N-carbamylglutamate and L-arginine Supplementation Improve the Intestinal Oxidative Resistance of the Intrauterine Growth-retarded Ovine Fetuses

Hao Zhang

Yangzhou University

Yi Ma

Yangzhou University

Mengzhi Wang

Yangzhou University

Elsabagh Mabrouk

Kafrelsheikh University

Hongrong Wang (✉ 76780096@qq.com)

Laboratory of Metabolic Manipulation of Herbivorous Animal Nutrition, College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, P. R. China.

Research

Keywords: L-arginine, Intrauterine growth restriction, Intestinal function, N-carbamylglutamate, NO-dependent pathway

Posted Date: October 23rd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-95710/v1>

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Abstract

Background: The maternal under nutrition-induced intrauterine growth retardation (IUGR) is associated with intestinal oxidative injury in fetuses and neonates in various animal models. However, whether maternal dietary Arginine (Arg) and *N*-carbamylglutamate (NCG) supplementation during IUGR alters fetal small intestine redox status is unclear.

Objective: The ovine model of IUGR was used to elucidate whether dietary supplementation of rumen-protected Arg (RP-Arg) and NCG modulates the fetal intestinal oxidative resistance via the nitric oxide (NO) -dependent pathway.

Methods: On day 35 of gestation, 32 twin-bearing Hu ewes were randomly assigned into 4 treatment groups, 8 ewes each. The first and second groups received 100% (Control, CON) and 50% (restricted, RES) of NRC-recommended pregnancy nutrient requirements, respectively. The third and fourth treatment groups included the RES ewes supplemented with 20 g/day of RP-Arg (RES+ARG) or 5 g/day of NCG (RES+NCG), respectively. On day 110 of gestation, fetal blood and intestinal specimens were collected and assayed for oxidative damage biomarkers.

Results: The NCG or Arg-supplemented RES ewes elevated the fetal jejunal NO concentrations and NO synthase (NOS) activity, but decreased the fetal jejunal and plasma concentrations of interleukin (IL)-6 and tumor necrosis factor α (TNF- α) ($P < 0.05$) compared with those in the RES ewes. Further, the NCG or Arg treatment increased the contents of catalase (CAT), glutathione peroxidase 1 (GPx1), nuclear factor erythroid 2-related factor 2 (Nrf2), superoxide dismutase 2 (SOD2), heme oxygenase (HO-1), quinone oxidoreductase 1 (NQO1), claudin-1, zonula occludens-1 (ZO-1), epithelial NOS (eNOS) and inducible NOS (iNOS) in the fetal jejunum ($P < 0.05$).

Conclusion: These results indicate that both NCG and Arg supplementation for RES ewes help maintain intestinal function in fetuses experiencing IUGR through modulating the oxidation status.

Introduction

Optimal maternal nutrition during pregnancy is crucial for the development of a functional small intestine in the fetus (Cao et al., 2014; Meyer and Caton, 2016). The maternal undernutrition-induced intrauterine growth retardation (IUGR) is associated with abnormal gut development and function in the fetuses and neonates in various animal models (Gao et al., 2008; Meyer et al., 2013). IUGR has been shown to adversely affect the pre- and post-natal development and function of the small intestine in various mammals (Meyer and Caton, 2016; Huang et al., 2019).

Newborn IUGR animals have remarkably decreased antioxidant abilities at birth and weaning (Biri et al., 1985a; Gupta et al., 2004). Oxidative stress (OS) results from the imbalance between the antioxidant capacity and the production of reactive oxygen species (ROS) (Yin et al., 2013). As intestinal lumen is in direct contact with oxidants either from microbiota or nutrient intake, it is likely to be affected by oxidative

injury induced by the IUGR (Zheng et al., 2018; Marseglia et al., 2015). The IUGR has been shown also to adversely affect the protein abundance in the fetal small intestine at various stages of development (Wang et al., 2014). These changes collectively comprise the intestinal digestive and absorptive functions (Wang et al., 2005; Xu et al., 1994) which might be responsible in part for the postnatal growth retardation of IUGR newborns (Hu et al., 2016; Zhang et al., 2019a; Zhang et al., 2019b).

Arginine (Arg), the essential amino acid among young mammals, particularly for animals that are subjected to stress conditions (Dasgupta et al., 2006), exerts a vital part among numerous metabolic pathways (Moncada and Higgs, 1993). N-carbamylglutamate (NCG) is the N-acetylglutamate (NAG) analogue with metabolic stability, which is suggested to elevate the plasma content of Arg as well as the synthesis of endogenous Arg through the activation of intestinal pyrroline-5-carboxylate synthase and carbamylphosphate synthase-1 (Wu et al., 2004). Another important function of arginine pertains to its role in mitochondrial biological function and biogenesis via the NO signal transduction pathway (Guo et al., 2018). Particularly, Arg enhances the synthesis of NO in normal conditions within the small intestine (Weckman et al., 2019), thus, it exerts a vital part in regulating the antioxidant defense (Liang et al., 2018). Recent evidence emphasized the importance of arginine/NO pathway during pregnancy as indicated by the increase in the circulating levels of arginine and its metabolites in feed-restricted ewes bearing multiple-fetuses (Berlinguer et al., 2020). However, whether maternal dietary Arg or NCG supplementation during IUGR alters fetal small intestine redox status is unclear.

We hypothesized that maternal dietary Arg or NCG supplementation would improve the development of the fetal intestine through reducing oxidant status in fetuses exposed to IUGR. Specific objectives were to elucidate the benefits of maternal dietary Arg or NCG supplementation on intestinal dysfunction in IUGR ovine fetuses and to offer a theoretical basis for using NCG or Arg as a functional component in the feeds of pregnant ewes at risk of undernutrition.

Materials And Methods

Animals, housing and management

All procedures were carried out according to the protocols from the Guide for the Care and Use of Laboratory Animals formulated by the Ethics Committee of Yang Zhou University (SYXK2013-0057). Forty-eight multiparous Hu ewes [with the mean body weight (BW) of 40.1 ± 1.2 kg] that had similar body condition score (BCS; 2.55 ± 0.18 ; range, 0 (emaciation) - 5 (obesity) and similar age (18.5 ± 0.5 months) (Russel et al., 1969) were chosen for the study. The animals were housed in an indoor barn at the Jiangyan Experimental Station in Taizhou of Jiangsu (China). The barn was equipped with heating radiators to maintain the environmental temperature at an average of $15.3 \pm 0.9^\circ\text{C}$. The barn lighting was automatically controlled for mimicking the normal environmental light cycle. The animals were drenched against parasites using ivermectin (0.2 mg kg^{-1}). Afterward, the animals were subjected to estrus synchronization using the intravaginal progestogen sponge protocol (30mg; Pharnp) for 12 days. Starting 2 days following the removal of the sponge, the ewes were subjected to 3 vasectomized rams (at

08:00 and 16:00) for detecting the estrus behavior and artificial insemination was performed accordingly. The inseminated ewes (at day 0 of gestation) were then put into individual pens (1.05 m×1.60 m) for 35 days. On day 35 of gestation, the ewes were tested by ultrasonography (Asonics Microimager 1000 sec for scanning instrument; Ausonics) for pregnancy diagnosis and twins determination. Accordingly, 32 ewes carrying twin fetuses were selected for the present research. Diet composition (Table 1) was formulated to meet 100% of the nutrient requirements for pregnant ewes (NRC, 1985). From day 0 to day 35 of the study, each animal was given 100% of the nutrient requirements (NRC, 1985). Then, the animal was fed once every day at 08:00 and could drink clean water freely.

Experimental design

On day 35 of gestation, the animals were divided randomly into 4 treatment groups, 8 animals each, the first group served as a control (CON) and received 100% of nutrient requirements. In the second group, the feeding allowance was restricted to 50% of nutrient requirements (restricted feeding; RES). The third and fourth treatment groups included the RES ewes supplemented with 20 g/day of rumen-protected (RP) Arg (RES+ARG) or 5 g/day of NCG (RES+NCG), respectively. The RP-Arg product (Beijing Feeding Feed Science Technology Co., Beijing, China) and the NCG product (Institute of Subtropical Agriculture, The Chinese Academy of Sciences, Changsha, China) contain 50% Arg and NCG, respectively (Zhang et al., 2016a) and, thus actual supplemented amounts of RP-Arg and NCG were 10 g/day and 2.5 g/day, respectively. The RP-Arg product is made up of a matrix of glycerides and phospholipids prepared via the spray congealing and spray drying procedures (Eldem et al., 1991). The ruminal protection of Arg is estimated to be at $\geq 85\%$, whereas the intestinal release of Arg is estimated at $\geq 90\%$ (Chacher et al., 2012). The Arg dose was determined based on prior research on pregnant ewes receiving parenteral Arg or RP-Arg supplementation (McCoard et al., 2013; Zhang et al., 2018a). The NCG dose was determined based on prior research on piglets (Zeng et al., 2012), dairy cows (Chacher et al., 2014), and pregnant sheep (Zhang et al., 2016b). The restricted feeding could be attained by giving 50% of the total diet determined to satisfy the 100% of pregnancy nutrient requirements set by the NRC (1985). From the 35th gestational day, BW was measured on 10-days intervals, and then the feed intake was adjusted accordingly. All diets were in the form of pellets and the experiment continued to day 110 of gestation.

Fetal blood and intestinal sample collection

On day 110 of gestation, each animal was stunned using the captive bolt gun (Supercash Mark 2; Aceles and Shelvoke), followed by euthanasia by exsanguination. A 5 mL blood sample was extracted from the fetal hearts at 0900 hours. All blood samples were immediately placed in heparinized tubes on ice, followed by 15 min of centrifugation at 4 °C and 3000 × *g*, and then kept at -80 °C for subsequent analysis. Then, 10 g duodenal, jejunal, and ileal samples (rinsed with cold PBS) were collected, respectively, immediately frozen within liquid nitrogen, and then stored at -80°C for subsequent analyses (Xu et al., 2018).

Intestinal morphological analyses and counting of goblet cells

The jejunal specimens that were fixed within the 4% paraformaldehyde were washed using the gradient xylene and ethanol, and then the dried specimens were subjected to paraffin embedding. Then, 5 jejunal slides with 3 respective sections (5 μm in thickness) were prepared, and then the samples were rehydrated by gradient ethanol dilutions and deparaffinized with xylene (5 μm). To observe the intestinal morphology, each slide was stained using the hematoxylin and eosin (H&E) through 20 well-oriented crypts and villi in every section (Optimus software, version 6.5, Media Cybergenetics). Moreover, the ratio of villi to crypt (VCR) was determined, and the number of goblet cells in each villus was calculated (NIS-Elements BR 2.3, Nikon France SAS). Then, values were collected from 10 villi of every small intestine segment, which were then averaged (Zhang et al., 2018b; Zhang et al., 2019c).

Analyses of insulin-like growth factor 1 (IGF-1), insulin, nitric oxide (NO), nitric oxide synthase (NOS) and protein in plasma and jejunum

The IGF-1 and insulin contents were determined by an ELISA micro-plate kit specific to ewes (Mercodia, Guangzhou, China). Later, the NOS and NO contents were determined by the commercial kit (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) following manufacturer protocols (Ando et al., 2016). Protein content was determined by a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.).

Cytokine analysis in plasma and jejunal tissues

Interleukin-1 (IL-1, BioSource/MED Probe, Camarillo, CA, U.S.A.), tumor necrosis factor- α (TNF- α , R&D Systems, Oxford, U.K.), and IL-6 (R&D Systems, Oxford, U.K.) were determined by respective commercial kits. The absorbance was measured at 450 nm by a BioTek synergy HT microplate reader (BioTek Instruments, Winooski, VT, U.S.A.). All values were presented as pg/mL of plasma or ng/g protein in jejunal tissues (Nikiforou et al., 2016; Zhang et al., 2019a).

Enzyme activity assays in jejunal tissues

Jejunal tissue was subjected to homogenization and centrifuged within the lysis buffer for 15 min at 4 °C and 15,000 $\times g$. Supernatants were extracted to determine the markers related to the OS as well as the activities of antioxidant enzymes. A bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.) was used to determine the protein content. Besides, the maleic dialdehyde (MDA) level, the total antioxidant capacity (T-AOC) degree, together with the activities of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) were determined in both homogenized jejunal tissue and plasma using respective commercial ELISA kits (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

mRNA levels determined through real-time PCR assay in jejunal tissues

The total RNA was extracted by the RNA isolation kit (Qiagen, Frankfurt, Germany) following manufacturer protocol, and detected by real-time PCR according to the previous description (Zhang et al., 2018b). The RNA content was determined by spectrophotometry, and the RNA integrity was assessed using the agarose gel electrophoresis and spectrophotometry. Afterward, the reverse transcription was carried out using the PrimeScript RT reagent kit (TaKaRa) following the manufacturer protocol. Later, the

qPCR was carried out using the FastStart Universal SYBR Green Master kit (Roche, Mannheim, Germany) in the Step One Plus Real-Time PCR System (Applied Biosystems, CA, USA). All primers were designed with the Primer 5.0 (Table 2), among which, β -actin was used to be a housekeeping normalizing gene. The reaction mixture (10 μ L) contained freshly prepared SYBR Premix Ex Taq II (5 μ L, Tli RNaseH Plus), RT products (1 μ L), ROX Reference Dye II (0.2 μ L, 50 $\times$), diethylpyrocarbonate-treated water (3 μ L), and primers (0.8 μ L). The PCR conditions were as follows: 30 s at 95 $^{\circ}$ C for 1 cycle; followed by 5 s at 95 $^{\circ}$ C and then 31 s at 60 $^{\circ}$ C for 40 cycles; and 15 s at 95 $^{\circ}$ C, 1 min at 60 $^{\circ}$ C, and 15 s at 95 $^{\circ}$ C for 1 cycle. The efficiency of amplification was determined according to the previously reported method (Zhang et al., 2019a). Each correlation coefficient (r) for the standard curve was ≥ 0.99 , whereas the amplification efficiency value was 90-110%. Meanwhile, the amplification specificity was verified using the melting curves, and the mRNA expression was determined according to the $2^{-\Delta\Delta C_t}$ method. According to our results, the difference in the β -actin level was not statistically significant across the 4 treatments. Besides, the mRNA expression of a target gene within the CON group was used to normalize that in different treatment groups (namely, the fold-change) (Zhang et al., 2019a).

Western blotting

The total protein was extracted from the jejunal tissues by the commercial kit (Beyotime Biotechnology, Jiangsu, China) following the manufacturer protocol. Afterward, the content of protein was determined by a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.). In this study, the primary antibodies used included anti-glutathione peroxidase 1 (anti-GPx1, diluted at 1:2000, Abcam), anti-catalase (CAT, diluted at 1:2000, Protein Tech), anti-heme oxygenase-1 (anti-HO-1, diluted at 1:1000, Abcam), anti-SOD2 (diluted at 1:5000, Novus), anti-Nrf2 (diluted at 1:2000, Protein Tech), anti-p65 (diluted at 1:300, Cell Signaling), anti-quinone oxidoreductase 1 (anti-NQO1, diluted at 1:1000, Abcam), anti-IL-1 β (diluted at 1:300, Santa Cruz Biotechnology), anti-pp65 (diluted at 1:300, Cell Signaling), anti-ZO-1 (diluted at 1:1000, Santa Cruz Biotechnology), anti-TNF- α (diluted at 1:300, Santa Cruz Biotechnology), anti-inducible NO synthase (anti-iNOS, diluted at 1:1000, Cell Signaling), anti- β -actin (diluted at 1:1500, Santa Cruz), anti-epithelial NO synthase (anti-eNOS, diluted at 1:1000, Cell Signaling), and anti-claudin-1 (diluted at 1:1000, Santa Cruz Biotechnology). Thereafter, the protein was subjected to 1 h of incubation using the secondary antibody horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (diluted at 1:5000, Antgene Biotech). Then, the signals were measured through the enhanced chemiluminescence kits (ECL-Plus, Thermo, Waltham, MA, USA), and later scanned for detecting the intensity of fluorescence using a BioRad gel detection system. Each densitometric value was standardized based on β -actin, which was then reported as fold-change in comparison with the control. All measurements were repeated six times.

Statistical methods

Statistical comparisons among the 4 groups of ewes killed on d 110 of gestation were completed using a 1-way ANOVA. Results were presented as least squares means $\pm$ SEM. Data analyses among different groups of fetuses were carried out by the one-way ANOVA following the SAS (version 9.2) PROC GLM procedure. The sex of the fetus was incorporated into the initial model, and no significance was found

($P > 0.05$). Therefore, it was eliminated from that eventual model that only included the nutrition treatments in the ewes. The difference among different groups was determined using Duncan's multiple range test. A difference of $P \leq 0.05$ declared significance.

Results

Maternal BW, fetal BW and small intestinal development

We previously reported that maternal BW in CON ewes was greater compared with RES, RES + ARG, and RES + NCG ewes (Fig.1) but maternal dietary NCG or Arg treatment did not affect the BW of RES ewes. Dietary Arg or NCG treatment during pregnancy from d 35 to d 110 improved the BW of IUGR ovine fetuses and enhanced the small intestinal development (Zhang et al., 2016).

Fetal intestinal morphology and density of goblet cells

Relative to the CON group, the number of goblet cells per villus, villus height, and VCR were reduced ($P < 0.05$) in the jejunum the fetuses of the RES group (Figure 2). In contrast, these variables were increased ($P < 0.05$) in the jejunum of the fetuses of the RES ewes subjected to dietary NCG or Arg supplementation compared with the RES group.

Insulin content, NOS activity and NO level in fetal plasma

Relative to the CON group, insulin content, NOS activity and NO level in fetal plasma of RES ewes were reduced ($P < 0.05$) (Figure 3), but these parameters were increased ($P < 0.05$) in RES + ARG or NCG group relative to those in the RES group.

Protein, NO, IGF-1, insulin and NOS contents in fetal jejunum

Compared with the CON, the RES feeding resulted in a reduction of the concentrations of protein, IGF-1, insulin, NOS, and NO in the fetal jejunum ($P < 0.05$) (Figure 4). Relative to RES group, a marked increase in protein, IGF-1, insulin, NOS, and NO contents were observed in the jejunum of fetuses of RES ewes supplemented with Arg or NCG ($P < 0.05$).

Fetal jejunal and plasma levels of cytokines

Compared with the CON, the RES group showed an increase of the fetal jejunal and plasma IL-6 and TNF- α contents ($P < 0.05$) (Figure 5). By contrast, RES + NCG or ARG reduced the fetal jejunal and plasma IL-6 and TNF- α as well as the fetal plasma IL-1 contents ($P < 0.05$) compared with the RES group.

Jejunal and plasma antioxidant enzyme activities in fetuses

Relative to the CON group, the fetal jejunal and plasma T-AOC activities were reduced ($P < 0.05$) in the RES group (Figure 6); nonetheless, ewes subjected to NCG or Arg treatment showed an increase ($P < 0.05$) in the fetal jejunal and plasma T-AOC activities compared with the RES ewes. The NCG or Arg treatment

resulted in a reduction in the fetal jejunal and plasma MDA concentrations ($P < 0.05$) relative to those in the RES group. The SOD and GSH-Px activities within fetal jejunum and the plasma of the RES group were reduced relative to those in the CON group ($P < 0.05$). The NCG or Arg treatment resulted in increasing the SOD activity of the fetal jejunum and plasma ($P < 0.05$) relative to those in the RES group.

mRNA level in fetal jejunum

Relative to the CON, the TLR-2, MyD88, TLR-9, TLR-4, IL-1 β , IL-6, TNF- α , and NF- κ B mRNA levels were upregulated ($P < 0.05$) in the fetal jejunum of the RES group (Figure 7). The NCG or Arg treatment resulted in downregulation ($P < 0.05$) of the above genes relative to those in the RES group. Relative to the CON, GPx1, eNOS, CAT, Nrf2, SOD2, NQO1, HO-1, claudin-1, ZO-1, iNOS, and occludin levels were downregulated ($P < 0.05$) in the fetal jejunum of the RES group. Supplementation of the RES group with NCG or Arg resulted in upregulation ($P < 0.05$) of the above-mentioned genes relative to those in the RES group.

Protein contents in fetal jejunum

Compared with the CON, the RES feeding reduced the CAT, GPx1, Nrf2, SOD2, NQO1, HO-1, claudin-1, ZO-1, eNOS, and iNOS levels in the fetal jejunum ($P < 0.05$) (Fig. 8-10). Compared with the RES, the NCG or Arg treatment increased to CAT, GPx1, Nrf2, SOD2, NQO1, HO-1, claudin-1 ZO-1, eNOS, and iNOS protein levels ($P < 0.05$). The TNF- α , IL- β , p-p65, and p65 protein levels in fetal jejunum were reduced ($P < 0.05$) under NCG or Arg treatment compared with those in the unsupplemented RES ewes (Fig. 11).

Discussion

The development of the intestine is of crucial importance to neonate growth and survival. At the advanced gestation, fetal intestine develops more rapidly (Zhu et al., 2018). The integrity of the intestine is necessary to maintain appropriate digestive functions (Choksi et al., 2018), as a result, this reflects a crucial intestinal health aspect. Relative to the CON, the IUGR fetuses had reduced jejunal VCR and villus height, which was consistent with prior studies (Su et al., 2018; Zhu et al., 2018). Improvements in the IUGR fetal intestinal morphology of RES ewes under NCG or Arg treatment are related to the promoted enterocyte proliferation and maturation (Yang et al., 2013). Thus, these results might explain the damaged integrity of the intestine of the IUGR ovine fetus, which also strengthened the role of NCG or Arg in protection against fetal intestinal injury induced by IUGR.

Arginine is identified to be the potent factor that stimulates the release of insulin (Fisker et al., 1998). Consequently, the increased serum concentration of this anabolic hormone enhances the utilization efficiency of nutrients through enhancing tissue protein synthesis (Yao et al., 2011). The fetal jejunal protein concentration and jejunal and plasma insulin concentrations of RES ewes under Arg or NCG treatment were in line with the above-mentioned results.

IUGR animals are more susceptible to OS, an effect that is probably associated with the dysregulation between prooxidants and antioxidants. Besides, excess OS affects cell homeostasis and could

predispose to cell injuries (Biri et al., 2007; Celik et al., 2019). Such dysregulation leads to cell damage, which may even result in cell death as well as irreversible oxidative damage if it remains to be out of control, finally negatively affecting organism survival and function (Yin et al., 2013b). The OS has been identified as the critical factor contributing to bowel injury within animals afflicted by IUGR (Su et al., 2017). Consequently, the markedly reduced jejunal T-AOC content and antioxidant enzyme activities, together with the remarkably increased MDA content of IUGR ovine fetuses emphasize the occurrence of oxidative injury. The above adaptations may result in bowel-related disorders through inducing oxidative injury to lipids, proteins, and DNA, as well as increase the membrane permeability (Murphy, 2009). The increased TNF- α , IL-6, and IL-1 contents in the jejunum of IUGR ovine fetuses compared with those of the CON group confirmed that the OS was positively correlated with pro-inflammatory cytokines (Artimani et al., 2018). The TNF- α serves as the pro-inflammatory cytokine, which participates in the systemic inflammation (Liu et al., 2016). Besides, the TNF- α is known to result in the increased intestinal epithelial barrier permeability (Lee, 2015). Therefore, the reduction in the oxidative stress along with the lower TNF- α and IL-1 β protein and mRNA contents in the fetal jejunum due to supplementing with Arg or NCG supports the view of a protective effect of these compounds.

The barrier of the intestinal epithelium is comprised of a variety of junctional complexes, which ensures the close epithelial cell binding as well as the appropriate epithelial barrier maintenance (Zihni et al., 2016). Goblet cells can produce trefoil peptides and mucins to generate the physical barrier on the intestinal mucosal surface (Wang et al., 2007). The tight junction proteins (TJP) are considered to modulate the paracellular intestinal epithelial barrier function (Tokuda and Yu, 2019). The levels of the fetal intestinal TJP, including ZO-1 and claudin-1, were increased in the RES supplemented with NCG and Arg, and the numbers of goblet cells were also increased, which emphasized the favorable effects on the barrier function of the fetal intestine. The results of this study are consistent with the previous studies which indicated that the Arg treatment boosted the intestinal morphology of the weaned as well as the IUGR suckling piglets (He et al., 2011; Wang et al., 2012). These findings imply that NCG or Arg could enhance the antioxidative defense in IUGR animals by reducing the pro-inflammatory cytokines. Nuclear factor erythroid 2-related factor (Nrf2) is of crucial importance to improve our understanding towards the underlying mechanism of the association with the antioxidant responsive element (ARE)-activated antioxidation and detoxification under the anti-oxidative state, which suggests that oxidation resistance may serve as the synergistic action regulated via different antioxidative enzymes, instead of via an antioxidative reaction alone (Suzuki and Yamamoto, 2015; Liang et al., 2018). Arg has the key effect of up-regulating Nrf2, the antioxidant transcriptional factor, as well as the phase II metabolizing enzymes (such as heme oxygenase (HO-1) and quinone oxidoreductase 1 (NQO1)), together with the antioxidant enzymes (like catalase (CAT), glutathione peroxidase 1 (GPx1), as well as superoxide dismutase2 (SOD2) (Zhang et al., 2019a). In our experimental conditions, Arg or NCG treatment markedly increased the mRNA abundance of antioxidant/detoxification genes (HO-1, NQO1, SOD2, CAT, GPx1) by activating Nrf2, which participated in the endogenous defense response of antioxidant. Overall, it alleviates oxidative injury and protects the intestinal epithelial cells (IOECs) against apoptosis in the presence of bowel inflammatory challenge (Zhang et al., 2019d). The NCG or Arg supplementation positively affected the Nrf2 protein and

mRNA levels, elevated the SOD2, CAT, GPx1, and phase II metabolizing enzymes (including HO-1 and NQO1) contents in fetal jejunum, but reduced the MDA content within IUGR ovine fetuses, which confirmed that the above-mentioned compounds had positive effects on the intestinal functions. As far as we know, this is the first study to report that Arg or NCG can activate and upregulate ARE-induced antioxidant and detoxification genes controlled by the Nrf2 signaling pathway within the fetal intestine from animals exposed to IUGR.

Nitric oxide, produced from arginine metabolism, can serve as the essential messenger, which plays various roles, including regulating cell proliferation and survival, immunologic function, as well as the cellular redox status (Thomas et al., 2008). The NO has been shown to play a role as a scavenger of free radicals (Az-Ma et al., 1996). In this study, maternal dietary supplementation with Arg or NCG had favorable action through promoting the jejunal oxidation resistance of IUGR ovine fetuses, which was ascribed to the fact that Arg exerted a role as the precursor of NO (El-Hattab et al., 2012).

To study whether the NO pathway was responsible for the Arg or NCG-enhanced fetal antioxidant capacity in the jejunum, both iNOS and eNOS protein and mRNA levels, NO content, and NOS activity were determined. In this study, maternal NCG or Arg treatment significantly increased the jejunal NOS protein and mRNA levels, NO level, or NOS activity in IUGR ovine fetuses. According to Dhar et al. (2012), Arg treatment decreased the OS induced by high glucose via the eNOS-independent signaling pathway. The NCG or Arg treatment had positive action on the intestinal health of IUGR fetuses in a NO pathway-dependent manner, which was partly ascribed to the enhanced jejunal oxidation resistance.

Conclusion

The fetal intestinal development dysfunction, together with the decreased oxidation resistance in jejunum induced by IUGR, is attenuated through the dietary NCG or Arg treatment. The above compounds enhance the intestinal barrier function partially through modulating the oxidation resistance through the NO-dependent pathway. The findings of this study expound the underlying mechanisms regarding the dysfunction in the small intestine of ovine fetuses suffering from IUGR.

Abbreviations

CON, ewes fed 100% of the National Research Council (NRC 1985)-recommended pregnancy nutrient requirements; CAT, catalase; eNOS, epithelial NO synthase; GPx1, glutathione peroxidase 1; HO-1, heme oxygenase-1; iNOS, inducible NO synthase; IL, interleukin; MyD88, myeloid differentiation factor 88; NF- κ B, nuclear factor kappa B (p65); Nrf2, nuclear factor erythroid 2-related factor 2; NQO1, quinone oxidoreductase 1; RES-ARG, ewes fed 50% of NRC (1985) recommendations and supplemented with 20 g per day RP-Arg; RES-NCG, ewes fed 50% of NRC (1985) recommendations and supplemented with 5 g per day NCG; SOD2, superoxide dismutase 2; TRAF-6, TNF receptor-associated factor 6; TLR, toll-like receptor; TNF- α , tumor necrosis factor α ; ZO-1, zonula occludens-1; RES, ewes fed 50% of NRC (1985) recommendations for pregnancy.

Declarations

Ethics approval and consent to participate

All animal experiments, which had gained approval from the Animal Care and Use Committee of the Yangzhou University, were carried out in accordance with the practical animal protection law and the Guide for the Care and Use of Laboratory Animals formulated by the National Research Council.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated or analyzed during current study are available from the corresponding author by request. All data that support the findings in of this study are included in the article.

Competing interests

The authors declare no competing financial interest.

Funding

The research was supported by the fund for the National Natural Science Foundation of China (31902180), the Research Project of Natural Science Foundation of Jiangsu Province (BK20170488), and China Postdoctoral Science Foundation (2017M610358).

Authors' contributions

HZ and HRW designed the research; YM and MZW conducted the research; YM and HZ analyzed the data; HZ and ME wrote the paper; HZ and HRW had primary responsibility for the final content. All authors read and approved the final manuscript.

Acknowledgement

HZ and HRW designed the research; YM and MZW conducted the research; YM and HZ analyzed the data; HZ and ME wrote the paper; HZ and HRW had primary responsibility for the final content. All authors read and approved the final manuscript. The authors thank all the members of the Hong Rong Wang's laboratory who contributed to sample determination.

References

Ando H, Nara Y, Kosaka Y, Arai M, Okamoto H. (2016). Neuroprotective effect of nitrite-derived NO in brain injury mediated through the NOS-independent but not the GC/COX/xanthine oxidase/PGIS-dependent pathways. *The Kitasato medical journal*, 46(1), 67-72.

- Artimani T, Karimi J, Mehdizadeh M, Yavangi M, Khanlarzadeh E, Ghorbani M, Asadi S, Kheiripour N. (2018). Evaluation of pro-oxidant-antioxidant balance (PAB) and its association with inflammatory cytokines in polycystic ovary syndrome (PCOS). *Gynecological endocrinology*, 34(2), 148-152.
- Az-Ma T, Fujii K, Yuge O. (1996). Self-limiting enhancement by nitric oxide of oxygen free radical-induced endothelial cell injury: evidence against the dual action of NO as hydroxyl radical donor/scavenger. *British journal of pharmacology*, 119(3), 455-462.
- Berlinguer, F., C. Porcu, G. Molle, A. Cabiddu, M. Dattena, M. Gallus, V. Pasciu, S. Succu, F.D. Sotgiu, P. Paliogiannis, S. Sotgia, A.A. Mangoni, A. Gonzalez-Bulnes, C. Carru, and A. Zinellu. 2020. Circulating Concentrations of Key Regulators of Nitric Oxide Production in Undernourished Sheep Carrying Single and Multiple Fetuses. *Animals* 10:65.
- Biri A, Bozkurt N, Turp A, Kavutcu M, Himmetoglu Ö, Durak, I. (2007). Role of oxidative stress in intrauterine growth restriction. *Gynecologic and obstetric investigation*, 64(4), 187-192.
- Birmani W, Raza A, Nawab A, Tang S, Waseem M, Li G, Xiao M, An L. (2019). Importance of Arginine as Immune Regulator in Animal Nutrition. *International Journal*, 5(1), 1-10.
- Celik E, Taysi S, Sucu S, Ulusal H, Sevincler E, Celik A. (2019). Urotensin 2 and Oxidative Stress Levels in Maternal Serum in Pregnancies Complicated by Intrauterine Growth Restriction. *Medicina*, 55(7), 328.
- Chacher B, Wang D, Liu H, Liu J. (2012). Degradation of L-arginine and N-carbamoyl glutamate and their effect on rumen fermentation in vitro. *Italian Journal of Animal Science*, 11(4), 4693-4696.
- Chacher B, Zhu W, Ye J, Wang D, Liu J. (2014). Effect of dietary N-carbamoylglutamate on milk production and nitrogen utilization in high-yielding dairy cows. *Journal of Dairy Science*, 97(4), 2338-2345.
- Choksi YA, Reddy VK, Singh K, Barrett CW, Short SP, Parang B, Keating CE, Thompson JJ, Verriere TG, Brown RE. (2018). BVES is required for maintenance of colonic epithelial integrity in experimental colitis by modifying intestinal permeability. *Mucosal immunology*, 11(5), 1363.
- Dasgupta T, Hebbel RP, Kaul DK, Medicine. (2006). Protective effect of arginine on oxidative stress in transgenic sickle mouse models. *Free Radic BiolMed*, 41(12), 1771-1780.
- Dhar I, Dhar A, Wu L, Desai K. (2012). Arginine attenuates methylglyoxal-and high glucose-induced endothelial dysfunction and oxidative stress by an endothelial nitric-oxide synthase-independent mechanism. *J Pharmacol Exp Ther*, 342(1), 196-204.
- El-Hattab AW, Hsu JW, Emrick LT, Wong L-JC, Craigen WJ, Jahoor F, Scaglia F. (2012). Restoration of impaired nitric oxide production in MELAS syndrome with citrulline and arginine supplementation. *Mol Genet Metab*, 105(4), 607-614.

- Eldem T, Speiser P, Hincal, A. (1991). Optimization of spray-dried and-congealed lipid micropellets and characterization of their surface morphology by scanning electron microscopy. *Pharm Res*, 8(1), 47-54.
- Fisker S, Jørgensen J, Orskov H, Christiansen J. (1998). I-Arginine and insulin-tolerance tests in the diagnosis of adult growth hormone deficiency: influence of confounding factors. *Clinical endocrinology*, 48(1), 109-115.
- Gao, F., X. Hou, and Y. Liu. 2008. Effect of intrauterine growth restriction on weight and cellularity of gastrointestinal tract in postnatal lambs. *Can. J. Anim. Sci.* 88:107–112.
- Guo Y, Nie H, Xu C, Zhang G, Sun L, Zhang T, Wang Z, Feng X, Wang, F. (2018). Effects of nutrient restriction and arginine treatment on oxidative stress in the ovarian tissue of ewes during the luteal phase. *Theriogenology*, 113, 127-136.
- Gupta P, Narang M, Banerjee B, Basu S. (2004). Oxidative stress in term small for gestational age neonates born to undernourished mothers: a case control study. *BMC Pediatr*, 4(1), 14.
- He Q, Tang H, Ren P, Kong X, Wu G, Yin Y, Wang Y. (2011). Dietary supplementation with l-arginine partially counteracts serum metabonome induced by weaning stress in piglets. *Journal of proteome research*, 10(11), 5214-5221.
- Hu L, Peng X, Chen H, Yan C, Liu Y, Xu Q, Fang Z, Lin Y, Xu S, Feng B. (2016). Effects of intrauterine growth retardation and *Bacillus subtilis* PB6 supplementation on growth performance, intestinal development and immune function of piglets during the suckling period. *European Journal of Nutrition*, 1-13.
- Huang, S., N. Li, C. Liu, T. Li, W. Wang, L. Jiang, Z. Li, D. Han, S. Tao, and J. Wang. 2019. Characteristics of the gut microbiota colonization, inflammatory profile, and plasma metabolome in intrauterine growth restricted piglets during the first 12 hours after birth. *J Microbiol.* 57:748–758.
- Lee SH. (2015). Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases. *Intestinal research*, 13(1), 11.
- Liang M, Wang Z, Li H, Cai L, Pan J, He H, Wu Q, Tang Y, Ma J, Yang L. (2018). l-Arginine induces antioxidant response to prevent oxidative stress via stimulation of glutathione synthesis and activation of Nrf2 pathway. *Food and chemical toxicology*, 115, 315-328.
- Liu C, Feng X, Li Q, Wang Y, Li Q, Hua M. (2016). Adiponectin, TNF- α and inflammatory cytokines and risk of type 2 diabetes: a systematic review and meta-analysis. *Cytokine*, 86, 100-109.
- Marseglia L, D'Angelo G, Manti S, Aversa S, Reiter RJ, Antonuccio P, Centorrino A, Romeo C, Impellizzeri P, Gitto E. (2015). Oxidative stress-mediated damage in newborns with necrotizing enterocolitis: a possible role of melatonin. *Am J Perinatol*, 32(10), 905-909.

- McCoard S, Sales F, Wards N, Sciascia Q, Oliver M, Koolaard J, Linden DVD. (2013). Parenteral administration of twin-bearing ewes with L-arginine enhances the birth weight and brown fat stores in sheep. *Springerplus*, 2(1), 684.
- Meyer, A.M., and J.S. Caton. 2016. Role of the Small Intestine in Developmental Programming: Impact of Maternal Nutrition on the Dam and Offspring. *Adv Nutr* 7:169–178.
- Meyer, A.M., T.L. Neville, J.J. Reed, J.B. Taylor, L.P. Reynolds, D.A. Redmer, C.J. Hammer, K.A. Vonnahme, and J.S. Caton. 2013. Maternal nutritional plane and selenium supply during gestation impact visceral organ mass and intestinal growth and vascularity of neonatal lamb offspring. *J Anim Sci* 91:2628–2639.
- Moncada S, Higgs A. (1993). The L-arginine-nitric oxide pathway. *N Engl J Med*, 329(27), 2002-2012.
- Murphy PM. (2009). How mitochondria produce reactive oxygen species. *Biochem. J*, 417(1), 1-13.
- NRC. 1985. Nutrient requirements of small ruminants: Sheep, goats, cervids and new world camelids. Natl. Acad. Press, Washington, DC.
- Nikiforou M, Kemp MW, Van Gorp RH, Saito M, Newnham JP, Reynaert NL, Janssen LEW, Jobe AH, Kallapur SG, Kramer BW. (2016). Selective IL-1 α exposure to the fetal gut, lung, and chorioamnion/skin causes intestinal inflammatory and developmental changes in fetal sheep. *Laboratory Investigation*, 96(1), 69.
- Russel A, Doney J, Gunn R. (1969). Subjective assessment of body fat in live sheep. *J. Agric. Sci*, 72(3), 451-454.
- Su W, Zhang H, Ying Z, Li Y, Zhou L, Wang F, Zhang L, Wang T. (2018). Effects of dietary L-methionine supplementation on intestinal integrity and oxidative status in intrauterine growth-retarded weanling piglets. *Eur. J. Nutr*, 57(8), 2735-2745.
- Suzuki T, Yamamoto M. (2015). Molecular basis of the Keap1–Nrf2 system. *Free Radic.Biol. Med*, 88, 93-100.
- Thomas DD, Ridnour LA, Isenberg JS, Flores-Santana W, Switzer CH, Donzelli S, Hussain P, Vecoli C, Paolocci N, Ambs S. (2008). The chemical biology of nitric oxide: implications in cellular signaling. *Free Radic Biol Med*, 45(1), 18-31.
- Tokuda S, Yu ASL. (2019). Regulation of Epithelial Cell Functions by the Osmolality and Hydrostatic Pressure Gradients: A Possible Role of the Tight Junction as a Sensor. *International journal of molecular sciences*, 20(14), 3513.
- Wang T, Huo YJ, Shi FX, Xu RJ, Hutz RJ. (2005). Effects of intrauterine growth retardation on development of the gastrointestinal tract in neonatal pigs. *88*(1), 66-72.

- Wang X, Lin G, Liu C, Feng C, Zhou H, Wang T, Li D, Wu G, Wang J. (2014). Temporal proteomic analysis reveals defects in small-intestinal development of porcine fetuses with intrauterine growth restriction. *J Nutr Biochem*, 25(7), 785-795.
- Wang X, Qiao S, Yin Y, Yue L, Wang Z, Wu G. (2007). A deficiency or excess of dietary threonine reduces protein synthesis in jejunum and skeletal muscle of young pigs. *J. Nutr*, 137(6), 1442-1446.
- Wang Y, Zhang L, Zhou G, Liao Z, Ahmad H, Liu W, Wang T. (2012). Dietary L-arginine supplementation improves the intestinal development through increasing mucosal Akt and mammalian target of rapamycin signals in intra-uterine growth retarded piglets. *British Journal of Nutrition*, 108(8), 1371-1381.
- Weckman AM, McDonald CR, Baxter J-AB, Fawzi WW, Conroy AL, Kain KC. (2019). Perspective: L-arginine and L-citrulline Supplementation in Pregnancy: A Potential Strategy to Improve Birth Outcomes in Low-Resource Settings. *Advances in Nutrition*, 10(5), 765-777.
- Wu GY, Knabe DA, Kim SW. (2004). Arginine nutrition in neonatal pigs. *The Journal of nutrition*, 134(10), 2783S-2790S.
- Xu RJ, Mellor DJ, Birtles MJ, Reynolds GW, Simpson HV. (1994). Impact of intrauterine growth retardation on the gastrointestinal tract and the pancreas in newborn pigs. *18*(2), 231.
- Xu X, Chen S, Wang H, Tu Z, Wang S, Wang X, Zhu H, Wang C, Zhu J, Liu Y. (2018). Medium-chain TAG improve intestinal integrity by suppressing toll-like receptor 4, nucleotide-binding oligomerisation domain proteins and necroptosis signalling in weanling piglets challenged with lipopolysaccharide. *British Journal of Nutrition*, 119(9), 1019-1028.
- Yang H, Fu D, Kong X, Wang W, Yang X, Nyachoti C, Yin Y. (2013). Dietary supplementation with N-carbamylglutamate increases the expression of intestinal amino acid transporters in weaned Huanjiang mini-pig piglets. *91*(6), 2740-2748.
- Yao K, Guan S, Li T, Huang R, Wu G, Ruan Z, Yin Y. (2011). Dietary L-arginine supplementation enhances intestinal development and expression of vascular endothelial growth factor in weanling piglets. *Br. J. Nutr*, 105(5), 703-709.
- Yin J, Ren W, Liu G, Duan J, Yang G, Wu L, Li T, Yin Y. (2013b). Birth oxidative stress and the development of an antioxidant system in newborn piglets. *Free Radic Res*, 47(12), 1027-1035.
- Yin J, Ren W, Wu X, Yang G, Wang J, Li T, Ding J, Cai L, Su D. (2013a). Oxidative stress-mediated signaling pathways: a review. *Food Agric. Environ*, 11(2), 132-139.
- Zeng X, Huang Z, Mao X, Wang J, Wu G, Qiao S. (2012). N-carbamylglutamate enhances pregnancy outcome in rats through activation of the PI3K/PKB/mTOR signaling pathway. *PLoS One*, 7(7), e41192.

- Zhang H, Sun L, Wang Z, Deng M, Zhang G, Guo R, Ma T, Wang F. (2016). Dietary N-carbamylglutamate and rumen-protected L-arginine supplementation ameliorate fetal growth restriction in undernourished ewes. *Journal of Animal Science*, 94(5), 2072-2085.
- Zhang H, Zhao F, Nie H, Ma T, Wang Z, Feng W, Loo J. (2018a). Dietary N-carbamylglutamate and rumen-protected L-arginine supplementation during intrauterine growth restriction in undernourished ewes improve fetal thymus development and immune function. *Reproduction Fertility Development*, 30(11), 1522-1531.
- Zhang H, Dong L, Wang M, Yu L, Wang H. (2018b). Effects of dietary L-arginine and N-carbamylglutamate supplementation on intestinal integrity, immune function and oxidative status in intrauterine growth retarded suckling lambs. *J. Agric. Food Chem*, 66(16), acs.jafc.8b00726.
- Zhang H, Sun H, Peng A, Guo S, Wang M, Loo J, Wang H. (2019a). N-carbamylglutamate and L-arginine promote intestinal function in suckling lambs with intrauterine growth restriction by regulating antioxidant capacity via a nitric oxide-dependent pathway. *Food & Function*.
- Zhang H, Peng A, Yu Y, Guo S, Wang M, Coleman DN, Loo J, Wang H. (2019b). N-Carbamylglutamate and L-Arginine Promote Intestinal Absorption of Amino Acids by Regulating the mTOR Signaling Pathway and Amino Acid and Peptide Transporters in Suckling Lambs with Intrauterine Growth Restriction. *The Journal of nutrition*, 149(6), 923-932.
- Zhang H, Peng A, Guo S, Wang M, Wang H. (2019c). Dietary N-carbamylglutamate and L-arginine supplementation improve intestinal energy status in intrauterine-growth-retarded suckling lambs. *Food & Function*, 10.
- Zhang H, Peng A, Yu Y, Guo S, Wang M, Wang H. (2019d). L-Arginine Protects Ovine Intestinal Epithelial Cells from Lipopolysaccharide-Induced Apoptosis through Alleviating Oxidative Stress. *J. Agric. Food Chem*, 67(6), 1683-1690.
- Zheng P, Song Y, Tian Y, Zhang H, Yu B, He J, Mao X, Yu J, Luo Y, Luo J. (2018). Dietary arginine supplementation affects intestinal function by enhancing antioxidant capacity of a nitric oxide-independent pathway in low-birth-weight piglets. *The Journal of nutrition*, 148(11), 1751-1759.
- Zhu Y, Li T, Huang S, Wang W, Dai Z, Feng C. (2018). Maternal L-glutamine supplementation during late gestation alleviates intrauterine growth restriction-induced intestinal dysfunction in piglets. *Amino acids*, 50(9), 1289-1299.
- Zihni C, Mills C, Matter K, Balda MS. (2016). Tight junctions: from simple barriers to multifunctional molecular gates. *Nature reviews Molecular cell biology*, 17(9), 564.

Tables

Table 1. Ingredient and nutrient composition of the experimental diets on a dry matter basis¹.

	Diet 1 (0–90 days gestation)	Diet 2 (91–110 days gestation)
Ingredient (%)		
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
Mineral/vitamin premix	0.30	0.30
Total	100	100
Nutrient composition (analysed)		
Gross energy (MJ kg ⁻¹)	17.63	18.49
Crude protein (%)	9.98	13.59
Ether extract (%)	4.21	4.59
NDF (%)	37.12	32.57
ADF (%)	20.98	18.93
Ca (%)	0.57	0.81
P (%)	0.45	0.69

The premix provided the following nutrients 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. NDF, neutral detergent fibre; ADF, acid detergent fibre.

Table 2 Primer sequences used in the real-time PCR

Gene	Sequences (5'-3')	GenBank accession number
CAT	F: CACTCAGGTGCGGGATTCT R: ATGCGGGAGCCATATTCAGG	GQ204786.1
GPX1	F: GCAACCAGTTTGGGCATCAG R: GCCATTACCTCGCACTTTT	JF728302.1
SOD2	F: GTGAACAACCTCAACGTCGC R: GCGTCCCTGCTCCTTATTGA	XM_013966636.1
Nrf2	F: ATCCAGATGCTCACCATGCG r: CCCAATGCAGGACTTGGTCT	XM_005674733.2
HO-1	F: GAACGCAACAAGGAGAAC R: CTGGAGTCGCTGAACATAG	001014912.1
NQO1	F: CAACAGACCAGCCAATCA R: ACCTCCCATCCTTTCCTC	001034535.1
MyD88	F: ATGGTGGTGGTTGTCTCTGAC R: GGAACTCTTCTTCATTGGCTTGT	GQ221044.1
TLR-2	F: CAAGAGGAAGCCCAGGAAG R: TGGACCATGAGGTTCTCCA	DQ890157.1
TLR-4	F: TGCTGGCTGCAAAAAGTATG R: CCCTGTAGTGAAGGCAGAGC	HQ343416.1
TLR-9	F: ATGGGCCCTACTGTG R: CTATTCGGCTGTCGTGG	HQ263217.1
TRAF-6	F: TCAGAGAACAGATGCCCAAT R: GCGTGCCAAGTGATTCCT	XM_012134166.2
IL-6	F: AGGAAAAAGATGGATGCTTCCA R: GACCAGCAGTGGTTTTGATCAA	NM_001009392
IL-1 β	F: CGTCTTCCTGGGACGTTTTAG R: CTGCGTATGGCTTCTTTAGGG	NM_001009465
NF- κ B	F: ATACGTCGGCCGTGTCTAT R: GGAAGTGTGATCCGTGTAG	XM_005226864.2
TNF- α	F: ACACCATGAGCACCAAAAGC	NM_001024860.1

	R: AGGCACAAGCAACTTCTGGA	
ZO-1	F: AGAAGATAGCCCTGCAGCCAA R: CCT CTC CTT TGT TAA AAC TAA GTC	AJ313188
Occludin	F: CTGGATCAGGGAATATCCACC R: ACTCTTCACTTTCTTCTCTATAGT	AJ313191
Claudin-1	F: CACCCTTGGCATGAAGTGTA R: AGCCAATGAAGAGAGCCTGA	HM117762.1
iNOS	F: TGAGAATGGCAGCTACTGGGTCAA R: GGTGATGTCCAGGAAATGGGTGA	AF223942.1
eNOS	F: AGCATCACCTACGACACTCTG R: ACTGGTTGATGAAGTCCCTGG	NM_001129901.1
β -actin	F: GCTCTTCCAGCCGTCCTT R: TGAAGGTGGTCTCGTGAATGC	NM_001009784.1

CAT, catalase; GPx1, glutathione peroxidase 1; SOD2, superoxide dismutase 2; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO1, quinone oxidoreductase 1; MyD88, myeloid differentiation factor 88; TRAF-6, TNF receptor-associated factor 6; TLR, toll-like receptor; IL, interleukin, TNF- α , tumor necrosis factor α ; NF- κ B, nuclear factor kappa B (p65); ZO-1, zonula occludens-1; eNOS, epithelial NO synthase; iNOS, inducible NO synthase; F, forward; R, reverse.

Figures

Figure 1

Maternal BW, fetal BW, and small intestinal weights in CON and RES ewes and in RES ewes supplemented with Arg or NCG on day 110 of gestation. Maternal BW (A), fetal weight (B), and fetal small intestine (C) were determined. BW, body weight; CON, ewes fed 100% of the National Research Council (NRC 1985)-recommended pregnancy nutrient requirements; RES, ewes fed 50% of NRC (1985) recommendations for pregnancy; RES+ARG, ewes fed 50% of NRC (1985) recommendations and supplemented with 20 g per day RP-Arg; RES+NCG, ewes fed 50% of NRC (1985) recommendations and supplemented with 5 g per day NCG. Values are means, with standard errors represented by vertical bars ($n = 8/\text{group}$ for ewes, $n = 16/\text{group}$ for the fetus). Labeled means without a common letter differ, $P < 0.05$.

Figure 2

Fetal villus morphology in the jejunum in control and RES ewes and in RES ewes supplemented with Arg or NCG on day 110 of gestation. Villous height and crypt depth (A), and VCR and number per villus (B) were determined. VCR, villous height: crypt depth ratio; CON, ewes fed 100% of the National Research Council (NRC 1985)-recommended pregnancy nutrient requirements; RES, ewes fed 50% of NRC (1985) recommendations for pregnancy; RES+ARG, ewes fed 50% of NRC (1985) recommendations and supplemented with 20 g per day RP-Arg; RES+NCG, ewes fed 50% of NRC (1985) recommendations and supplemented with 5 g per day NCG. Values are means, with standard errors represented by vertical bars ($n = 8/\text{group}$ for ewes, $n = 16/\text{group}$ for the fetus). Labeled means without a common letter differ, $P < 0.05$.

Figure 3

Fetal plasma insulin concentration, the content of NO, and activity of NOS in control and RES ewes and in RES ewes supplemented with Arg or NCG on day 110 of gestation. Insulin, NO and NOS (A) were determined. NO, nitric oxide; NOS, NO synthase; CON, ewes fed 100% of the National Research Council (NRC 1985)-recommended pregnancy nutrient requirements; RES, ewes fed 50% of NRC (1985) recommendations for pregnancy; RES+ARG, ewes fed 50% of NRC (1985) recommendations and supplemented with 20 g per day RP-Arg; RES+NCG, ewes fed 50% of NRC (1985) recommendations and supplemented with 5 g per day NCG. Values are means, with standard errors represented by vertical bars ($n = 8/\text{group}$ for ewes, $n = 16/\text{group}$ for the fetus). Labeled means without a common letter differ, $P < 0.05$.

Figure 4

Fetal jejunal protein concentration, insulin, IGF-1 level, the content of NO, and activity of NOS in control and RES ewes and in RES ewes supplemented with Arg or NCG on day 110 of gestation. Protein, insulin and NO (A), and IGF-1 and NOS (B) were determined. IGF-1, insulin-like growth factor 1; NO, nitric oxide; NOS, NO synthase; CON, ewes fed 100% of the National Research Council (NRC 1985)-recommended pregnancy nutrient requirements; RES, ewes fed 50% of NRC (1985) recommendations for pregnancy; RES+ARG, ewes fed 50% of NRC (1985) recommendations and supplemented with 20 g per day RP-Arg; RES+NCG, ewes fed 50% of NRC (1985) recommendations and supplemented with 5 g per day NCG. Values are means, with standard errors represented by vertical bars (n = 8/group for ewes, n = 16/group for the fetus). Labeled means without a common letter differ, P < 0.05.

Figure 5

The cytokine concentrations in the plasma and jejunum of the fetus in control and RES ewes and in RES ewes supplemented with Arg or NCG on day 110 of gestation. TNF- α , IL-1 and IL-6 concentrations in the fetal plasma (A) and jejunum (B) were determined. TNF- α , tumor necrosis factor α ; IL, interleukin; CON, ewes fed 100% of the National Research Council (NRC 1985)-recommended pregnancy nutrient requirements; RES, ewes fed 50% of NRC (1985) recommendations for pregnancy; RES+ARG, ewes fed 50% of NRC (1985) recommendations and supplemented with 20 g per day RP-Arg; RES+NCG, ewes fed 50% of NRC (1985) recommendations and supplemented with 5 g per day NCG. Values are means, with standard errors represented by vertical bars ($n = 8/\text{group}$ for ewes, $n = 16/\text{group}$ for the fetus). Labeled means without a common letter differ, $P < 0.05$.

Figure 6

The antioxidant activity in the plasma and jejunum of the fetus in control and RES ewes and in RES ewes supplemented with Arg or NCG on day 110 of gestation. T-AOC, MDA, GSH-Px and SOD activities in the fetal plasma and jejunum (A; B) were determined. T-AOC, total antioxidant capacity; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CON, ewes fed 100% of the National Research Council (NRC 1985)-recommended pregnancy nutrient requirements; RES, ewes fed 50% of NRC (1985) recommendations for pregnancy; RES+ARG, ewes fed 50% of NRC (1985) recommendations and supplemented with 20 g per day RP-Arg; RES+NCG, ewes fed 50% of NRC (1985) recommendations and supplemented with 5 g per day NCG. Values are means, with standard errors represented by vertical bars (n = 8/group for ewes, n = 16/group for the fetus). Labeled means without a common letter differ, P < 0.05.

Figure 7

The mRNA abundance of genes in the jejunum of the fetus in control and RES ewes and in RES ewes supplemented with Arg or NCG on day 110 of gestation. The mRNA abundance of antioxidant related genes (A), immune function-related genes (B), intestinal integrity-related genes (C), and nitric oxide-dependent pathway-related genes (D) were determined. CAT, catalase; GPx1, glutathione peroxidase 1; SOD2, superoxide dismutase2; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO1, quinone oxidoreductase 1; MyD88, myeloid differentiation factor 88; TRAF-6, tumor necrosis factor receptor-associated factor 6; TLR, toll-like receptor; IL, interleukin, TNF-α, tumor necrosis factor α; NF-κB, nuclear factor kappa B (p65); ZO-1, zonula occludens-1; iNOS, inducible NO synthase; eNOS, epithelial NO synthase; CON, ewes fed 100% of the National Research Council (NRC 1985)-recommended pregnancy nutrient requirements; RES, ewes fed 50% of NRC (1985) recommendations for pregnancy; RES+ARG, ewes fed 50% of NRC (1985) recommendations and supplemented with 20 g per day RP-Arg; RES+NCG, ewes fed 50% of NRC (1985) recommendations and supplemented with 5 g per day NCG. Values are

means, with standard errors represented by vertical bars (n = 8/group for ewes, n = 16/group for the fetus). Labeled means without a common letter differ, P < 0.05.

Figure 8

Fetal jejunal antioxidant defense-related protein expression in control and RES ewes and in RES ewes supplemented with Arg or NCG on day 110 of gestation. Representative charts of Western blot results (A) and CAT, GPx1, SOD2, Nrf2, HO-1, and NQO1 (B) were determined. CAT, catalase; GPx1, glutathione peroxidase 1; SOD2, superoxide dismutase2; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO1, quinone oxidoreductase 1; CON, ewes fed 100% of the National Research Council (NRC 1985)-recommended pregnancy nutrient requirements; RES, ewes fed 50% of NRC (1985) recommendations for pregnancy; RES+ARG, ewes fed 50% of NRC (1985) recommendations and supplemented with 20 g per day RP-Arg; RES+NCG, ewes fed 50% of NRC (1985) recommendations and supplemented with 5 g per day NCG. Values are means, with standard errors represented by vertical bars (n = 8/group for ewes, n = 16/group for the fetus). Labeled means without a common letter differ, P < 0.05.

Figure 9

Fetal jejunal immune function related protein expression in control and RES ewes and in RES ewes supplemented with Arg or NCG on day 110 of gestation. Representative charts of Western blot results (A) and TNF- α , IL-1 β , p65 and p-p65 (B) were determined. IL, interleukin; TNF- α , tumor necrosis factor α ; NF- κ B, nuclear factor kappa B (p65); CON, ewes fed 100% of the National Research Council (NRC 1985)-recommended pregnancy nutrient requirements; RES, ewes fed 50% of NRC (1985) recommendations for pregnancy; RES+ARG, ewes fed 50% of NRC (1985) recommendations and supplemented with 20 g per day RP-Arg; RES+NCG, ewes fed 50% of NRC (1985) recommendations and supplemented with 5 g per day NCG. Values are means, with standard errors represented by vertical bars ($n = 8/\text{group}$ for ewes, $n = 16/\text{group}$ for the fetus). Labeled means without a common letter differ, $P < 0.05$.

Figure 10

Fetal jejunal integrity-related protein expression in control and RES ewes and in RES ewes supplemented with Arg or NCG on day 110 of gestation. Representative charts of Western blot results (A) and ZO-1 and claudin-1 (B) were determined. ZO-1, zonula occludens-1; CON, ewes fed 100% of the National Research Council (NRC 1985)-recommended pregnancy nutrient requirements; RES, ewes fed 50% of NRC (1985) recommendations for pregnancy; RES+ARG, ewes fed 50% of NRC (1985) recommendations and supplemented with 20 g per day RP-Arg; RES+NCG, ewes fed 50% of NRC (1985) recommendations and supplemented with 5 g per day NCG. Values are means, with standard errors represented by vertical bars ($n = 8/\text{group}$ for ewes, $n = 16/\text{group}$ for the fetus). Labeled means without a common letter differ, $P < 0.05$.

Figure 11

Fetal jejunal nitric oxide-dependent pathway-related protein expression in control and RES ewes and in RES ewes supplemented with Arg or NCG on day 110 of gestation. Representative charts of Western blot results (A) and iNOS and eNOS (B) were determined. iNOS, inducible NO synthase; eNOS, epithelial NO synthase; CON, ewes fed 100% of the National Research Council (NRC 1985)-recommended pregnancy nutrient requirements; RES, ewes fed 50% of NRC (1985) recommendations for pregnancy; RES+ARG, ewes fed 50% of NRC (1985) recommendations and supplemented with 20 g per day RP-Arg; RES+NCG, ewes fed 50% of NRC (1985) recommendations and supplemented with 5 g per day NCG. Values are means, with standard errors represented by vertical bars (n = 8/group for ewes, n = 16/group for the fetus). Labeled means without a common letter differ, $P < 0.05$.