$ per group).
[2] CON: the normal birth weight group given a control diet; IUGR: the intrauterine-growth-retarded group given a control diet; IUGR + Arg: IUGR supplemented with 1% L-arginine; IUGR + NCG: IUGR supplemented with 0.1% *N*-carbamylglutamate.

using a QuantStudio 5 Real-time PCR System (Applied Biosystems, CA, USA). Target genes were estimated for relative expression levels via the $2^{-\Delta\Delta Ct}$ approach, followed by normalization against β-actin, the reference gene (Zhang et al., 2018). Appendix Table 2 details the entire qRT-PCR primers used.

2.12. Western blotting

A radio-immunoprecipitation assay buffer containing a protease inhibitor cocktail (Beyotime Institute of Biotechnology, Jiangsu, China) was used to separate total protein from colonic mucosa. Concentrations were then assayed with the BCA protein kit. Resolution of protein aliquots was via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel followed by transfer onto Polyvinylidene fluoride (PVDF) membranes. This was followed by 1 h membrane incubation using blocking buffer (5% bovine albumin in 1% TBST involving Tris-buffered saline) at ambient temperature and a subsequent overnight probing using primary antibody at 4 °C. Afterwards, the membranes were washed in 0.05% TBST saline buffer with Tris and a subsequent 1 h incubation with an appropriate HRP–labelled anti-rabbit goat IgG secondary antibody (1:5,000, ProteinTech, Chicago, USA) at ambient temperature. Following relative expression normalization of target proteins against β-actin (internal protein), a Multi-Imaging System (Tanon 5200; Tanon Science & Technology, Shanghai, China) was utilized to visualize the target bands and Image-Pro Plus 6.0 was employed for their analysis.

2.13. Statistical analyses

Results are presented as means plus pooled standard errors of the mean (SEM) and were processed statistically via the SPSS 16.0 (Chicago, IL, USA). The fixed effect of lamb sex was initially included in the statistical model but was removed later from the final model because of the non-significant effect ($P > 0.05$) and thus, the treatment was the sole fixed effect. For determination of inter-treatment statistical disparities, one-way ANOVA was employed while multiple comparisons were accomplished by Tukey's post hoc test. $P < 0.05$ was used for statistical significance.

3. Results

3.1. Protein content, insulin, IGF-1, NO levels and NOS activity in the colon

The tNOS, iNOS and cNOS activities and the NO, IGF-1, protein and insulin levels were significantly reduced ($P < 0.05$) in the colon of IUGR lambs compared to the CON group (Table 1). The IUGR

Table 2
Effect of dietary Arg or NCG supplementation on oxidative status and mitochondrial ROS production in the colon of IUGR suckling lambs.[1]

Item	Groups[2]				SEM	*P*-value
	CON	IUGR	IUGR + Arg	IUGR + NCG		
ROS production, fold change	1.00[c]	2.29[a]	1.58[b]	1.63[b]	0.112	0.008
T-AOC, U/mg protein	1.99[a]	1.21[c]	1.57[b]	1.51[b]	0.141	0.023
MDA, nmol/mg protein	0.35[c]	0.52[a]	0.47[b]	0.44[b]	0.038	0.009
GSH-Px, U/mg protein	15.3[a]	7.37[c]	10.2[b]	11.0[b]	1.03	0.012
SOD, U/mg protein	111[a]	57.9[c]	84.3[b]	109[a]	5.39	0.005
Protein carbonyl, nmol/mg protein	1.87[c]	2.76[a]	2.21[b]	2.19[b]	0.132	0.017
GR, U/g protein	4.13[c]	6.13[a]	4.98[b]	5.02[b]	0.356	0.028
GSH, nmol/mg protein	1.58[a]	0.81[c]	1.14[b]	1.19[b]	0.102	0.005
GSSG, nmol/mg protein	0.10	0.12	0.11	0.13	0.031	0.089
GSH:GSSG ratio	15.8[a]	6.75[c]	10.4[b]	9.16[b]	1.149	0.008
H_2O_2, mmol/g of protein	11.2[c]	18.3[a]	14.4[b]	14.6[b]	1.67	0.016

Arg = L-arginine; NCG = *N*-carbamylglutamate; ROS = reactive oxygen species; IUGR = intrauterine growth retardation; T-AOC = total antioxidant capacity; MDA = malondialdehyde; GSH-Px = glutathione peroxidase; SOD = superoxide dismutase; GR = glutathione reductase; GSH = reduced glutathione; GSSG = oxidized glutathione; H_2O_2 = hydrogen peroxide.
[a,b,c] Within a row, mean values without a common letter differ significantly ($P < 0.05$).
[1] Mean values with SEM ($n = 12$ per group).
[2] CON: the normal birth weight group given a control diet; IUGR: the intrauterine-growth-retarded group given a control diet; IUGR + Arg: IUGR supplemented with 1% L-arginine; IUGR + NCG: IUGR supplemented with 0.1% *N*-carbamylglutamate.

Table 3
Effect of dietary Arg or NCG supplementation on the caspase 3, caspase 8, caspase 9 activities, and the levels of cytochrome C in the colon of IUGR suckling lambs.[1]

Item	Groups[2]				SEM	*P*-value
	CON	IUGR	IUGR + Arg	IUGR + NCG		
Caspase 3, U/mg protein	6.15^{c}	11.8^{a}	9.03^{b}	8.94^{b}	0.472	0.009
Caspase 8, U/mg protein	5.08^{c}	10.9^{a}	7.79^{b}	7.84^{b}	0.691	0.023
Caspase 9, U/mg protein	4.67^{b}	6.08^{a}	5.67^{ab}	5.53^{ab}	0.376	0.031
Cytochrome C, mmol/L	0.21^{c}	0.57^{a}	0.39^{b}	0.31^{b}	0.083	0.006

Arg = L-arginine; NCG = *N*-carbamylglutamate; IUGR = intrauterine growth retardation.
^{a,b,c} Within a row, mean values without a common letter differ signficantly ($P < 0.05$).
[1] Mean values with SEM ($n = 12$ per group).
[2] CON: the normal birth weight group given a control diet; IUGR: the intrauterine-growth-retarded group given a control diet; IUGR + Arg: IUGR supplemented with 1% L-arginine; IUGR + NCG: IUGR supplemented with 0.1% *N*-carbamylglutamate.

lambs fed with NCG or Arg had greater ($P < 0.05$) NO, IGF-1, protein and insulin levels and activities of tNOS, iNOS and cNOS in their colon compared with the IUGR lambs, but there was no difference ($P > 0.05$) between NCG and Arg and their administration did not restore ($P < 0.05$) the profile of the mentioned parameters to that of CON group.

3.2. Mitochondrial ROS generation and oxidative status in the colon

Colonic ROS production and levels of MDA, GR, H_2O_2 and protein carbonyl were higher ($P < 0.05$) but SOD, GSH-Px, GSH and T-AOC activities and GSH:GSSG ratio were lower ($P < 0.05$) in IUGR lambs compared with CON lambs (Table 2). Relative to the IUGR lambs, colonic SOD, GSH-Px, T-AOC and GSH activities and GSH:GSSG ratio were higher ($P < 0.05$), whereas ROS production and levels of MDA, GR, H_2O_2 and protein carbonyl were lower ($P < 0.05$) in the IUGR + Arg and IUGR + NCG lambs. There was no difference ($P > 0.05$) between Arg and NCG on mitochondrial ROS generation and oxidative status in the colon nor they did not restore ($P < 0.05$) the oxidative status to the normality of the CON group except for SOD activity which was returned ($P > 0.05$) to the CON profile by NCG administration.

3.3. Caspase 3, caspase 8 and caspase 9 activities and cytochrome C levels in the colon

The colonic caspase 3, caspase 8 and caspase 9 activities and the cytochrome C levels were greater ($P < 0.05$) in IUGR lambs compared to the CON ones (Table 3). Caspase 3 and caspase 8 activities and cytochrome C content were lower ($P < 0.05$) in the colon of IUGR + NCG and IUGR + Arg groups relative to the IUGR group, but their levels did not return ($P < 0.05$) to normality of the CON group. The activity of caspase 9 did not change ($P > 0.05$) with dietary treatments compared with CON and IUGR groups.

3.4. DNA level, protein:DNA ratio, apoptotic cell count and proliferation index in the colon

Colonic DNA content, protein:DNA ratio and proliferation index were lower ($P < 0.05$) but apoptotic cells per HPF (400×) were higher ($P < 0.05$) in the IUGR group compared with the CON group (Table 4). Although dietary Arg or NCG administration improved the foregoing IUGR-impacted parameters, it failed to restore them to the levels in the CON group ($P < 0.05$). Moreover, there was no difference ($P > 0.05$) between Arg and NCG groups for the aforementioned measurements.

3.5. Colonic mRNA abundance

The IUGR lambs exhibited greater ($P < 0.05$) colonic mRNA levels of *ATF4*, *ATF6*, *Bax*, caspase 3, caspase 8, caspase 9, *CHOP10*, *Fas* and *GRP78* compared with the CON lambs (Table 5). The IUGR + Arg or NCG groups had lower ($P < 0.05$) colonic mRNA expression of the aforementioned genes compared with the IUGR group. Relative to the CON group, the IUGR group had lower ($P < 0.05$) colonic mRNA levels of *CAT*, *GPx1*, *iNOS*, *eNOS*, *Bcl-2* and *Fasl*. In comparison with the IUGR group, the Arg or NCG group exhibited greater ($P < 0.05$) colonic mRNA levels of the foregoing genes.

3.6. Colonic protein levels

The protein expression of CAT, GPx1, SOD2 and Bcl-2 was lower ($P < 0.05$), while that of Bax, Fas, caspase 3, CHOP10, GRP78 and ATF6 was higher ($P < 0.05$) in the colon of IUGR lambs compared to those of the CON lambs (Figs. 1–3). The protein expression of the foregoing genes was reversed ($P < 0.05$) upon the administration of Arg or NCG compared to the IUGR treatment but it did not return ($P < 0.05$) to the normal level except for the CHOP10 gene which was normalized ($P > 0.05$) by the NCG treatment (see Fig. 4).

Table 4
Effect of dietary Arg or NCG supplementation on the DNA contents, protein: DNA ratio, apoptotic cell numbers, and the proliferation index in the colon of IUGR suckling lambs.[1]

Item	Groups[2]				SEM	*P*-value
	CON	IUGR	IUGR + Arg	IUGR + NCG		
DNA contents, mg	1895^{a}	985^{c}	1315^{b}	1328^{b}	59.2	0.011
Protein:DNA ratio	9.54^{a}	5.36^{c}	7.04^{b}	7.13^{b}	0.641	0.008
Proliferation index, %	4.62^{a}	2.28^{c}	3.46^{b}	3.29^{b}	0.208	0.009
Apoptotic cell per HPF	198^{c}	279^{a}	243^{b}	239^{b}	8.7	0.019

Arg = L-arginine; NCG = *N*-carbamylglutamate; IUGR = intrauterine growth retardation; HPF = high-power field (400×).
^{a,b,c} Within a row, mean values without a common letter differ significantly ($P < 0.05$).
[1] Mean values with SEM ($n = 12$ per group).
[2] CON: the normal birth weight group given a control diet; IUGR: the intrauterine-growth-retarded group given a control diet; IUGR + Arg: IUGR supplemented with 1% L-arginine; IUGR + NCG: IUGR supplemented with 0.1% *N*-carbamylglutamate.

Table 5
Effects of dietary Arg or NCG supplementation on the mRNA abundance of genes in the colon of IUGR suckling lambs.[1]

Item	Groups[2]				SEM	*P*-value
	CON	IUGR	IUGR + Arg	IUGR + NCG		
Antioxidant-related genes						
CAT	1.00^{a}	0.57^{c}	0.73^{b}	0.93^{a}	0.061	0.007
GPx1	1.00^{a}	0.44^{c}	0.65^{b}	0.68^{b}	0.073	0.012
SOD2	1.00^{a}	0.72^{b}	0.87^{ab}	0.83^{ab}	0.087	0.026
NO-dependent pathway related genes						
iNOS	1.00^{a}	0.51^{c}	0.76^{b}	0.79^{b}	0.082	0.006
eNOS	1.00^{a}	0.46^{c}	0.69^{b}	0.72^{b}	0.058	0.018
Pro-apoptotic and anti-apoptotic related genes						
Bax	1.00^{c}	1.99^{a}	1.44^{b}	1.39^{b}	0.132	0.005
Bcl-2	1.00^{a}	0.53^{c}	0.71^{b}	0.93^{a}	0.081	0.008
Fas	1.00^{c}	2.35^{a}	1.58^{b}	1.63^{b}	0.112	0.009
Fasl	1.00^{a}	0.42^{c}	0.69^{b}	0.72^{b}	0.146	0.012
Caspase 3	1.00^{c}	1.79^{a}	1.35^{b}	1.38^{b}	0.094	0.007
Caspase 8	1.00^{c}	2.14^{a}	1.52^{b}	1.12^{c}	0.121	0.014
Caspase 9	1.00^{c}	2.67^{a}	1.72^{b}	1.69^{b}	0.143	0.006
ER stress-related genes						
CHOP10	1.00^{c}	3.12^{a}	1.78^{b}	1.81^{b}	0.168	0.021
GRP78	1.00^{c}	2.97^{a}	1.58^{b}	1.62^{b}	0.129	0.006
ATF4	1.00^{c}	2.31^{a}	1.51^{b}	1.48^{b}	0.152	0.013
ATF6	1.00^{c}	2.03^{a}	1.45^{b}	1.12^{c}	0.104	0.009

Arg = L-arginine; NCG = *N*-carbamylglutamate; IUGR = intrauterine growth retardation; *CAT* = catalase; *GPx1* = glutathione peroxidase 1; *SOD2* = superoxide dismutase 2; *iNOS* = inducible nitric oxide synthase; *eNOS* = epithelial nitric oxide synthase; *Bax* = Bcl-2-associated X protein; *Bcl-2* = B-cell lymphoma/leukaemia 2; *Fas* = apoptosis antigen 1; *Fasl* = Fas ligand; ER = endoplamic reticulum; *CHOP10* = C/EBP homologous protein 10; *GRP78* = glucose-regulated protein 78; *ATF4* = activating transcription factor 4; *ATF6* = activating transcription factor 6.
[a,b,c] Within a row, mean values without a common letter differ significantly ($P < 0.05$).
[1] Mean values with SEM ($n = 12$ per group).
[2] CON: the normal birth weight group given a control diet; IUGR: the intrauterine-growth-retarded group given a control diet; IUGR + Arg: IUGR supplemented with 1% L-arginine; IUGR + NCG: IUGR supplemented with 0.1% *N*-carbamylglutamate.

4. Discussion

Arginine is a powerful stimulator of insulin secretion (Ragy and Ahmed, 2019). Hence, nutrient use efficiency can be improved in part by enhancing concentrations of anabolic hormones in the intestine and the ensuing increase in tissue protein synthesis (Yao et al., 2011). This function of Arg is indicated by the elevated colonic levels of insulin in the IUGR + Arg or NCG groups. NO has been universally acknowledged to be a signal molecule related to the host defence, immunomodulation, neurotransmission, as well as vascular homeostasis (Tan et al., 2010). It has been reported that Arg metabolism contributes – through a complex mechanism at the cellular, tissue and systemic levels – to NO synthesis through cNOS and iNOS (Tan et al., 2010). Thus, dietary supplementation with Arg is a possible prerequisite for sustaining health benefits associated with NO signalling (Tan et al., 2010). This point is supported by our results concerning the elevated levels of NO, iNOS, cNOS, tNOS and NO-dependent pathway-related genes (iNOS and eNOS) in the colon of IUGR suckling lambs receiving Arg or NCG compared to the untreated IUGR lambs.

Oxidation stress (OS) occurs upon an imbalance of oxidant–antioxidant status in part due to a reduction in activities of antioxidant enzymes and elevated levels of oxygen radicals. Antioxidant enzymes (CAT, GSH-Px and SOD) constitute the front line of the cellular defence against ROS (Ighodaro and Akinloye, 2018). Oxidative stress was reported as a crucial contributor to intestinal injury among IUGR offspring (Wang et al., 2010). Based on our findings, high colonic protein levels of carbonyl and MDA among non-supplemented suckling lambs with IUGR imply extensive lipid peroxidation or protein oxidation. Our findings are consistent with those reported in IUGR weanling piglets (Su et al., 2018). As a major ROS, H_2O_2 can diffuse across the cellular membrane, and at low levels can serve as a physiological signalling molecule within cells, resulting in proliferation and differentiation (Arakaki et al., 2013).

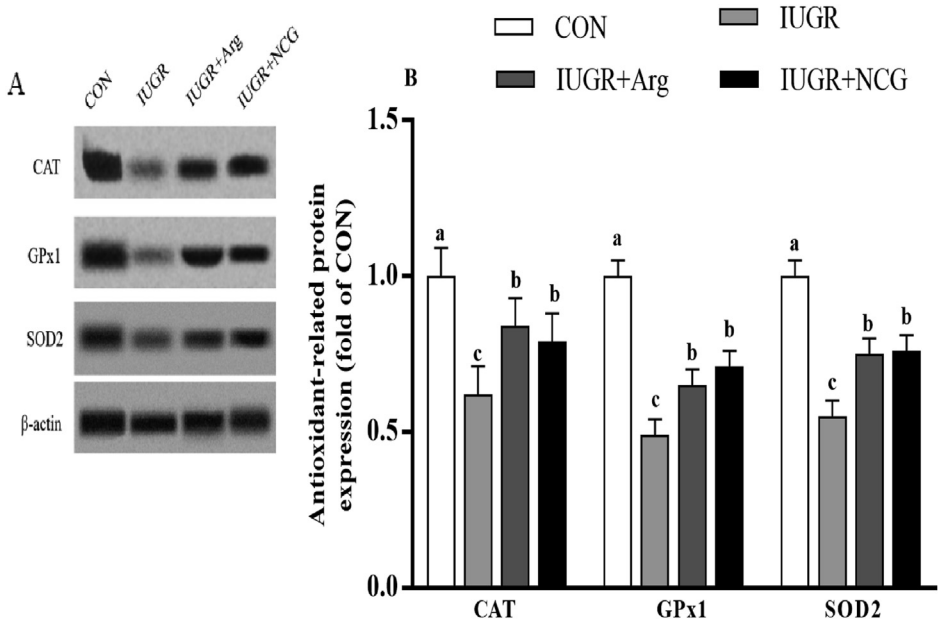

Fig. 1. Effects of dietary Arg or NCG supplementation on antioxidant related protein expression in the colon of IUGR suckling lambs. Representative charts of western blot results (A) and related protein expression of CAT, GPx1, and SOD2 (B) were obtained. Values are presented as mean ± SEM ($n = 12$). [a,b,c] Mean values in bars without a common letter differ significantly ($P < 0.05$). Arg = L-arginine; NCG = *N*-carbamylglutamate; IUGR = intrauterine growth retardation; CAT = catalase; GPx1 = glutathione peroxidase 1; SOD2 = superoxide dismutase 2. CON: the normal birth weight group given a control diet; IUGR: the intrauterine-growth-retarded group given a control diet; IUGR + Arg: IUGR supplemented with 1% L-arginine; IUGR + NCG: IUGR supplemented with 0.1% *N*-carbamylglutamate.

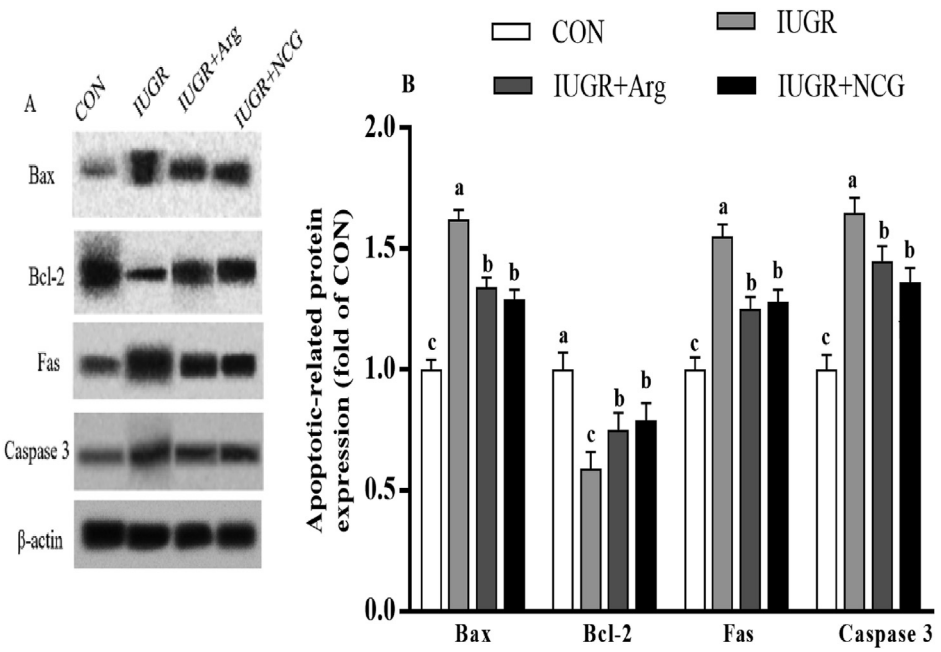

Fig. 2. Effects of dietary Arg or NCG supplementation on mitochondrial and death receptor-dependent pathways related protein expression in the colon of IUGR suckling lambs. Representative charts of western blot results (A) and related protein expression of Fas, Caspase 3, Bax, and Bcl-2 (B) were obtained. Values are presented as mean ± SEM ($n = 12$). [a,b,c] Mean values in bars without a common letter differ significantly ($P < 0.05$). Arg = L-arginine; NCG = *N*-carbamylglutamate; IUGR = intrauterine growth retardation; Bax = Bcl-2-associated X protein; Bcl-2 = B-cell lymphoma/leukaemia 2; Fas = apoptosis antigen 1. CON: the normal birth weight group given a control diet; IUGR: the intrauterine-growth-retarded group given a control diet; IUGR + Arg: IUGR supplemented with 1% L-arginine; IUGR + NCG: IUGR supplemented with 0.1% *N*-carbamylglutamate.

At abnormally high levels, however, concentrations of H_2O_2 suppresscellular differentiation, ultimately resulting in necrosis or apoptosis (Kan et al., 2021). As a major member of the antioxidant defence system, GSH can scavenge ROS; the GSH:GSSG ratio is altered by OS toward a lower GSH level and a greater GSSG level (Prasai et al., 2018). Arginine facilitates the small intestinal generation of NO, which exerts a crucial function on the antioxidant defence system (Dai et al., 2013). In the current study, dietary Arg or NCG supplementation positively impacted GSH and the ratio of GSH:GSSG, and Arg or NCG decreased the H_2O_2 concentration in the colon, highlighting the potential of Arg and NCG in relieving the IUGR-triggered excess ROS.

Fas- and mitochondria-reliant apoptosis has been demonstrated to crucially impact the onset of intraepithelial OS-triggered apoptosis (Turillazzi et al., 2017). The effects of Bcl-2, an anti-apoptotic mitochondrial molecule, on suppressing cytochrome C

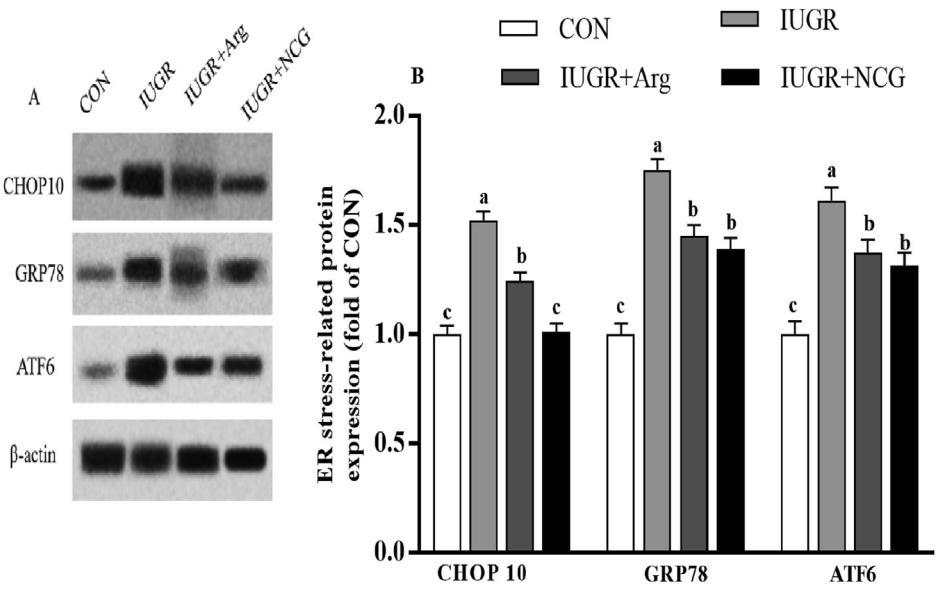

Fig. 3. Effects of dietary Arg or NCG supplementation on the ER-stress related protein expression in the colon of IUGR suckling lambs. Representative charts of western blot results (A) and related protein expression of CHOP10, GRP78, and ATF6 (B) were obtained. Values are presented as mean ± SEM ($n = 12$). [a,b,c,] Mean values in bars without a common letter differ significantly ($P < 0.05$). Arg = L-arginine; NCG = *N*-carbamylglutamate; IUGR = intrauterine growth retardation; CHOP10 = C/EBP homologous protein 10; GRP78 = glucose-regulated protein 78; ATF6 = activating transcription factor 6. CON: the normal birth weight group given a control diet; IUGR: the intrauterine-growth-retarded group given a control diet; IUGR + Arg: IUGR supplemented with 1% L-arginine; IUGR + NCG: IUGR supplemented with 0.1% N-carbamylglutamate.

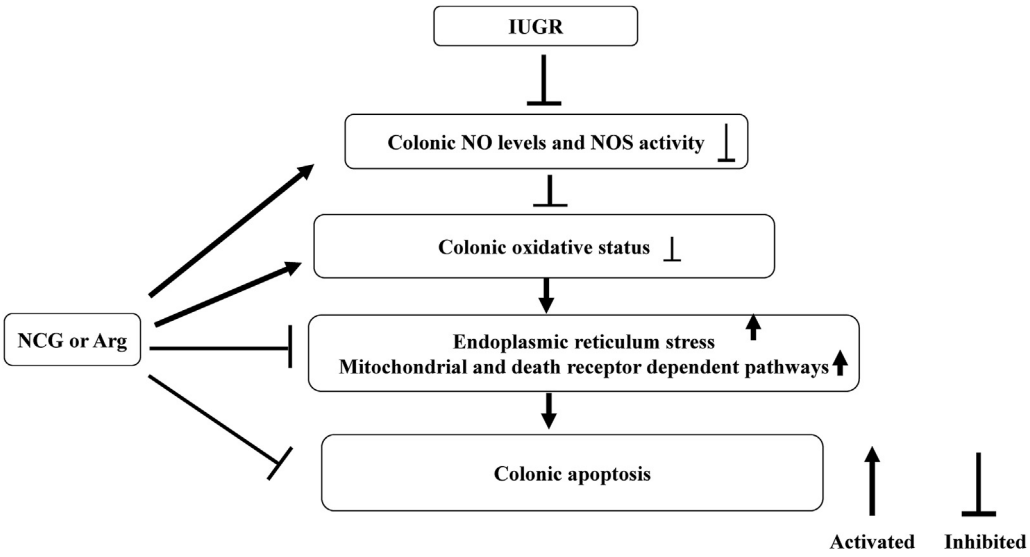

Fig. 4. The possible mechanism of Arg or NCG on the regulation of colonic redox status and apoptosis in IUGR Hu lambs. Arg = L-arginine; IUGR = intrauterine growth retardation; NCG = *N*-carbamylglutamate; NO = nitric oxide; NOS = NO synthase.

secretion and resisting OS-triggered apoptosis also have been reported (Takahashi et al., 2004). In addition, some ROS scavengers (e.g. SOD and GSH) were capable of inhibiting Fas-reliant apoptosis within diverse types of cells (Villalpando-Rodriguez and Gibson, 2021). In our work, elevated levels of Bax, Fas, caspase 3, caspase 8 and caspase 9 were in line with the lower activities of CAT, SOD and GSH-Px within the colonic tissue of IUGR lambs, but dietary Arg or NCG administration reversed these trends. Accordingly, Arg and NCG suppressed OS-triggered apoptosis via both the Fas- and mitochondria-reliant pathways.

Participation of OS in apoptosis through the ER signalling and mitochondria-reliant pathways has been reported extensively (Han et al., 2021). The ER is vulnerable to OS, and prolonged production of ROS can adversely influence healthy cellular functions via impaired ER regulation thereby leading to induction of UPR and initiation of apoptosis (Wang et al., 2021). According to Adams et al. (2019), UPR is controlled via 3 axes: PERK-eIF2α-ATF4 (suppressing protein synthesis), IRE1 (decreasing protein load) and ATF6 (synthesizing chaperones). In case of unsuccessful homeostatic reestablishment inside cells, the 3 axes achieve apoptotic chain activation through ER stress. As the ER stress persists, CHOP is activated by UPR sensor-induced direct stimulation of downstream signalling. Subsequently, after moving into the nucleus, CHOP elicits the extrinsic apoptotic axis by elevating death receptor (DR) 2/Fas/CD95, DR4 and DR5 levels. As a regulator of the cascade mediated by caspase-8, DR2 triggers apoptosis by eliciting caspase 3 (Hu et al., 2019). *CHOP* expression, however, necessitates the translation of *ATF4*. According to the present findings, there is a correlation between ER stress and colonic oxidative damage induced by IUGR. It has been reported in previous studies of sepsis or LPS-induced acute lung injury that ER stress activation can be induced by CHOP (Chen et al., 2018; Zeng et al., 2017). Similarly, colonic ER stress was attributable to CHOP activation by ER stress sensors, a novel biomarker identified in IUGR lambs. Our findings, therefore, are consistent with the above-mentioned ones, where Arg or NCG incorporation relieved ER stress-triggered colonic apoptosis among lambs with IUGR. This was manifested by the downregulation of *ATF6*, *ATF4*, *CHOP10* and *GRP78* in our study.

Activation of such apoptosis was probably due to intrinsic (mitochondrial) or extrinsic (DR) pathways (Su et al., 2016). The extrinsic apoptotic axis deals with direction interplay within the DRs such as Fas, which can elicit the catalysis of caspase 8 (Nunes et al., 2014). Similarly, the intrinsic apoptotic axis, as a mitochondria-reliant pathway, can elicit one or more of the pro-apoptotic Bcl-2 members thereby acting as the apoptotic gatekeeper inside mitochondria (Mohamed et al., 2021). The DR-mediated axis is crucially influential in apoptosis (Shaukat et al., 2021). Following binding between Fas and FasL, they recruit the Fas-associated via death domain (FADD) thereby further recruiting caspase 8, caspase 10 and cFLIP, the downstream molecules of FADD (Horn et al., 2017). The role of caspase 8 in the DR apoptotic axis is exceptionally important (Jin et al., 2009). A previous report demonstrated FADD-related apoptosis, which is mediated via ER stress (Lindner et al., 2020). Nonetheless, the effect of NCG and Arg on resisting ER stress within colonic tissue from suckling IUGR lambs by DR-mediated apoptosis has never been investigated. The present study suggested that IUGR lambs had marked elevations in FasL, Fas and caspase 8 levels. Thus, these data suggest that Arg or NCG protect suckling IUGR lambs from colonocyte apoptosis through the DR axis mediated by ER stress.

For apoptosis mediated by ER stress, the downstream regulators include Bcl-2 members and caspase proteases (Han et al., 2021). Localizing to ER membranes, Bcl-2 proteins are partitioned into anti-apoptotic Bcl-2 and pro-apoptotic Bax (Levine et al., 2008). After Bax knockdown, ER stress-elicited apoptosis was restored in murine embryonic fibroblasts (Seervi et al., 2018). Apoptosis-related caspase proteases can be categorized under initiator type (caspase 9, etc.) and effector type (caspase 3, etc.) (Julien and Wells, 2017). Pro-apoptotic activators initially activate caspase 9 and then caspase 3. During apoptosis onset, diverse cellular substrates are cleaved via caspase 3 and as a consequence, apoptosis-specific morphological and biochemical alterations occur (Elmore, 2007). In our work, IUGR upregulated caspase 3, caspase 9 and Bax but downregulated Bcl-2, implying the induction of apoptosis by IUGR via mediating Bcl-2 proteins and caspase proteases in the ovine colon. The alterations of the above-mentioned proteins and proteases in the colon of IUGR suckling lambs were counteracted by dietary Arg or NCG supplementation. The colonic protective activity of Arg or NCG, thus, was demonstrated in lambs with IUGR, which was achieved by suppressing ER stress, as well as downstream mediators of the mitochondria-reliant axis of apoptosis.

5. Conclusion

The current work verified that Arg or NCG improves redox status and protects against apoptosis induced by ER stress in the colon of IUGR suckling lambs. The protective effects of Arg or NCG might be through inhibition of ER stress-induced apoptosis by suppressing the death receptor-dependent and mitochondrial apoptotic pathways. Thus, dietary NCG and Arg supplementation in early life nutrition could counteract the IUGR-induced intestinal dysfunction in animals and humans.

Author contributions

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

Declaration of competing 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.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.08.009.

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