

Effects of dietary L-methionine supplementation on intestinal integrity and oxidative status in intrauterine growth-retarded weanling piglets

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Abstract

Purpose The present study investigated whether dietary methionine supplementation might protect against intrauterine growth retardation (IUGR)-induced damage in the intestine of piglets.

Methods Thirty normal birth weight (NBW) female piglets and sixty same-sex IUGR piglets were weaned at 21 days of postnatal age and fed the control diet (4.0 g methionine per kg of feed, NBW-CON, and IUGR-CON groups) or the methionine-supplemented diet (5.2 g methionine per kg of feed, IUGR-MET group) for 28 days ($n = 6$).

Results Piglets in the IUGR-CON group showed decreased average daily feed intake and average daily gain and an increased feed conversion ratio than those in the NBW-CON group. Compared with NBW-CON piglets, IUGR-CON piglets had decreased villus height (VH) and villus height-to-crypt depth ratio in both the jejunum and ileum. In addition, in comparison with the NBW-CON piglets, IUGR increased the concentration of malondialdehyde (MDA) and the index of apoptosis, while it decreased the concentrations of methionine and reduced glutathione (GSH), the ratio of reduced glutathione/oxidized glutathione (GSH/GSSG), and the protein expression of occludin (OCLN) in both the jejunum and ileum. Dietary methionine supplementation decreased the MDA and protein carbonyl concentrations

and the apoptotic index, while it increased the VH level, methionine and GSH concentrations, GSH/GSSG ratio, and the OCLN protein expression in the jejunum of IUGR-MET piglets.

Conclusions Methionine may have beneficial effects in improving intestinal integrity and oxidative status in IUGR weanling piglets.

Keywords Methionine · Intrauterine growth retardation · Intestinal damage · Oxidative stress · Piglets

Introduction

Uteroplacental insufficiency, the major cause of intrauterine growth retardation (IUGR), limits the availability of critical substrates such as amino acids, glucose, and hormones to the fetus during the gestation period [1, 2]. When a fetus is exposed to a limited nutrient supply, the growth of the brain may be protected at the expense of other organs [3], such as the small intestine. However, the small intestine is an important organ responsible for the digestion, absorption, and metabolism of nutrients, including amino acids [4]. Accumulating evidence shows that IUGR is associated with abnormal intestinal mucosal growth, as indicated by decreased villus height (VH), villus surface area (VSA), and the ratio of villus height to crypt depth (VH/CD) [5, 6]. A previous study found that IUGR offspring exhibited increased levels of heat-shock proteins and glutathione S-transferase omega in the jejunum, which provide a line of evidence for the presence of oxidative stress during postnatal life [7]. In addition, several studies with rodents showed that oxidative stress may contribute to increased intestinal paracellular permeability by impairing intestinal integrity [8, 9]. Therefore, increasing the intestinal antioxidant capacity of

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IUGR animals is a potential approach to alleviate intestinal damage.

Methionine is a sulfur amino acid (SAA) with numerous biological functions, including protein metabolism, methylation, the synthesis of cysteine, and reduced glutathione (GSH), and as a component of antioxidant systems [10]. A recent report pointed out that a dietarily adequate methionine level improved intestinal integrity in weanling piglets [11]. The previous studies have verified that IUGR piglets have decreased feed intake compared with their heavier littermates [12, 13], and therefore, SAA is provided at insufficient levels. It has been reported that SAA deficiency could impair the growth and development of the small intestine [14]. SAA deficiency also significantly increased intestinal oxidative stress in piglets, as indicated by diminished cellular cysteine and GSH concentrations [15]. Therefore, increasing the methionine level in IUGR infants' diet may help to maintain normal growth and basic function in their intestines.

However, to our knowledge, data are lacking about the effects of dietary methionine supplementation in the intestine of IUGR infants. Thus, the current study was conducted to investigate the effects of dietary methionine supplementation on intestinal integrity and oxidative status in IUGR weanling piglets. In swine production, piglets usually weaned at 21 days [13, 16, 17]. Weaning is a critical process for piglets due to dramatic changes in diets and environment [18]. IUGR piglets have more severe weaning stress because of decreased feed intake compared with their heavier littermates [13]. Therefore, study on the potential role of methionine supplementation in regulating intestinal homeostasis in the IUGR weanling piglets may be helpful in solving the complications. Considering the biological similarity between humans and pigs [19], this study may provide some guidance to guarantee the appropriate development of IUGR offspring during the early periods after weaning.

Materials and methods

Experimental design, diets, and management

All experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (NJAU-CAST-2015-098). Approximately 90 healthy pregnant sows with similar expected dates of confinement and parity (second or third) were chosen during pregnancy. In each litter, one newborn normal birth weight (NBW) (~ 1.52 kg) and two IUGR (~ 0.87 kg) female piglets were carefully selected based on guidelines laid out in the previous studies [20, 21]. At weaning (21 days of postnatal age), 30 pairs of NBW (6.55 ± 0.14 kg) and IUGR (4.85 ± 0.10 kg) piglets were allocated to receive

a control diet (4.0 g methionine per kg of feed, NBW-CON, and IUGR-CON groups), and the remaining IUGR (4.83 ± 0.08 kg) piglets were allocated to receive a methionine-supplemented diet (5.2 g methionine per kg of feed, IUGR-MET group) for 28 days. Thereafter, all piglets were divided into three treatments \times six replicates (pens) \times five piglets per replicate. The inclusion of methionine in the control diet was determined according to the recommendation of National Research Council (2012) [22], while the inclusion of methionine in the diet for IUGR-MET piglets was confirmed according to an independent study by colleagues. A total of 200 21-day-old NBW female piglets were randomly distributed into five treatments, and each treatment consisted of five replicates with eight piglets per replicate. Piglets were fed a control diet (4.0 g methionine per kg of feed) supplemented with 0, 0.6, 1.2, 1.8, and 2.4 g methionine per kg diet, respectively. In the preliminary study, the optimum effects of methionine on growth performance and plasma antioxidant capacity of piglets were observed when its inclusion was 5.2 g/kg of the diet (unpublished). The dietary supplementation of methionine was achieved by supplementing L-methionine (99%; CJ CheilJedang, Seoul, Korea) into the piglets' feed, replacing the equivalent weight of L-alanine; the concentrations of other nutrients were maintained at a constant level in all experimental diets. The composition and nutrient levels of the diets are shown in Supplementary Table 1. The average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) of piglets were recorded carefully.

Sample collection

At 49 days of postnatal age, the piglet whose weight was the nearest to the average weight of each pen was selected (one pig per pen). The piglets were sacrificed by intramuscular injection of sodium pentobarbital (50 mg/kg body weight) at 8 h after the last meal. The entire small intestine starting from the pyloric sphincter to the ileocecal valve was removed from the abdominal cavity and divided into three segments, including the duodenum, jejunum, and ileum. The jejunal and ileal segments were immediately flushed with ice-cold physiological saline to remove luminal contents, and the mesenteric attachments were carefully removed. Sections of approximately 1 cm in length were carefully collected from the mid of each segment, and fixed in 4% (w/v) paraformaldehyde in 100 mmol/L phosphate-buffered saline (PBS), pH 7.4 for 24 h for histological analyses. Jejunal and ileal mucosae were scraped from the rest of the tissue using a glass microscope slide. The intestinal mucosae were then rapidly frozen in liquid nitrogen and stored at -80 °C for further analysis.

Determination of intestinal amino acids

Approximately 150 mg of jejunal and ileal samples were weighed and detected using a Hitachi L-8900 amino acid analyzer (Hitachi, Tokyo, Japan) as described in a previous study [23]. The amount of each amino acid in the samples was calculated with reference to their corresponding standard solution. Intestinal amino acid concentrations included both protein-bound and free amino acids and were expressed as g/100 g wet weight.

Intestinal morphology analysis

The jejunal and ileal samples fixed in 4% paraformaldehyde were dried using a graded series of xylene and ethanol, and embedded in paraffin. The samples (5 μm) were then deparaffinized using xylene and rehydrated with graded dilutions of ethanol. The slides were stained with hematoxylin and eosin. Eight slides for each tissue were prepared, and the images were acquired using an optical binocular microscope with a digital camera (Nikon ECLIPSE 80i, Tokyo, Japan). The morphological measurements of the VH, villus width (VW), crypt depth (CD), and VH/CD ratio were calculated using the Image-Pro Plus software, and the VSA was calculated using the previously reported equations [24]:

$$VSA = \pi \times \frac{VW}{2} \sqrt{\left(\frac{VW}{2}\right)^2 + VH^2}.$$

Measurement of intestinal enzyme activities

The activities of the sucrase, maltase, lactase, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase (GR) and the concentrations of malondialdehyde (MDA), protein carbonyls, GSH, and oxidized glutathione (GSSG) were determined using the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). All results were normalized to total protein concentration in each sample for inter-sample comparison. The protein concentrations were quantified using the bicinchoninic acid protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

TUNEL staining

Apoptosis was evaluated using a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay with the TUNEL Bright-Green Apoptosis Detection Kit (Vazyme Biotech, Nanjing, Jiangsu, China). In brief, the jejunal and ileal specimens were de-waxed and incubated with Proteinase K (20 μg/ml) for 20 min at room temperature. The specimens were then

treated with the terminal deoxynucleotidyl transferase (TdT) buffer including BrightGreen Labeling Mix and recombinant TdT enzyme for 60 min at 37 °C followed by a thorough washing with PBS to stop the reaction. Finally, the specimens were stained with 4,6-diamidino-2-phenylindole solution (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) for 5 min to detect cell nuclei. The number of positive cells was counted using an LSM 700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). The apoptotic index was defined as the ratio of apoptotic cells to total cells.

Total RNA extraction and mRNA quantification

Total RNA was extracted from frozen intestinal mucosae using the TRIzol Reagent (Invitrogen Life Technologies, Gaithersburg, MD, USA). After the determination of RNA concentration, mRNA was reverse-transcribed into complementary DNA (cDNA) using a reverse transcription kit (TaKaRa Biotechnology, Dalian, Liaoning, China). Real-time PCR was performed using the ABI StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The reaction mixture was made by adding 0.4 μL of each of forward and reverse primers, 0.4 μL of ROX Reference Dye (TaKaRa Biotechnology, Dalian, Liaoning, China), 10 μL of SYBR Premix Ex Taq™ (TaKaRa Biotechnology, Dalian, Liaoning, China), 6.8 μL of double-distilled H₂O, and 2 μL of cDNA template. Each sample was assayed in triplicate. The reaction condition was as follows: 30 s at 95 °C, 40 cycles of 5 s at 95 °C, and 30 s at 60 °C. The relative mRNA expression levels were calculated by the 2^{-ΔΔCt} method after normalization against the reference gene β-actin [25]. The values of NBW-CON group were used as a calibrator. The primer sequences for superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPx1), occludin (OCLN), claudin-2 (CLDN-2), zonula occludens-1 (ZO-1), B-cell lymphoma/leukaemia 2 (Bcl-2), Bcl-2-associated X protein (Bax), and β-actin are shown in Supplementary Table 2.

Western blot

Proteins were extracted from approximately 40 mg of frozen intestinal mucosae by grinding with RIPA lysis buffer (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) and phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China). The protein concentrations were measured using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China). Equal amounts of protein (40 μg/lane) were electrophoresed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred on to polyvinylidene difluoride membranes. After blocking with Tris-buffered

saline Tween-20 buffer (TBST; 0.1% Tween-20, 100 mmol/L Tris-HCl, and 150 mmol/L NaCl, pH 8.0) containing 5% non-fat dry milk for 90 min at room temperature, the membranes were washed in TBST three times and incubated overnight with the primary antibodies. The primary antibodies were OCLN (1:1000; Novus Biologicals, Littleton, CO, USA) and β -actin (1:1000; Cell Signaling Technology, Danvers, MA, USA). The membranes were washed in TBST three times and processed with a secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG, 1:2000; Cell Signaling Technology, Danvers, MA, USA) for 60 min at room temperature. The blots were developed using an enhanced chemiluminescence reagents (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) followed by autoradiography. Images were recorded with a Luminescent Image Analyzer LAS-4000 system (Fujifilm, Tokyo, Japan) and quantified by Gel-Pro Analyzer 4.0 software (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

Data were analyzed using SPSS 16.0 statistical software (SPSS, Chicago, IL, USA). Statistical differences between different groups were determined via one-way analysis of variance and Tukey's post hoc test for multiple comparisons. *P* values less than 0.05 were considered statistically significant. Results are expressed as mean \pm SE.

Results

Growth performance

IUGR decreased ($P < 0.05$) the ADFI and ADG and increased the FCR of piglets in the IUGR-CON group when compared with the NBW-CON group (Table 1). There were no significant differences ($P > 0.05$) in the ADFI, ADG, and

Table 1 Effects of dietary L-methionine supplementation on growth performance in intrauterine growth-retarded weanling piglets

Items	NBW-CON	IUGR-CON	IUGR-MET
ADFI (g/d)	572 \pm 16 ^a	422 \pm 15 ^b	445 \pm 15 ^b
ADG (g/d)	385 \pm 11 ^a	252 \pm 11 ^b	287 \pm 10 ^b
FCR	1.49 \pm 0.04 ^b	1.68 \pm 0.05 ^a	1.55 \pm 0.04 ^{ab}

Data are presented as mean \pm SE, *n* = 6/group

Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$)

NBW-CON normal birth weight group given a control diet, *IUGR-CON* intrauterine growth retardation group given a control diet, *IUGR-MET* intrauterine growth retardation group given a methionine-supplemented diet, *ADFI* average daily feed intake, *ADG* average daily gain, *FCR* feed conversion ratio

FCR of piglets in the IUGR-MET group when compared with the IUGR-CON group.

Intestinal concentrations of amino acids

In comparison with NBW-CON piglets, IUGR decreased ($P < 0.05$) the concentrations of methionine, cysteine, and valine in the jejunum of IUGR-CON piglets (Table 2). In addition, IUGR-CON piglets showed a decrease ($P < 0.05$) in the concentration of methionine in the ileum compared with NBW-CON piglets. Dietary methionine supplementation increased ($P < 0.05$) the concentration of methionine in the jejunum of IUGR-MET piglets. There were no differences in other parameters among the groups ($P > 0.05$).

Intestinal mucosal morphology

IUGR induced obvious decreases ($P < 0.05$) in VH and VH/CD ratio in both the jejunum and ileum of IUGR-CON piglets compared with the NBW-CON data (Table 3). Compared with NBW-CON piglets, IUGR-CON piglets had decreased ($P < 0.05$) VSA in the jejunum. An increased

Table 2 Effects of dietary L-methionine supplementation on amino acid concentrations in the intestine of intrauterine growth-retarded weanling piglets (g/100 g wet weight)

Items	NBW-CON	IUGR-CON	IUGR-MET
Jejunum			
Methionine	0.24 \pm 0.01 ^a	0.19 \pm 0.01 ^b	0.24 \pm 0.01 ^a
Cysteine	0.22 \pm 0.01 ^a	0.17 \pm 0.01 ^b	0.19 \pm 0.01 ^{ab}
Lysine	0.97 \pm 0.03	1.01 \pm 0.05	0.98 \pm 0.04
Threonine	0.57 \pm 0.02	0.56 \pm 0.02	0.60 \pm 0.02
Leucine	1.09 \pm 0.05	0.99 \pm 0.04	1.06 \pm 0.03
Isoleucine	0.50 \pm 0.02	0.53 \pm 0.03	0.49 \pm 0.04
Valine	0.69 \pm 0.03 ^a	0.58 \pm 0.02 ^b	0.60 \pm 0.04 ^{ab}
Phenylalanine	0.47 \pm 0.02	0.48 \pm 0.03	0.47 \pm 0.02
Ileum			
Methionine	0.23 \pm 0.02 ^a	0.17 \pm 0.01 ^b	0.19 \pm 0.01 ^{ab}
Cysteine	0.21 \pm 0.01	0.20 \pm 0.02	0.19 \pm 0.01
Lysine	0.94 \pm 0.04	0.99 \pm 0.05	1.00 \pm 0.03
Threonine	0.54 \pm 0.02	0.55 \pm 0.02	0.60 \pm 0.03
Leucine	1.04 \pm 0.05	1.00 \pm 0.03	1.05 \pm 0.03
Isoleucine	0.49 \pm 0.03	0.52 \pm 0.04	0.48 \pm 0.04
Valine	0.63 \pm 0.03	0.60 \pm 0.02	0.61 \pm 0.03
Phenylalanine	0.48 \pm 0.02	0.49 \pm 0.02	0.47 \pm 0.02

Data are presented as mean \pm SE, *n* = 6/group

Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$)

NBW-CON normal birth weight group given a control diet, *IUGR-CON* intrauterine growth retardation group given a control diet, *IUGR-MET* intrauterine growth retardation group given a methionine-supplemented diet

Table 3 Effects of dietary L-methionine supplementation on villus morphology in the intestine of intrauterine growth-retarded weanling piglets

Items	NBW-CON	IUGR-CON	IUGR-MET
Jejunum			
Villus height (μm)	465 \pm 20 ^a	377 \pm 17 ^b	442 \pm 15 ^a
Villus width (μm)	88 \pm 4	84 \pm 2	85 \pm 2
Crypt depth (μm)	193 \pm 9	201 \pm 13	214 \pm 9
Villus height-to-crypt depth ratio	2.51 \pm 0.08 ^a	1.98 \pm 0.13 ^b	2.14 \pm 0.06 ^b
Villous surface area (mm^2)	0.065 \pm 0.005 ^a	0.050 \pm 0.003 ^b	0.060 \pm 0.003 ^{ab}
Ileum			
Villus height (μm)	348 \pm 7 ^a	310 \pm 11 ^b	319 \pm 12 ^{ab}
Villus width (μm)	86 \pm 2	86 \pm 1	84 \pm 1
Crypt depth (μm)	174 \pm 4	181 \pm 3	169 \pm 3
Villus height-to-crypt depth ratio	2.08 \pm 0.04 ^a	1.76 \pm 0.05 ^b	1.97 \pm 0.09 ^{ab}
Villous surface area (mm^2)	0.047 \pm 0.001	0.042 \pm 0.002	0.043 \pm 0.002

Data are presented as mean \pm SE, $n = 6/\text{group}$

Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$)

NBW-CON normal birth weight group given a control diet, *IUGR-CON* intrauterine growth retardation group given a control diet, *IUGR-MET* intrauterine growth retardation group given a methionine-supplemented diet

Table 4 Effects of dietary L-methionine supplementation on disaccharidases activities in the intestine of intrauterine growth-retarded weanling piglets

Items	NBW-CON	IUGR-CON	IUGR-MET
Jejunum (U/mg protein)			
Sucrase	64.3 \pm 4.6	53.8 \pm 4.8	63.1 \pm 4.6
Maltase	271.7 \pm 11.3 ^a	203.6 \pm 15.2 ^b	264.0 \pm 19.7 ^a
Lactase	71.0 \pm 6.5	58.7 \pm 4.4	79.4 \pm 7.0
Ileum (U/mg protein)			
Sucrase	49.4 \pm 6.4 ^a	25.0 \pm 4.2 ^b	41.6 \pm 6.8 ^{ab}
Maltase	235.6 \pm 14.4	197.4 \pm 14.7	231.1 \pm 16.1
Lactase	39.7 \pm 6.1	36.2 \pm 4.1	42.9 \pm 4.2

Data are presented as mean \pm SE, $n = 6/\text{group}$

Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$)

NBW-CON normal birth weight group given a control diet, *IUGR-CON* intrauterine growth retardation group given a control diet, *IUGR-MET* intrauterine growth retardation group given a methionine-supplemented diet

($P < 0.05$) VH in the jejunum was observed in IUGR-MET piglets as compared with their IUGR-CON counterparts. There were no differences in other parameters among the groups ($P > 0.05$).

Disaccharidases activities

IUGR significantly decreased ($P < 0.05$) maltase activity in the jejunum of the IUGR-CON group in comparison with the NBW-CON group (Table 4). In addition, a remarkable decrease ($P < 0.05$) in sucrase activity was observed in the

ileum of IUGR-CON piglets. The IUGR-MET group had increased maltase activity ($P < 0.05$) in the jejunum when compared with the IUGR-CON data. There were no differences in other parameters among the groups ($P > 0.05$).

Intestinal oxidative status

The IUGR-CON piglets had significantly increased ($P < 0.05$) MDA and protein carbonyl concentrations and decreased ($P < 0.05$) GSH and GSSG concentrations in the jejunum in comparison with the NBW-CON piglets (Table 5). In addition, IUGR significantly increased ($P < 0.05$) the concentration of MDA, whereas it decreased ($P < 0.05$) the GSH and GSSG concentrations in the ileum of IUGR-CON piglets when compared with the NBW-CON piglets. In contrast, decreased ($P < 0.05$) MDA and protein carbonyl concentrations and an increased ($P < 0.05$) GSH concentration and GSH/GSSG ratio were observed in the jejunum of IUGR-MET piglets in comparison with the IUGR-CON piglets. Furthermore, methionine treatment alleviated ($P < 0.05$) the increased MDA concentration and decreased GSH/GSSG ratio in the ileum of IUGR piglets in comparison with the piglets who received a control diet. There were no differences in other parameters among the groups ($P > 0.05$).

Apoptotic index

Piglets in the IUGR-CON group showed a greater ($P < 0.05$) apoptotic percentage in both the jejunum and ileum than the NBW-CON group (Fig. 1). In contrast, piglets treated with

Table 5 Effects of dietary L-methionine supplementation on oxidative status in the intestine of intrauterine growth-retarded weanling piglets

Items	NBW-CON	IUGR-CON	IUGR-MET
Jejunum			
MDA (nmol/mg protein)	0.47 ± 0.08 ^b	0.71 ± 0.03 ^a	0.51 ± 0.04 ^b
Protein carbonyl (nmol/mg protein)	2.14 ± 0.21 ^b	2.99 ± 0.23 ^a	2.21 ± 0.15 ^b
SOD (U/mg protein)	144.3 ± 6.2	129.9 ± 5.3	136.6 ± 6.9
GSH-Px (U/mg protein)	19.5 ± 0.8	20.7 ± 0.9	21.7 ± 0.9
GR (U/g protein)	5.7 ± 1.0	5.6 ± 0.8	6.2 ± 0.9
GSH (nmol/mg protein)	2.55 ± 0.16 ^a	1.89 ± 0.13 ^b	2.45 ± 0.11 ^a
GSSG (nmol/mg protein)	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.01
GSH/GSSG	19.13 ± 1.01 ^a	14.64 ± 0.83 ^b	17.77 ± 0.66 ^a
Ileum			
MDA (nmol/mg protein)	0.53 ± 0.05 ^b	0.76 ± 0.07 ^a	0.56 ± 0.05 ^b
Protein carbonyl (nmol/mg protein)	2.09 ± 0.27	2.34 ± 0.25	2.31 ± 0.18
SOD (U/mg protein)	108.7 ± 4.7	95.0 ± 6.7	100.4 ± 5.5
GSH-Px (U/mg protein)	20.9 ± 1.1	19.2 ± 0.9	20.8 ± 0.9
GR (U/g protein)	5.2 ± 1.0	5.6 ± 1.0	6.0 ± 1.1
GSH (nmol/mg protein)	1.88 ± 0.19 ^a	1.29 ± 0.11 ^b	1.64 ± 0.15 ^{ab}
GSSG (nmol/mg protein)	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.01
GSH/GSSG	18.04 ± 1.13 ^a	11.86 ± 0.75 ^b	15.87 ± 0.78 ^a

Data are presented as mean ± SE, $n = 6$ /group

Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$)

NBW-CON normal birth weight group given a control diet, *IUGR-CON* intrauterine growth retardation group given a control diet, *IUGR-MET* intrauterine growth retardation group given a methionine-supplemented diet, *MDA* malondialdehyde, *SOD* superoxide dismutase, *GSH-Px* glutathione peroxidase, *GR* glutathione reductase, *GSH* reduced glutathione, *GSSG* oxidized glutathione

methionine had an increased ($P < 0.05$) apoptotic percentage in both the jejunum and ileum than the IUGR-CON piglets.

Messenger RNA expressions

IUGR down-regulated ($P < 0.05$) the mRNA expression levels of OCLN and Bcl-2 in the jejunum of the IUGR-CON group in comparison with the NBW-CON group (Table 6). In addition, administering methionine up-regulated ($P < 0.05$) the mRNA abundances of OCLN and Bcl-2 in the jejunum of the IUGR-MET group when compared with the IUGR-CON group. However, there were no differences in the mRNA expression levels of SOD1, GPx1, CLDN-2, ZO-1, and Bax among the groups ($P > 0.05$).

Protein expression

The protein expression of OCLN was decreased ($P < 0.05$) in the jejunum of the IUGR-CON piglets compared to the NBW-CON piglets (Fig. 2). Dietary methionine supplementation increased ($P < 0.05$) the protein expression of OCLN in the jejunum of the IUGR-MET piglets. However, there was no difference in the protein expression of OCLN in the ileum among the groups ($P > 0.05$).

Discussion

It has been reported that insufficient intake of SAA is detrimental in both humans and animals [15, 26]. Methionine is an essential amino acid not only for whole body (protein biosynthesis, redox balance, and transsulfuration and transmethylation reactions) but also for enterocyte metabolism (GSH and cysteine synthesis) [27, 28]. Its importance as a key amino acid is reflected by the fact that it is usually absorbed from the diet with highest fractional absorption rates among all proteinogenic amino acid [28]. Therefore, dietary methionine supply is important for humans and animals to provide health benefits. A considerable amount of evidence has shown that IUGR delays postnatal growth in infants and is associated with the impaired integrity and antioxidant capacity in the intestine [7, 29, 30]. The present study corroborated these findings and found that methionine treatment increased the GSH content and GSH/GSSG ratio and concomitantly decreased the apoptotic percentage, increased the VH, and up-regulated the protein expression of OCLN in the jejunum of IUGR piglets. These observations might further strengthen the potential effect of methionine to protect against the intestinal damage of weanling piglets induced by IUGR and also provide an applicable rationale for properly targeted methionine supply in IUGR humans.

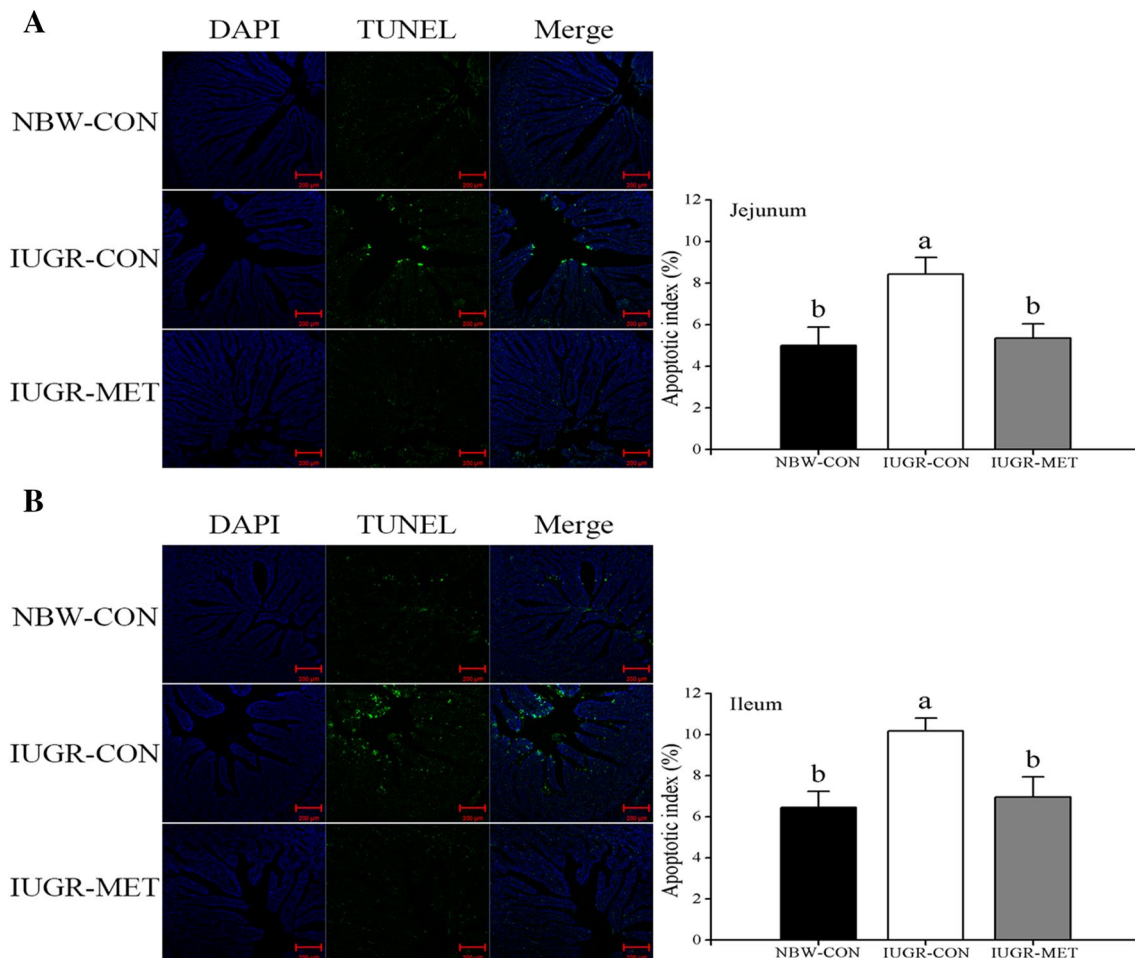


Fig. 1 Effects of dietary L-methionine supplementation on apoptotic indices in the jejunum (a) and ileum (b) of intrauterine growth-retarded weanling piglets. *NBW-CON* normal birth weight group given a control diet, *IUGR-CON* intrauterine growth retardation

group given a control diet, *IUGR-MET* intrauterine growth retardation group given a methionine-supplemented diet. Data are presented as mean \pm SE, $n = 6$ /group. Mean values in columns with unlike superscript letters were significantly different ($P < 0.05$)

Intestinal integrity is a key factor that reflects gut health in humans and animals [31]. The present study found that IUGR impaired intestinal morphology in weanling piglets, as indicated by the decreased VH, VSA, and VH/CD ratio. The structural damage of the intestinal villus indicates a decreased ability to absorb nutrients and a fragile mechanical barrier in IUGR piglets [24]. Moreover, the VH/CD ratio is widely considered to be a good indicator of mucosal turnover [32]. In keeping with our findings, Wang et al. [6] found that IUGR offspring had a decreased VH and VH/CD ratio. Abnormal expressions of tight junctions obviously affect paracellular permeability and lead to many pathological states [33]. In the present study, IUGR decreased OCLN at both transcription and translation levels in the jejunum. Wang et al. [30] also found that decreased OCLN expression was correlated with increased paracellular permeability in IUGR piglets.

The results obtained in this study may provide an explanation for impaired intestinal integrity in IUGR piglets.

Disaccharidases enzymes have crucial roles in facilitating mucosal maturation and gut digestive function [34]. An in vivo trial showed that IUGR decreased the activities of lactase and maltase in rabbits [35]. Likewise, D’Inca et al. [36] found that preterm IUGR piglets had significantly lower sucrase and maltase activities at birth compared with their normal littermates. In the present study, IUGR obviously reduced the activity of maltase in the jejunum. Similarly, a decreased sucrase activity was also presented in the ileum of IUGR weanling piglets. Thus, the results obtained herein may provide another explanation for the impaired development of the small intestine in IUGR weanling piglets.

Until now, the exact mechanisms underlying the intestinal damage of IUGR piglets remained unknown. However, it is likely associated with the increased level of cell apoptosis

Table 6 Effects of dietary L-methionine supplementation on mRNA expressions in the intestine of intrauterine growth-retarded weanling piglets

Items	NBW-CON	IUGR-CON	IUGR-MET
Jejunum			
SOD1	1.00 ± 0.10	0.79 ± 0.14	0.92 ± 0.18
GPx1	1.00 ± 0.11	1.07 ± 0.12	0.89 ± 0.09
OCLN	1.00 ± 0.14 ^a	0.52 ± 0.05 ^b	0.92 ± 0.11 ^a
CLDN-2	1.00 ± 0.13	0.68 ± 0.08	0.74 ± 0.13
ZO-1	1.00 ± 0.15	0.61 ± 0.09	0.67 ± 0.09
Bcl-2	1.00 ± 0.08 ^a	0.56 ± 0.07 ^b	0.90 ± 0.11 ^a
Bax	1.00 ± 0.16	0.71 ± 0.13	0.82 ± 0.15
Ileum			
SOD1	1.00 ± 0.15	0.81 ± 0.10	0.72 ± 0.09
GPx1	1.00 ± 0.13	0.71 ± 0.12	0.95 ± 0.06
OCLN	1.00 ± 0.13	1.09 ± 0.15	1.45 ± 0.13
CLDN-2	1.00 ± 0.15	0.74 ± 0.12	1.19 ± 0.14
ZO-1	1.00 ± 0.16	0.97 ± 0.15	1.23 ± 0.08
Bcl-2	1.00 ± 0.20	0.94 ± 0.18	1.07 ± 0.16
Bax	1.00 ± 0.16	0.80 ± 0.14	1.10 ± 0.16

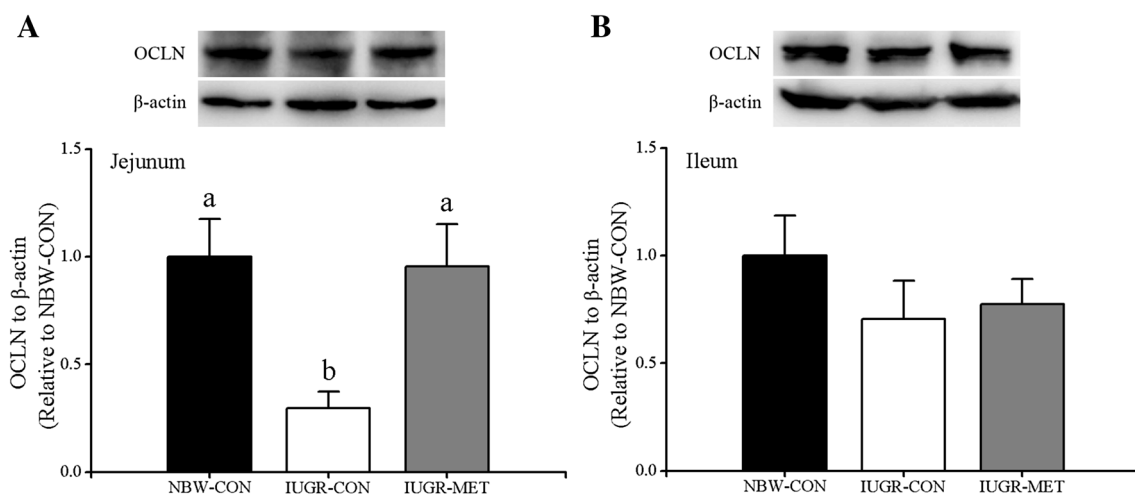
Data are presented as mean ± SE, $n = 6$ /group

Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$)

NBW-CON normal birth weight group given a control diet, *IUGR-CON* intrauterine growth retardation group given a control diet, *IUGR-MET* intrauterine growth retardation group given a methionine-supplemented diet, *SOD1* superoxide dismutase 1, *GPx1* glutathione peroxidase 1, *ZO-1* zonula occludens-1, *Bcl-2* B-cell lymphoma/leukaemia 2, *Bax* Bcl-2-associated X protein

induced by IUGR. Although apoptosis is essential for epithelial turnover and tissue homeostasis in the intestine, excessive apoptosis could result in intestinal barrier dysfunction [37]. In the present study, the increased apoptotic indices of both the jejunum and ileum induced by IUGR were observed using a TUNEL assay. Similarly, IUGR increased the apoptotic index with a concomitant decrease in mRNA expression levels of Bcl-2 in rats [38]. The present study showed that the mRNA expression of Bcl-2 was down-regulated by IUGR, which was consistent with the previous findings [13, 38]. Bcl-2 contributes significantly to apoptosis regulation and tissue homeostasis [39]. Hence, this result also indicates that IUGR might affect intestinal development through increasing cell apoptosis in weanling piglets.

Oxidative stress can induce apoptosis and affect cellular homeostasis. As previously mentioned, oxidative stress is a pivotal factor contributing to the intestinal damage of IUGR offspring [7]. In this work, IUGR was found to increase the concentrations of MDA and protein carbonyl in the jejunum, which reflects the degree of lipid peroxidation or protein oxidation, respectively. Likewise, Zhang et al. [40] reported that IUGR increased plasma MDA and protein carbonyl concentrations in piglets. As a scavenger of reactive oxygen species (ROS), GSH plays an important role in antioxidant defense [41]. Oxidative stress shifts the GSH/GSSG ratio towards lower GSH content and higher GSSG content [42]. A previous study observed decreased GSH content in IUGR piglets compared with their heavier counterparts [43]. In this study, significant differences were found for GSH content and the GSH/GSSG ratio in both the jejunum and ileum between NBW-CON and IUGR-CON piglets. These investigations

**Fig. 2** Effects of dietary L-methionine supplementation on OCLN protein contents in the jejunum (a) and ileum (b) of intrauterine growth-retarded weanling piglets. *NBW-CON* normal birth weight group given a control diet, *IUGR-CON* intrauterine growth retar-

dation group given a control diet, *IUGR-MET* intrauterine growth retardation group given a methionine-supplemented diet. Data are presented as mean ± SE, $n = 6$ /group. Mean values in columns with unlike superscript letters were significantly different ($P < 0.05$)

illustrate that IUGR infants have an impaired antioxidant capacity and reveal widespread oxidative damages.

It has been demonstrated that SAA deficiency decreases the total GSH content in the proximal jejunum of piglets [14]. The present study observed decreased methionine and cysteine concentrations in the jejunum of IUGR piglets, which is consistent with the previous results [44, 45]. Cysteine is required for the synthesis of GSH and taurine, which are essential compounds for host defense against oxidative stress [27]. However, in a previous study, cysteine administration did not accelerate the GSH synthesis rate and its concentration in preterm infants in the early life [46], possibly because cysteine undergoes rapid spontaneous oxidation at neutral pH to form cystine and hydrogen peroxide, and the production of large amounts of hydrogen peroxide at certain extracellular and intracellular sites has negative consequences [47]. Thus, there seems to be a particular need for the nutritional and functional role of methionine in the intestinal development of IUGR infants. In the current study, dietary methionine supplementation increased the methionine concentration with a concomitant increase of GSH content and the GSH/GSSG ratio in the jejunum of IUGR piglets. Likewise, higher GSH and lower GSSG concentrations were also observed in the duodenum and jejunum of methionine-supplemented piglets [11]. The underlying mechanisms may involve at least two possible processes. First, a variety of ROS react readily with methionine residues in proteins to generate methionine sulfoxide [48], and, therefore, decrease the consumption of GSH. Second, methionine provides sulfur for cysteine synthesis and may increase the efficiency of the GSH redox cycle [49]. Moreover, IUGR piglets given a methionine-supplemented diet had decreased concentrations of MDA and protein carbonyl in the jejunum. The decreased percentage of apoptosis and the increased Bcl-2 mRNA expression were also observed in the jejunum of methionine-supplemented piglets. The enhanced efficiency of methionine metabolism, and, consequently, an improved GSH redox cycle in the intestine, may explain the attenuated oxidative stress and cell apoptosis in methionine-supplemented piglets [41].

In this study, methionine intervention improved VH in the jejunum of IUGR piglets. Similarly, a higher VH in the jejunum and a lower CD in the duodenum were also noted in methionine-supplemented weanling piglets [11]. A previous report demonstrated that SAA deficiency suppressed intestinal mucosal growth, correlated with villus atrophy, and reduced epithelial cell proliferation in piglets [15]. The effectiveness of methionine as an antioxidant and on the GSH redox cycle may explain the improved villus development in the intestine [15, 50]. Here, the up-regulated expression of OCLN in the jejunum induced by methionine was also observed at both mRNA and protein levels, which was consistent with a previous finding [11]. Hou et al. [51]

revealed that the dietary supplementation of *N*-acetylcysteine, a precursor of cysteine, could increase the OCLN protein expression in the ileum of piglets challenged with lipopolysaccharide, which indirectly supports the present results. Therefore, dietary methionine supplementation may contribute to the epithelial cell metabolism and gut function through the regulation of cysteine synthesis. In the current study, piglets in the IUGR-MET group showed an increased activity of maltase in the jejunum. Fang et al. [52] reported that SAA are critical for the gut to maintain its functions including the digestion, absorption, and metabolism of nutrients. Thus, increasing the methionine level in their diet may be favorable to maintain the gut health of IUGR piglets.

In conclusion, methionine treatment has therapeutic potential for improving intestinal integrity and oxidative status in IUGR weanling piglets. The results of this study demonstrate the nutritional and functional importance of intestinal methionine metabolism, and may be helpful in the development of new nutritional strategies for IUGR offspring to attenuate intestinal damage during the early life.

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Compliance with ethical standards

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

Ethical statement The use of animals for this research was approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University.

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