Intrauterine growth retardation promotes fetal intestinal autophagy in rats via the mechanistic target of rapamycin pathway

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Abstract. Intrauterine growth retardation (IUGR) impairs fetal intestinal development, and is associated with high perinatal morbidity and mortality. However, the mechanism underlying this intestinal injury is largely unknown. We aimed to investigate this mechanism through analysis of intestinal autophagy and related signaling pathways in a rat model of IUGR. Normal weight (NW) and IUGR fetuses were obtained from primiparous rats via ad libitum food intake and 50% food restriction, respectively. Maternal serum parameters, fetal body weight, organ weights, and fetal blood glucose were determined. Intestinal apoptosis, autophagy, and the mechanistic target of rapamycin (mTOR) signaling pathway were analyzed. The results indicated that maternal 50% food restriction reduced maternal serum glucose, bilirubin, and total cholesterol and produced IUGR fetuses, which had decreased body weight; blood glucose; and weights of the small intestine, stomach, spleen, pancreas, and kidney. Decreased Bcl-2 and increased Casp9 mRNA expression was observed in IUGR fetal intestines. Analysis of intestinal autophagy showed that the mRNA expression of WIPI1, MAP1LC3B, Atg5, and Atg14 was also increased, while the protein levels of p62 were decreased in IUGR fetuses. Compared to NW fetuses, IUGR fetuses showed decreased mTOR protein levels and enhanced mRNA expression of ULK1 and Beclin1 in the small intestine. In summary, the results indicated that maternal 50% food restriction on gestational days 10–21 reduced maternal serum glucose, bilirubin, and total cholesterol contents, and produced IUGR fetuses that had low blood glucose and reduced small intestine weight. Intestinal injury of IUGR fetuses caused by maternal food restriction might be due to enhanced apoptosis and autophagy via the mTOR signaling pathway.

Key words: Autophagy, Intestinal injury, Intrauterine growth retardation, Maternal food restriction, mTOR

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I ntrauterine growth retardation (IUGR) is defined as a fetal or birth weight of less than the 10th percentile in a given population or less than 2 SD of the mean body weight at the same gestational age [1, 2], and it is closely associated with inhibition of embryonic/fetal development, smaller organs, and higher perinatal morbidity and mortality [3, 4]. In America, more than 8% of infants have IUGR, which is caused by many factors, including maternal undernutrition, genetics, environmental stress, and dysfunction of the placenta or uterus. The rate of IUGR in pigs is 5–10% [5–7]. IUGR is thought to be a prevalent, severe problem both in humans and in animals used for production, especially multiparous animals.

The small intestine is an important organ for both immunity and nutrient absorption, and IUGR is closely associated with intestinal injuries, such as necrotizing enterocolitis [8, 9]. Because of ethical restrictions, studies on infant pathologies are commonly dependent on the use of appropriate animal models, such as pig and rat. Our previous studies revealed that IUGR affects intestinal growth and morphology in neonatal piglets and alters the gene expression of growth-related proteins [10, 11]. D’Inca et al. [12] showed that IUGR reduced the intestinal structure, leading to a longer and thinner small intestine in piglets and reduced villous size in term IUGR piglets. IUGR fetuses from 60% maternal food-restricted ewes had reduced body weights and small intestine weights as well as lower protein and protein:DNA contents in the jejunum [13]. Wang et al. [14] reported that IUGR affected small intestinal mucosal permeability and the mRNA expression of redox-sensitive genes. Altered intestinal enzymes (sucrase and maltase) and proteomes have been demonstrated in IUGR piglets, and feeding improved postnatal intestinal adaptation and necrotizing enterocolitis in preterm IUGR pigs [12, 15, 16]. However, the mechanism underlying intestinal injury in IUGR neonates and fetuses is still largely unknown.

Many factors can affect fetal intestinal development, and the largest contributors are nutrients, oxygen, and growth factors (e.g., insulin and insulin-like growth factors [IGFs]) [17–19]. Glucose is the most important source of fuel for oxidation in tissues, and it is mainly supplied by maternal glucose and transported through placenta to the fetus with the help of IGFs, especially IGF-1 and IGF-2 [17, 19]. The proportion of energy produced via anaerobic metabolic pathways from glucose alone in the rat small intestine is 78%, whereas that produced from glucose and glutamine is 95% [20]. During pregnancy, multiple adaptations occur in organs and tissues, such as the liver and adipose tissue, to enhance glucose synthesis, attenuate glucose utilization, and provide adequate uteroplacental blood glucose for normal fetal growth [17, 21]. Once maternal uteroplacental uptake
of glucose is decreased, fetal blood glucose will decrease, which subsequently activates fetal endogenous gluconeogenesis from the principal substrates (e.g., amino acids) [17]. Mechanistic target of rapamycin (mTOR) is a critical sensor of nutritional status and growth factors, and can modulate autophagy via Beclin1 or ULK1 in mammals [22, 23]. Although adequate autophagy can improve cell and organ survival, excessive autophagy may lead to cell death and tissue injury [24, 25].

Recently, Xia et al. [26] demonstrated that renal injury in IUGR rat fetuses was associated with mTOR-Beclin1 signaling-induced autophagy, which might enhance renal apoptosis and inhibit renal development. Wang et al. [11] also reported that dietary L-arginine improved intestinal development by increasing mucosal mTOR signaling in IUGR piglets. Based on these observational studies, we hypothesized that intestinal injury in IUGR fetuses may be associated with autophagy via the mTOR signaling pathway. To test this hypothesis, a rat model of IUGR was established by maternal 50% food restriction during late gestation. The IUGR offspring from these dams had restricted growth performance, impaired organs, and showed postnatal catch-up growth, similar to preterm IUGR infants and piglets [4, 12, 16, 27]. Once the decreased small intestine weight showed postnatal catch-up growth, similar to preterm IUGR infants FR dams had restricted growth performance, impaired organs, and tissue injury [24, 25].

Materials and Methods

Ethical procedures

The study was approved by and conducted under the supervision of the Institutional Animal Care and Use Committee of Nanjing Agricultural University, China.

Animals and experimental design

Primiparous Sprague Dawley rats obtained from the Experimental Animal Center of Soochow University (Jiangsu, China) were used to construct a rat model of IUGR as described previously [4, 27]. Briefly, the rats were housed in a temperature-controlled facility (20 ± 2°C) under a 12-h light-dark cycle. On day 10 of gestation, 16 healthy pregnant rats were randomly divided into 2 treatment groups, with 8 rats per group. From gestational day 10 to day 21, dams in the 2 treatment groups were fed commercial rat chow either ad libitum (Adlib) or at 50% food restriction (FR). On gestational day 21, the dams were anesthetized with sodium pentobarbital after a 4-h fast, the rats were housed in a temperature-controlled facility (20 ± 2°C) under a 12-h light-dark cycle. On day 10 of gestation, 16 healthy pregnant rats were randomly divided into 2 treatment groups, with 8 rats per group. From gestational day 10 to day 21, dams in the 2 treatment groups were fed commercial rat chow either ad libitum (Adlib) or at 50% food restriction (FR). On gestational day 21, the dams were anesthetized with sodium pentobarbital after a 4-h fast, and fetuses were obtained by cesarean section. Maternal serum samples were collected by centrifugation at 3000 × g for 15 min and stored at −80°C until analysis.

Analysis of maternal serum parameters

The concentrations of glucose, bilirubin, total cholesterol, and triglycerides in maternal serum were determined with commercial kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer’s instructions.

Determination of mRNA expression levels

Total RNA was extracted from the small intestine with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. After RNA quality was verified with a Nano-drop 2000 (as A260/A280 and A260/A230 values of 1.90–2.05) and by agarose gel electrophoresis, 2 µg of RNA was incubated with Random Primers (Promega, Belgium) at 72°C for 5 min. Then, a reverse transcription premix (TaKaRa, Dalian, China), containing 5× M-MLV-RT buffer, M-MLV reverse transcriptase, and dNTPs was added, and the mixture was incubated for 1 h. Finally, the reverse transcription reaction was inactivated by incubation at 85°C for 15 min.

The sequences of the primers used for the target genes (Bcl-2, Bax, Casp3, Casp9, WIP1b, MAP1LC3A, MAP1LC3B, Atg5, Atg14, ULK1, Beclin1, and GAPDH) are listed in Table 1. The housekeeping gene GAPDH was included as a control. Real-time PCR assays were conducted with an ABI 7500 RT-PCR system (Applied Biosystems, Foster City, CA, USA) using the SYBR Premix Ex Taq™ Kit (TaKaRa) according to the manufacturer’s instructions. Relative mRNA expression was determined with ABI software and calculated by the 2−ΔΔCt method as described by Livak and Schmittgen [28].

Western blotting analysis

Western blotting analysis was conducted as described by Xu et al. [29], with some modifications. Briefly, intestinal tissues were homogenized, and protein concentrations were determined with the BCA Protein Assay Kit (Beyotime, Jiangsu, China). Then, protein from each sample (20 µg) was separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skimmed milk in blocking buffer containing 0.1% TBST for 1.5 h at room temperature, and then incubated with primary antibodies against p62 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mTOR, and β-actin (Cell Signaling Technology, Danvers, MA, USA) for 12 h at 4°C. After three consecutive washes with TBST, the membranes were incubated with the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G; Cell Signaling Technology). Antibody-bound protein bands were detected using enhanced chemiluminescence reagents (ECL-Kit; Beyotime) followed by autoradiography. The blots were scanned using a LAS-4000 Luminescent Image Analyzer (Fuji Film, Tokyo, Japan), and the antigen-antibody complexes were quantified with Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

All data were evaluated with Student’s t-test using the SPSS statistical package for Windows (Version 20.0; SPSS, Chicago, IL, USA). Results are shown as means ± SE. P values less than 0.05 were considered statistically significant.
Results

**Maternal serum parameters**

As shown in Table 2, triglyceride levels were not affected by food restriction (P > 0.05). In contrast, the concentrations of glucose, bilirubin, and total cholesterol in the maternal serum of rats from the FR group were significantly lower than those in rats from the Adlib group (P < 0.01).

**Fetal body weight and blood glucose**

As shown in Fig. 1, IUGR fetuses from FR dams had significantly (P < 0.01) lower body weights (Fig. 1A) and blood glucose concentrations (Fig. 1B) than normal weight (NW) fetuses from Adlib dams.

**Weights of selected organs**

The absolute and relative weights of selected organs were presented in Table 3. The results indicated that there was no difference in the weights of the brains between the two groups (P > 0.05). However, the absolute and relative weights of the spleen, pancreas, kidney, small intestine, and stomach in IUGR fetuses were significantly lower than those in normal weight NW fetuses (P < 0.01). The absolute
Heart weight of IUGR fetuses was lower than that of NW fetuses (P < 0.05), whereas there was no difference in the relative weight of the heart between the two groups (P > 0.05).

| Table 3. Selected organ weights in IUGR and NW fetuses |
|---------------------------------|---|---|---|
| Organ 1  | NW      | IUGR    | P    |
| Absolute weight, g          |     |         |      |
| Brain                          | 0.212 ± 0.014 | 0.201 ± 0.003 | 0.47 |
| Heart                          | 0.037 ± 0.002 | 0.031 ± 0.001 * | 0.01 |
| Spleen                         | 0.023 ± 0.002 | 0.013 ± 0.002 ** | < 0.01 |
| Pancreas                        | 0.041 ± 0.004 | 0.021 ± 0.001 ** | < 0.01 |
| Kidney                          | 0.067 ± 0.003 | 0.048 ± 0.003 ** | < 0.01 |
| Small intestine                 | 0.132 ± 0.006 | 0.091 ± 0.003 ** | < 0.01 |
| Stomach                         | 0.052 ± 0.004 | 0.027 ± 0.002 ** | < 0.01 |
| Relative weight, %             |     |         |      |
| Brain                          | 3.67 ± 0.23 | 4.04 ± 0.10 | 0.15 |
| Heart                          | 0.64 ± 0.03 | 0.63 ± 0.02 | 0.78 |
| Spleen                         | 0.40 ± 0.04 | 0.26 ± 0.03 ** | < 0.01 |
| Pancreas                        | 0.70 ± 0.07 | 0.42 ± 0.02 ** | < 0.01 |
| Kidney                          | 1.17 ± 0.04 | 0.95 ± 0.04 ** | < 0.01 |
| Small intestine                 | 2.30 ± 0.10 | 1.82 ± 0.05 ** | < 0.01 |
| Stomach                         | 0.91 ± 0.07 | 0.53 ± 0.03 ** | < 0.01 |

1 Data are expressed as mean ± SE (n = 16); single and double asterisks indicate a significant (* P < 0.05 and ** P < 0.01) difference between the IUGR and NW groups.

The mRNA expression of apoptosis-related genes in the small intestine

The mRNA expression levels of Bcl-2, Bax, Casp3, and Casp9 in the small intestine are shown in Fig. 2, and the results showed that IUGR did not affect the mRNA expression of Bax and Casp3 (P > 0.05). However, Bcl-2 and Casp9 mRNA expression levels in the small intestine of IUGR fetuses were significantly lower and higher, respectively, than the corresponding expression levels in NW fetuses (P < 0.01).

Intestinal autophagy analysis

As shown in Fig. 3, the mRNA expression levels of WIPI1, MAP1LC3B, Atg5, and Atg14 in the small intestine of IUGR fetuses were significantly higher than those in the small intestine of NW fetuses (P < 0.05), whereas there was no difference in the MAP1LC3A mRNA expression levels between the IUGR and NW groups (P > 0.05). Consistently, p62 protein levels were significantly lower in the small intestine of IUGR fetuses than those in NW fetuses (Fig. 4).

The mTOR signaling pathway

As shown in Fig. 5, IUGR fetuses had lower mTOR protein levels in the small intestine than NW fetuses (P < 0.05). ULK1 and Beclin1 mRNA expression levels in the small intestine of IUGR fetuses were significantly higher than those in NW fetuses (P < 0.05).
Discussion

In the present study, we showed that maternal 50% food restriction in late gestation reduced maternal serum glucose, bilirubin, and total cholesterol levels and that these dams produced IUGR fetuses with impaired organs, including the small intestine. To our knowledge, this is the first study demonstrating that intestinal injury in IUGR fetuses might be due to enhanced autophagy via the mTOR signaling pathway. As there are current clinical trials seeking strategies to manipulate autophagy [30–32], our findings may provide information for early diagnosis of IUGR and could be beneficial for preventing intestinal diseases and improving the growth and development of IUGR infants and animals.

Maternal nutrition is critical for fetuses, as the nutrients and biologically active substances transferred through the placenta affect fetal development [7, 33, 34]. Gupta et al. [35] showed that maternal magnesium deficiency in mice caused fetal growth restriction and altered lipid metabolism. Conversely, maternal magnesium supplementation reduced the IUGR rate in a rat model of bilateral uterine artery ligation-induced IUGR [36]. Protein restriction in maternal rats or sows has been used as a model of IUGR [37, 38]. Desai et al. [37] demonstrated that maternal protein restriction caused organ-selective growth defects in IUGR rat offspring, which showed smaller decreases in lung and brain weight; proportionate reductions in the weights of the heart, kidney, and thymus; and greater decreases in the weights of the pancreas, spleen, muscle, and liver. Desai et al. [4, 39] demonstrated that IUGR newborns had reduced leptin and increased plasma ghrelin levels and showed postnatal catch-up growth and programmed obesity. In agreement with previous studies, we found that maternal food restriction in late gestation (gestational days 10–21) caused IUGR, and these fetuses showed decreased organ weights (small intestine, stomach, spleen, pancreas, and kidney). Meyer et al. [40] showed that nutrient restriction during early to mid-gestation significantly decreased the maternal total gastrointestinal tract as well as omasal and pancreatic weights, but it did not affect fetal body weight or the weights of various organs, such as the gastrointestinal tract, liver, and pancreas. The authors suggested that the non-significant effects on fetal organ weights might be due to the insensitivity of these weights to maternal

Fig. 3. The mRNA expression levels of autophagy-related genes in the small intestine of IUGR and NW fetuses. The mRNA expression levels of WIP1, MAP1LC3B, MAP1LC3A, Atg5, and Atg14 were determined by qPCR. Data are expressed relative to the levels of the housekeeping gene GAPDH and normalized to the NW group (n = 12). * P < 0.05, ** P < 0.01.

Fig. 4. Protein expression of p62 in the small intestine of IUGR and NW fetuses. Data are expressed relative to β-actin and normalized to the NW group (n = 4). ** P < 0.01.
increased Bcl-2 and The results showed that IUGR fetuses had decreased the mRNA expression of apoptosis-related genes was determined. To further elucidate the molecular mechanism of intestinal impairment, small intestine in human fetuses and newborn piglets [10, 46]. To in IUGR fetuses, it has been reported that IUGR also impairs the cholesterol might be helpful markers for the diagnosis of IUGR. Recent studies, we found that IUGR fetuses showed reduced blood glucose concentrations, which could partially explain the decreased organ weights. In our study, maternal serum glucose, bilirubin, and total cholesterol in the FR group were significantly lower than those in the Adlib group, which was in agreement with the decreased fetal blood glucose level. Edison et al. [45] also showed that mothers with low cholesterol give birth to lower birth weight infants. These results suggested that the maternal serum glucose, bilirubin, and total cholesterol might be helpful markers for the diagnosis of IUGR.

In agreement with our observation of lower small intestine weights in IUGR fetuses, it has been reported that IUGR also impairs the small intestine in human fetuses and newborn piglets [10, 46]. To further elucidate the molecular mechanism of intestinal impairment, the mRNA expression of apoptosis-related genes was determined. The results showed that IUGR fetuses had decreased Bcl-2 and increased Casp9 mRNA expression in the small intestine, indicating that Casp9-related intestinal apoptosis was upregulated in IUGR fetuses. In agreement with our present study, Xia et al. [26] reported that renal Bel-2 protein was decreased and apoptosis was enhanced in hypoxia-induced IUGR fetuses. Additionally, inhibition of intestinal growth caused by uteroplacental insufficiency was associated with altered apoptosis in IUGR rat pups [47]. In a model of IUGR piglets, it was reported that intestinal growth impairment may be caused by a change in the balance between cell proliferation and apoptosis [48]. These observations suggest that intestinal impairment in IUGR is strongly linked to the expression of genes and proteins directly related to intestinal apoptosis [49–51].

Recently, it was revealed that the functional relationship between autophagy and apoptosis is complex, which may play a role in nervous system disorders [52–54]. Xia et al. [26] suggested that renal impairment in hypoxia-induced IUGR rat fetuses was associated with enhanced autophagy. Therefore, we analyzed intestinal autophagy in our model system. The results indicated that the mRNA expression levels of WIP11, MAP1LC3B, Atg5, and Atg14 were increased, and the p62 protein level was decreased in the small intestine of IUGR fetuses. Tsuyuki et al. [55] showed that the detection of WIP11 and MAP1LC3B mRNA is a convenient method for monitoring autophagosome formation in a wide range of cell types. The p62 protein (also known as sequestosome1/SQSTM1) is one of the most important specific substrates degraded in autophagy, and p62 protein levels are inversely correlated with autophagic activity [56–58]. Therefore, our autophagy analysis findings indicated that small intestinal autophagy was enhanced in IUGR fetuses. Moderate autophagy might provide amino acids through lysosomal protein degradation that can be used as substrates for fetal endogenous gluconeogenesis or protein synthesis for cell survival [17, 59]. However, excessive autophagy can lead to apoptosis and death, which could cause further organ impairment [24, 48]. As intestinal apoptosis was significantly enhanced in IUGR fetuses, the mechanism of intestinal injury might be associated with enhanced intestinal autophagy [26, 48, 51].

As a sensor of nutritional status (i.e., the levels of amino acids and glucose), stress, and growth factor signals (e.g., insulin and IGF-1), mTOR can regulate autophagy through direct phosphorylation of ULK1, which induces autophagy by phosphorylating Beclin1 and activating VPS34 lipid kinase [22, 60, 61]. Tsuyuki et al. [55] showed that ULK1 mRNA can be used as an indicator for monitoring autophagosome formation, and the mRNA expression of ULK1 and Beclin1 was increased as autophagy activity increased [58,60]. Wang et al. [11] reported that IUGR decreased the activities of Akt and
mTOR in the impaired small intestine of piglets, and Roos et al. [62] also found that IUGR reduced human placental mTOR activity. In addition, glucose and insulin/IGF-1 was shown to alter mTOR signaling to regulate placental transport of amino acids [22]. After we observed decreased blood glucose and enhanced intestinal autophagy in IUGR fetuses, we evaluated the mTOR signaling pathway. The results showed that IUGR decreased protein mTOR levels and enhanced the mTORC1 signaling pathway in the impaired small intestine of piglets and Roos of interest.

Conflict of interest: The authors declare that they have no conflict of interest.

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