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Original Research Article

Dietary dihydroartemisinin supplementation alleviates intestinal inflammatory injury through TLR4/NOD/NF-κB signaling pathway in weaned piglets with intrauterine growth retardation

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ABSTRACT

The aim of present study was to evaluate whether diets supplemented with dihydroartemisinin (DHA) could alleviate intestinal inflammatory injury in weaned piglets with intrauterine growth retardation (IUGR). Twelve normal birth weight (NBW) piglets and 12 piglets with IUGR were fed a basal diet (NBW-CON and IUCR-CON groups), and another 12 piglets with IUGR were fed the basal diet supplemented with DHA at 80 mg/kg (IUGR-DHA group) from 21 to 49 d of age. At 49 d of age, 8 piglets with similar body weight in each group were sacrificed. The jejunal and ileal samples were collected for further analysis. The results showed that IUGR impaired intestinal morphology, increased intestinal inflammatory response, raised enterocyte apoptosis and reduced enterocyte proliferation and activated transmembrane toll-like receptor 4 (TLR4)/nucleotide-binding and oligomerization domain (NOD)/nuclear factor-κB (NF-κB) signaling pathway. Dihydroartemisinin inclusion ameliorated intestinal morphology, indicated by increased villus height, villus height-to-crypt depth ratio, villus surface area and decreased villus width of piglets with IUGR ($P < 0.05$). Compared with NBW piglets, IUGR piglets supplemented with DHA exhibited higher apoptosis index and caspase-3 expression, and lower proliferation index and proliferating cell nuclear antigen expression in the intestine ($P < 0.05$). Dihydroartemisinin supplementation attenuated the intestinal inflammation of piglets with IUGR, indicated by increased concentrations of intestinal inflammatory cytokines and lipopolysaccharides ($P < 0.05$). In addition, DHA supplementation down-regulated the related mRNA expressions of TLR4/NOD/NF-κB signaling pathway and upregulated mRNA expressions of negative regulators of TLR4 and NOD signaling pathway in the intestine of piglets with IUGR ($P < 0.05$). Piglets in the IUGR-DHA group showed lower protein expressions of TLR4, phosphorylated NF-κB (pNF-κB) inhibitor α, nuclear pNF-κB, and higher protein expression of cytoplasmic pNF-κB in the intestine than those in the IUGR-CON group ($P < 0.05$). In conclusion, DHA supplementation could improve intestinal morphology, regulate enterocyte proliferation and apoptosis, and alleviate intestinal inflammation through TLR4/NOD/NF-κB signaling pathway in weaned piglets with IUGR.

1. Introduction

Intrauterine growth retardation (IUGR) is a common syndrome in the perinatal period, which can be defined as impaired growth and development of the mammalian embryo/fetus or its organs

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during pregnancy (Wu et al., 2006). As multi-fetal animals, pigs exhibit an incidence of IUGR as high as 15% to 20%, which have been used as a model for human IUGR studies (Dong et al., 2016). IUGR leads to increased risk for neonatal and long-term morbidities affecting multiple organ systems including the intestine (Fung et al., 2016; Garite et al., 2004). Infants with IUGR often display impaired intestinal morphology and function (Fung et al., 2016). Study also demonstrated that infant with IUGR was at a risk for intestinal inflammatory diseases (Longo et al., 2013).

Dihydroartemisinin (DHA) is a kind of derivative of artemisinin, which is extracted from the traditional Chinese herb *Artemisia annua* L. (Yin et al., 2018). Dihydroartemisinin is mainly used to treat malaria for decades. Besides anti-malaria activity, DHA also possesses anti-inflammatory activity and immunomodulatory effect (Ho et al., 2014). Numerous studies have certificated that DHA attenuates inflammatory injury through suppressing nuclear factor-κB (NF-κB) signaling pathway (Jiang et al., 2016; Li et al., 2006; Yang et al., 2015). Transmembrane toll-like receptor (TLR) and nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) are the key protein families of pattern recognition receptors, which are involved in mediating inflammatory process and expressed in many tissues including the intestine (Sanderson and Allan, 2007). Transmembrane toll-like receptor 4 (TLR4) is a significant member of TLR, which plays an important role in innate immunity and inflammation by sensing pathogen-associated molecular patterns, such as lipopolysaccharide (LPS) (Fang et al., 2013). When stimulated by LPS, TLR4 with the accessory proteins causes the activation of NF-κB via a series of signaling cascade reactions (Wang et al., 2017). The typical components of NLR are NOD1 and NOD2, which can also activate NF-κB (Fritz et al., 2006). NF-κB is a key transcription factor which modulates a large array of genes involved in the process of immunity, inflammation and cell proliferation (Baldwin, 2001). The activation of NF-κB regulates downstream targets and promotes the release of pro-inflammatory cytokines, finally resulting in tissue injury. However, no information is available about the effect and mechanism of DHA on intestinal inflammatory injury in piglets with IUGR.

Accordingly, we hypothesized that (1) IUGR impaired intestinal integrity and increased intestinal inflammation of piglets, (2) dietary supplementation of DHA could improve intestinal integrity and reduce intestinal inflammation of piglets with IUGR via TLR4/NOD/NF-κB signaling pathway. Therefore, the aim of this study was to estimate whether DHA could attenuate intestinal injury in weaned piglets with IUGR and to explore its mechanism. This research may provide a reference for treatment of IUGR in humans.

2. Materials and methods

2.1. Preparation of dihydroartemisinin

Dihydroartemisinin ($C_{15}H_{24}O_5$, MW, 284.35), a derivative of artemisinin, is one of the largest groups of sesquiterpene lactones. Dihydroartemisinin used in this experiment was purchased from DASF Biotechnology Co., Ltd (Nanjing, Jiangsu, China). It was freshly prepared every day and then mixed into the basal diet of piglets in a proper proportion. The concentration of DHA was up to 99% as determined by high performance liquid chromatography (HPLC) analysis.

2.2. Animals and experimental design

Institutional Animal Care and Use Committee of Nanjing Agricultural University approved all the animal protocols (NJAU-CAST-2018-146) in this study. At 114 d (SD 1) of gestation, 12 litters of neonatal piglets (Duroc × [Landrace × Yorkshire]) were selected and the birth weight of each piglet was recorded. From each litter, one piglet with NBW (1.56 ± 0.02 kg) and 2 piglets with IUGR (0.99 ± 0.03 kg) were marked by different tags. The criteria for the selection of piglets with IUGR and NBW in this experiment were similar to those in previous studies (Wang et al., 2005). All the newborn piglets ($n = 12$, half male and half female) suckled their mothers until they were weaned at 21 d of age. In each litter, one weaned piglet with NBW and one weaned piglet with IUGR were fed the basal diet (NBW-CON and IUGR-CON groups), and another weaned piglet with IUGR was fed the basal diet supplemented with DHA at 80 mg/kg (IUGR-DHA group) until 49 d of age. The chemical composition and nutrient level of the basal diet (Table 1) were based on the NRC (2012) recommendations. Piglets were housed in individual pens (1 m × 0.6 m) with the ambient temperature ranging from 25 to 28 °C and relative humidity ranging from 50% to 70%. All the piglets had free access to feed and water.

2.3. Sample collection

At the 49 d of age, 8 piglets with similar body weight from each group (half male and half female) were euthanized by intravenous injection of sodium pentobarbital (50 mg/kg BW). Blood samples were collected from jugular vein puncture in a nonheparinized tube and centrifuged at 3,000 × *g* for 15 min at 4 °C and then stored at −80 °C until analysis. The small intestine without the mesentery was immediately collected and allocated into duodenum, jejunum and ileum as described by Wang et al. (2008). The jejunal and ileal segments measuring approximately 1 cm were fixed in 4% paraformaldehyde solution for analysis of intestinal morphology. The mucosal samples of jejunum and ileum were collected and stored at −80 °C for analysis of inflammatory cytokine and lipopolysaccharide concentrations, gene and protein expressions in the intestine.

Table 1
Ingredients and nutrient level of the basal diet (%, air-dry basis).

Item	Content
Ingredients	
Corn	57.70
Soybean meal (46%)	12.50
Expanded corn	8.00
Full-fat soybean	8.00
Fermented soybean meal	4.00
Whey powder	3.00
Fish meal (crude protein 67%)	3.00
Dicalcium phosphate	1.80
Limestone	0.50
L-Lysine (78%)	0.30
L-Threonine	0.10
DL-Methionine	0.08
Wheat middling	0.02
Premix[1]	1.00
Total	100
Calculated nutrient levels	
Digestible energy, MJ/kg	14.04
Crude protein	18.31
Lysine	1.31
Methionine	0.40
Methionine + Cystine	0.70
Threonine	0.80
Calcium	0.85
Total phosphorus	0.72

[1] The premix provided the following per kilogram complete diet: vitamin A, 12,000 IU; vitamin D_3, 3,000 IU; α-tocopherol, 50 mg; vitamin K_3, 4 mg; vitamin B_1, 4 mg; vitamin B_2, 10 mg; vitamin B_6, 7 mg; vitamin B_{12}, 0.05 mg; niacin, 30 mg; pantothenic acid, 15 mg; folic acid, 0.3 mg; biotin, 0.08 mg; choline chloride, 500 mg; Fe ($FeSO_4 \cdot H_2O$), 110 mg; Cu ($CuSO_4 \cdot 5H_2O$), 7 mg; Zn (ZnO), 110 mg; I (KIO_3), 0.3 mg; Mn ($MnSO_4 \cdot H_2O$), 5 mg; Se (Na_2SeO_3), 0.3 mg.

2.4. Intestinal histological analysis

The jejunal and ileal samples stored in paraformaldehyde solution were dehydrated, embedded, sliced and performed with hematoxylin eosin staining, and then observed under the optical microscope. Random fields of vision were selected to take photos. Villus height (VH), crypt depth (CD) and villus width (VW) of jejunum and ileum were determined by an Image-Pro Plus software. Villus height-to-crypt depth ratio (VCR) was equal to VH divided by CD. Villus surface area (VSA) were calculated by the following equation:

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

2.5. Immunohistochemistry analysis

We assessed villus cell apoptosis status using terminal deoxynucleotidyl transferase (TdT)-mediated 2′-deoxyuridine-5′-triphosphate (dUTP) nick end labeling (TUNEL) assay. Briefly, the paraffin sections were dewaxed to water with xylene and alcohol, pretreated with protease K for antigen retrieval and rinsed with PBS butter (pH = 7.4). Then the sections were incubated with TdT:dUTP (1:9, vol:vol) and converter-peroxidase according to the TUNEL kit (Roche Corporation, Basel, Switzerland). Finally, the slides were stained with diaminobenzidine dye and counterstained with hematoxylin for 3 min. The number of positive cells (stained cells) was counted from 10 villi of each slide using a morphometric system. The definition of apoptosis index (AI) was the ratio of the number of apoptotic TUNEL positive cells to the total cell numbers multiplied by 100.

The nuclear antigen Ki-67 is a biomarker for crypt cell proliferative activity (Scholzen and Gerdes, 2000). Samples for intestinal morphology determination were used for immunohistochemistry analysis. The jejunal and ileal slices (5 μm thick) were dewaxed to water with xylene and alcohol, microwave-pretreated with citrate buffer for antigen retrieval and rinsed with PBS buffer (pH = 7.4). The tissue slices were incubated with 3% H_2O_2 in dark for 25 min and blocked with bovine serum albumin for 30 min. Then the sections were incubated with the primary antibody (rabbit polyclonal to Ki67, Abcam, Cambridge, UK; 1:500) overnight at 4 °C and with secondary antibody (goat anti-rabbit IgG, Abcam, Cambridge, UK; 1:1,000) conjugated with horseradish peroxidase for 50 min at room temperature. Subsequently, the slices were stained with diaminobenzidine dye under the microscope to control the color-development time and then counterstained with hematoxylin for 3 min. Finally, the sections were dehydrated with ethanol and mounted with neutral balsam. A morphometric system (Nikon Corporation, Tokyo, Japan) was used to measure the number of positive cells (stained cells) from 10 crypts per section. The proliferation index (PI) referred to the ratio of the number of Ki-67 positive cells to total cell numbers multiplied by 100.

2.6. Concentrations of intestinal inflammatory cytokine and analysis

The systemic inflammatory biomarkers can be evaluated by intestinal pro-inflammatory cytokines including interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α). The concentrations of IL-1β, IL-6 and TNF-α in the jejunum and ileum were determined by ELISA methods using each antibody and biotinylated secondary antibody according to the instruction of manufacturer (YILI Biological Technology Co., Ltd, Shanghai, China).

2.7. Concentration of intestinal lipopolysaccharide analysis

The concentrations of LPS in the jejunum and ileum were measured by ELISA methods using each antibody and biotinylated secondary antibody according to the instruction of manufacturer (YILI Biological Technology Co., Ltd, Shanghai, China).

2.8. Gene expression analysis

RNA was isolated from the frozen intestinal mucosa by a TRIzol reagent (TaKaRa Biotechnology Co. Ltd, Dalian, Liaoning, China). The concentration and purity of RNA were measured using a spectrophotometer (NanoDrop, 2000c, Thermo Scientific, Waltham, MA, USA). Then 1 μg of total RNA was reverse transcribed into complementary DNA using the Perfect Real Time SYBR Premix Ex Taq kit (TaKaRa Biotechnology Co. Ltd, Dalian, China). After that, quantitative real-time polymerase chain reaction assays were conducted on an ABI StepOnePlus Real-Time PCR detection system (Applied Biosystems; Carlsbad, CA, USA) using a SYBR Premix Ex Taq Kit (TakaRa Biotechnology Co. Ltd; Dalian, Liaoning, China). The primer sequences for *TLR4*, myeloid differentiation factor 88 (*MyD88*), IL-1 receptor-associated kinase 1 (*IRAK1*), TNF receptor-associated factor 6 (*TRAF6*), nucleotide-binding oligomerization domain protein 1 (*NOD1*), nucleotide-binding oligomerization domain protein 2 (*NOD2*), receptor-interacting serine/threonine-protein kinase 2 (*RIPK2*), nuclear factor-κB p65 (*NF-κB* p65), radioprotective 105 (*RP105*), suppressor of cytokine signaling 1 (*SOCS1*), toll-interacting protein (*Tollip*), Erbb2 interacting protein (*ERBB2IP*), centaurin β1 (*CENTB1*) and β-actin were presented in Table 2. All sequences for these genes were designed according to Xu et al. (2018). The levels of mRNA expressions were calculated using $2^{-\Delta\Delta Ct}$ method after normalization with the reference gene β-actin.

2.9. Western blot analysis

Antibodies against caspase-3 (1:500), proliferating cell nuclear antigen (PCNA, 1:500), TLR4 (1:500) were purchased from Abcam plc. (Cambridge, UK). Antibodies against MyD88 (1:1,000), total nuclear factor κB (tNF-κB, 1:1,000), phosphorylated nuclear factor κB (pNF-κB, 1:1,000), total NF-κB inhibitor α (tIκBα, 1:1,000) and phosphorylated NF-κB inhibitor α (pIκBα, 1:1,000), β-actin (1:1,000) and Na, K-ATPase (1:1,000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The total, nuclear or cytoplasmic proteins of intestinal mucosal samples were extracted using corresponding assay kits according to the instructions of manufacturer (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). For western blot analysis, 60 μg protein of each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane. The membrane was blocked in 5% non-fat dry milk in Tris-Tween buffered saline at room temperature for 2 h. The membrane was then incubated with primary antibody overnight at 4 °C and with secondary antibody (goat anti-rabbit IgG or goat anti-mouse IgG, 1:2,000; Abcam, Cambridge, UK) for 1 h at room temperature. Reactive protein was detected using enhanced chemiluminescence system. Finally, the image of each membrane was quantified by the Gel-Pro Analyzer 4.0 software (Media Cybernetics, Silver Spring, MD, USA).

2.10. Statistical analysis

All data were assessed by one-way analysis of variance procedure using SPSS statistical software (Ver. 20.0 for windows, SPSS, Chicago, IL, USA). A Tukey's post hoc test was performed to

Table 2
Primer sequences of target genes.

Gene	Accession No.	Sequences	Product length, bp
β-actin	XM_003124280.4	F: CACGCCATCCTGCGTCTGGA R: AGCACCGTGTTGGCGTAGAG	380
TLR4	GQ503242.1	F: TCAGTTCTCACCTTCCTCCTG R: GTTCATTCCTCACCCAGTCTTC	166
MyD88	AB292176.1	F: GATGGTAGCGGTTGTCTCTGAT R: GATGCTGGGGAACTCTTTCTTC	148
IRAK1	XM_003135490.1	F: CAAGGCAGGTCAGGTTTCGT R: TTCGTGGGGCGTGTAGTGT	115
TRAF6	NM_001105286.1	F: CAAGAGAATACCCAGTCGCACA R: ATCCGAGACAAAGGGGAAGAA	122
NOD1	AB187219.1	F: CTGTCGTCAACACCGATCCA R: CCAGTTGGTGACGCAGCTT	57
NOD2	AB195466.1	F: GAGCGCATCCTCTTAACTTTCG R: ACGCTCGTGATCCGTGAAC	66
RIPK2	XM_003355027.1	F: CAGTGTCCAGTAAATCGCAGTTG R: CAGGCTTCCGTCATCTGGTT	206
NF-κB p65	EU399817.1	F: AGTACCCTGAGGCTATAACTCGC R: TCCGCAATGGAGGAGAAGTC	133
RP105	AB190767.1	F: CGAGGCTTCTGACTGTTGTG R: GGTGCTGATTGCTGGTGTC	245
SOCS1	NM_001204768.1	F: GCGTGTAGGATGGTAGCA R: GAGGAGGAGGAGGAGGAAT	101
Tollip	AB490123.1	F: GCAGCAGCAACAGCAGAT R: GGTCACGCCGTAGTTCTTC	133
ERBB2IP	GU990777.1	F: ACAATTCAGCGACAGAGTAGTG R: TGACATCATTGGAGGAGTTCTTC	147
CENTB1	XM_003358258.2	F: GAAGCCGAAGTGTCCGAATT R: AGGTCACAGATGCCAAGAATG	125

TLR4 = toll-like receptor 4; *MyD88* = myeloid differentiation factor 88; *IRAK1* = IL-1 receptor-associated kinase 1; *TRAF6* = TNF receptor-associated factor 6; *NOD* = nucleotide-binding oligomerization domain protein; *RIPK2* = receptor-interacting serine/threonine-protein kinase 2; *NF-κB p65* = nuclear factor-κB p65; *RP105* = radioprotective 105; *SOCS1* = suppressor of cytokine signaling 1; *Tollip* = toll-interacting protein; *ERBB2IP* = Erbb2 interacting protein; *CENTB1* = centaurin β1.

determine the statistical differences among treatment groups. A level of $P < 0.05$ indicated that the difference was statistically significant. $P < 0.01$ was considered as highly significant. Results were presented as means ± SEM.

3. Results

3.1. Intestinal morphology

IUGR decreased VH ($P < 0.001$ and $P < 0.001$), VCR ($P < 0.001$ and $P < 0.001$) and VSA ($P < 0.001$ and $P < 0.001$) and increased CD ($P < 0.001$ and $P < 0.001$) in the jejunum and ileum of piglets (Table 3). Dihydroartemisinin administration effectively exhibited higher VH ($P < 0.001$ and $P < 0.001$), VCR ($P < 0.001$ and $P < 0.001$) and VSA ($P < 0.001$ and $P = 0.001$) and lower CD ($P < 0.001$ and $P < 0.001$) in the jejunum and ileum of piglets with IUGR.

3.2. Cell proliferation and apoptosis

Piglets with IUGR showed higher AI ($P < 0.001$ and $P < 0.001$) (Fig. 1) and lower PI ($P = 0.001$ and $P < 0.001$) (Fig. 2) in the jejunum and ileum than piglets with NBW (Table 4). The level of caspase-3 is the marker of cell apoptosis and PCNA is the marker of cell proliferation. The results showed that IUGR decreased the protein expression of caspase-3 ($P < 0.001$ and $P < 0.001$) and (Fig. 3) and increased the protein expression of PCNA ($P < 0.001$ and $P < 0.001$)

Table 3
Effect of dihydroartemisinin (DHA) on intestinal morphology in weaned piglets with intrauterine growth retardation (IUGR).

Item	Treatment[1]			*P*-value	
	NBW-CON	IUGR-CON	IUGR-DHA	NBW-CON vs. IUGR-CON	IUGR-CON vs. IUGR-DHA
Jejunum					
VH, μm	466.35 ± 7.16	366.91 ± 4.06*	446.11 ± 5.21#	<0.001	<0.001
VW, μm	88.40 ± 1.01	85.68 ± 0.95	85.71 ± 0.96	0.146	1.000
CD, μm	170.52 ± 2.51	233.07 ± 2.85*	206.48 ± 2.38#	<0.001	<0.001
VCR, μm/μm	2.74 ± 0.05	1.58 ± 0.02*	2.16 ± 0.02#	<0.001	<0.001
VSA, mm^2	0.065 ± 0.002	0.050 ± 0.001*	0.060 ± 0.001#	<0.001	<0.001
Ileum					
VH, μm	369.16 ± 5.92	321.63 ± 2.37*	360.97 ± 4.63#	<0.001	<0.001
VW, μm	88.70 ± 1.54	85.11 ± 1.18	85.81 ± 1.12	0.147	0.922
CD, μm	156.05 ± 4.22	236.96 ± 4.89*	206.26 ± 3.10#	<0.001	<0.001
VCR, μm/μm	2.37 ± 0.05	1.36 ± 0.03*	1.75 ± 0.03#	<0.001	<0.001
VSA, mm^2	0.052 ± 0.001	0.043 ± 0.001*	0.049 ± 0.001#	<0.001	0.001

VH = villus height; VW = villus width; CD = crypt depth; VCR = villus height-to-crypt depth ratio; VSA = villus surface area.
*, a significant difference ($P < 0.05$) between NBW-CON group and IUGR-CON group; #, a significant difference ($P < 0.05$) between IUGR-DHA group and IUGR-CON group.
[1] NBW-CON, normal body weight group fed a basal diet; IUGR-CON, IUGR group fed a basal diet; IUGR-DHA, IUGR group fed a diet supplemented with DHA at 80 mg/kg. Results were showed as means ± SEM ($n = 8$).

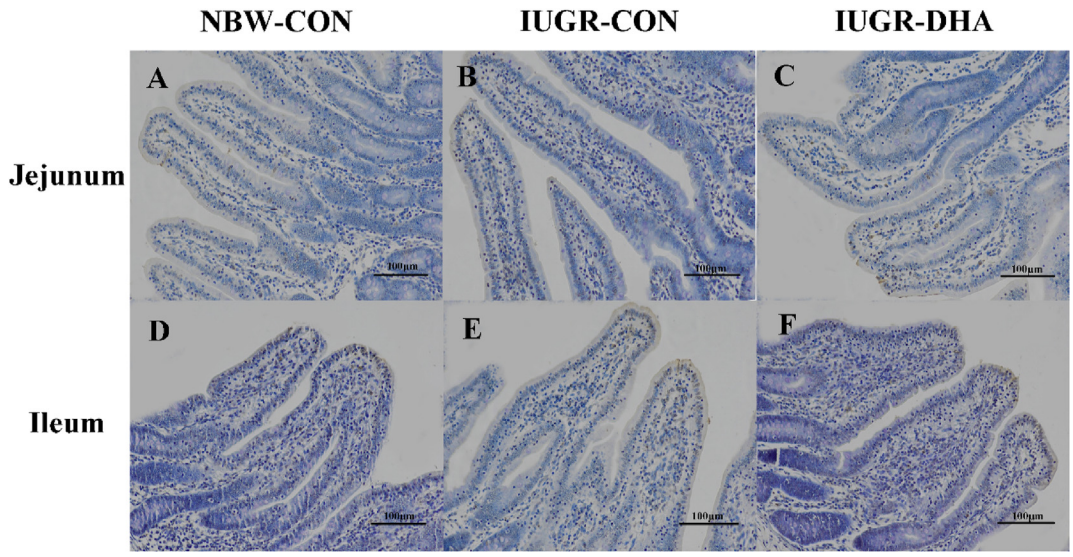

Fig. 1. Effects of dihydroartemisinin (DHA) on terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine-5′-triphosphate nick end labeling (TUNEL)-positive cells in the jejunum (A to C) and ileum (D to F) of weaned piglets with intrauterine growth retardation (IUGR). TUNEL immunohistochemical staining (200× magnification; scale bar, 100 μm). The apoptotic cells were stained yellow, or a brown and yellow color. NBW-CON, normal body weight group fed a basal diet; IUGR-CON, IUGR group fed a basal diet; IUGR-DHA, IUGR group fed a diet supplemented with DHA at 80 mg/kg.

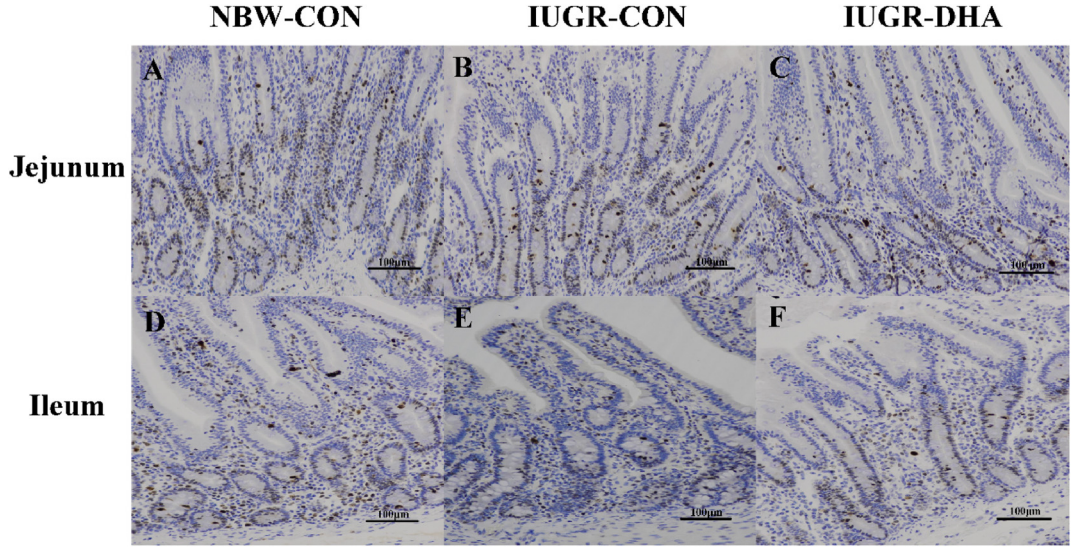

Fig. 2. Effects of dihydroartemisinin (DHA) on Ki-67 positive cells in the jejunum (A to C) and ileum (D to F) of weaned piglets with intrauterine growth retardation (IUGR). Ki-67 immunohistochemical staining (200× magnification, scale bar, 100 μm). The proliferative cells were stained yellow, or a brown and yellow color. NBW-CON, normal body weight group fed a basal diet; IUGR-CON, IUGR group fed a basal diet; IUGR-DHA, IUGR group fed a diet supplemented with DHA at 80 mg/kg.

Table 4
Effect of dihydroartemisinin (DHA) on enterocyte proliferation and apoptosis in weaned piglets with intrauterine growth retardation (IUGR).

Item	Treatment[1]			*P*-value	
	NBW-CON	IUGR-CON	IUGR-DHA	NBW-CON vs. IUGR-CON	IUGR-CON vs. IUGR-DHA
Jejunum					
AI, %	3.80 ± 0.25	9.72 ± 0.42*	6.62 ± 0.15#	<0.001	<0.001
PI, %	21.44 ± 1.22	15.07 ± 0.60*	20.08 ± 0.89#	0.001	0.005
Ileum					
AI, %	4.05 ± 0.24	7.13 ± 0.25*	5.31 ± 0.30#	<0.001	0.001
PI, %	30.10 ± 1.11	18.18 ± 1.03*	28.42 ± 1.41#	<0.001	<0.001

AI = apoptosis index; PI = proliferation index.
*, a significant difference ($P < 0.05$) between NBW-CON group and IUGR-CON group; #, a significant difference ($P < 0.05$) between IUGR-DHA group and IUGR-CON group.
[1] NBW-CON, normal body weight group fed a basal diet; IUGR-CON, IUGR group fed a basal diet; IUGR-DHA, IUGR group fed a DHA-supplemented diet at 80 mg/kg. Results were showed as means ± SEM ($n = 8$).

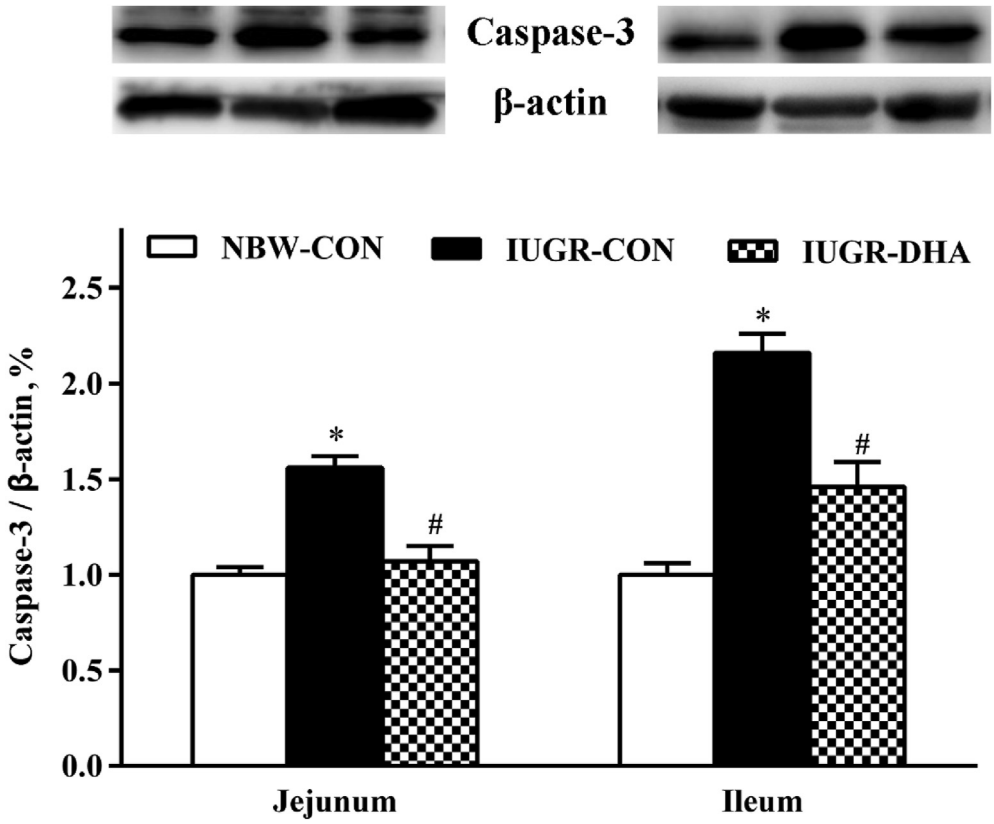

Fig. 3. Effect of dihydroartemisinin (DHA) on the protein expression of caspase-3 in the intestine of weaned piglets with intrauterine growth retardation (IUGR). Results were showed as means ± SEM ($n = 8$). NBW-CON, normal body weight group fed a basal diet; IUGR-CON, IUGR group fed a basal diet; IUGR-DHA, IUGR group fed a diet supplemented with DHA at 80 mg/kg. *, a significant difference ($P < 0.05$) between NBW-CON group and IUGR-CON group; #, a significant difference ($P < 0.05$) between IUGR-DHA group and IUGR-CON group.

(Fig. 4) in the jejunum and ileum of piglets ($P < 0.05$). Diets supplemented with DHA effectively enhanced PI ($P = 0.005$ and $P < 0.001$) and the level of PCNA ($P = 0.003$ and $P = 0.001$), and reduced AI ($P < 0.001$ and $P = 0.001$) and the level of caspase-3 ($P < 0.001$ and $P = 0.001$) in both jejunum and ileum of piglets with IUGR.

3.3. Concentrations of intestinal inflammatory cytokines

In the jejunum, the concentrations of IL-1β ($P < 0.001$), IL-6 ($P = 0.005$) and TNF-α ($P = 0.024$) were increased in the IUGR-CON group compared with those in the NBW-CON group (Table 5). Piglets with IUGR fed the DHA diet significantly reduced the levels of IL-1β ($P = 0.008$) and IL-6 ($P = 0.002$) compared with those fed the basal diet. In the ileum, the levels of IL-1β ($P = 0.037$) and IL-6 ($P = 0.003$) in the IUGR-CON group were higher than those of NBW-CON group. After DHA supplementation, piglets with IUGR decreased the concentrations of IL-1β ($P = 0.032$), IL-6 ($P = 0.029$) and TNF-α ($P = 0.032$).

3.4. Concentrations of intestinal lipopolysaccharide

Piglets with IUGR exhibited an increased concentration of LPS ($P = 0.002$ and $P = 0.006$) in the jejunum and ileum in comparison with piglets with NBW (Fig. 5). Dietary supplementation with DHA significantly reduced the concentrations of LPS ($P < 0.001$ and $P < 0.001$) in the jejunum and ileum of piglets with IUGR.

3.5. Intestinal mRNA expressions of TLR4/NOD/NF-κB signaling pathway

As presented in Table 6, piglets with IUGR upregulated the mRNA expressions of *TLR4* ($P < 0.001$), *MyD88* ($P < 0.001$), *IRAK1* ($P < 0.001$), *NOD1* ($P < 0.001$), *RIPK2* ($P = 0.001$), and *NF-κB p65* ($P = 0.001$) in the jejunum, and *TLR4* ($P < 0.001$), *NOD1* ($P < 0.001$), *NOD2* ($P < 0.001$), *RIPK2* ($P < 0.001$), and *NF-κB p65* ($P = 0.001$) in the ileum compared with piglets with NBW. Dietary supplementation with DHA down-regulated the mRNA expressions of jejunal *TLR4* ($P < 0.001$), *IRAK1* ($P < 0.001$), *NOD1* ($P < 0.001$), *RIPK2* ($P = 0.001$), and *NF-κB p65* ($P < 0.001$) and ileal *TLR4* ($P = 0.002$), *NOD1* ($P = 0.025$), *NOD2* ($P < 0.001$), *RIPK2* ($P < 0.001$), and *NF-κB p65* ($P < 0.025$) of piglets with IUGR.

3.6. Intestinal protein expressions of TLR4/NOD/NF-κB signaling pathway

As presented in Fig. 6, piglets with IUGR increased the protein expressions of TLR4 ($P < 0.001$ and $P < 0.001$), pIκBα ($P < 0.001$ and $P < 0.001$), nuclear pNF-κB ($P < 0.001$ and $P < 0.001$) and decreased cytoplasmic pNF-κB ($P = 0.002$ and $P < 0.001$) levels in both of the jejunum and ileum than piglets with NBW. Diets supplemented with DHA effectively decreased the protein expressions of TLR4 ($P < 0.001$ and $P < 0.001$), pIκBα ($P < 0.001$ and $P = 0.022$), nuclear pNF-κB ($P = 0.001$ and $P < 0.001$) and increased cytoplasmic pNF-κB ($P < 0.001$ and $P = 0.006$) in the jejunum and ileum of piglets with IUGR. The protein expression of jejunal MyD88 was also

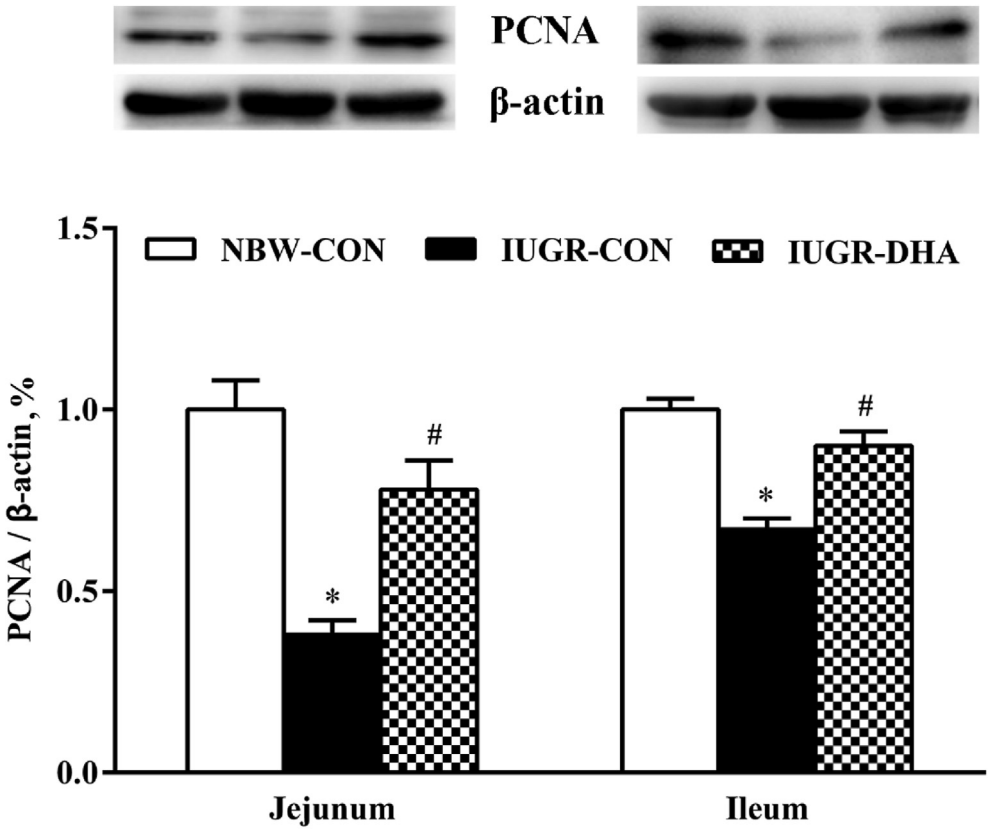

Fig. 4. Effect of dihydroartemisinin (DHA) on the protein expression of PCNA in the intestine of weaned piglets with intrauterine growth retardation (IUGR). Results were showed as means ± SEM ($n = 8$). NBW-CON, normal body weight group fed a basal diet; IUGR-CON, IUGR group fed a basal diet; IUGR-DHA, IUGR group fed a diet supplemented with DHA at 80 mg/kg. *, a significant difference ($P < 0.05$) between NBW-CON group and IUGR-CON group; #, a significant difference ($P < 0.05$) between IUGR-DHA group and IUGR-CON group. PCNA = proliferating cell nuclear antigen.

Table 5
Effect of dihydroartemisinin (DHA) on the concentrations of intestinal inflammatory cytokines in weaned piglets with intrauterine growth retardation (IUGR).

Item	Treatment[1]			*P*-value	
	NBW-CON	IUGR-CON	IUGR-DHA	NBW-CON vs. IUGR-CON	IUGR-CON vs. IUGR-DHA
Jejunum					
IL-1β, ng/g protein	33.61 ± 3.21	54.08 ± 3.00*	40.44 ± 1.65#	<0.001	0.008
IL-6, ng/g protein	64.73 ± 3.40	83.68 ± 2.96*	62.67 ± 3.63#	0.005	0.002
TNF-α, ng/g protein	17.93 ± 0.68	22.13 ± 1.13*	20.11 ± 1.12	0.024	0.350
Ileum					
IL-1β, ng/g protein	26.68 ± 1.86	33.66 ± 1.71*	26.59 ± 1.71#	0.037	0.032
IL-6, ng/g protein	56.67 ± 3.05	72.24 ± 2.55*	60.91 ± 2.73#	0.003	0.029
TNF-α, ng/g protein	15.13 ± 1.29	16.76 ± 0.62	13.14 ± 0.63#	0.433	0.032

IL-1β = interleukin 1β; IL-6 = interleukin 6; TNF-α = tumor necrosis factor α.
*, a significant difference (P < 0.05) between NBW-CON group and IUGR-CON group; #, a significant difference ($P < 0.05$) between IUGR-DHA group and IUGR-CON group.
[1] NBW-CON, normal body weight group fed a basal diet; IUGR-CON, IUGR group fed a basal diet; IUGR-DHA, IUGR group fed a diet supplemented with DHA at 80 mg/kg. Results were showed as means ± SEM ($n = 8$).

increased in the IUGR-CON group compared to that in the NBW-CON group ($P = 0.021$).

3.7. Intestinal mRNA expressions of negative regulators of TLR4/NOD signaling pathway

As shown in Table 7, the mRNA expressions of *Tollip* ($P < 0.001$), *ERBB2IP* ($P < 0.001$), and *CENTB1* ($P = 0.001$) in the jejunum and *SOCS1* ($P = 0.001$), *ERBB2IP* ($P < 0.001$) and *CENTB1* ($P < 0.001$) in the ileum were reduced in the IUGR-CON group when compared with those in the NBW-CON group. Diets supplemented with DHA increased the mRNA expressions of *Tollip* ($P < 0.001$), *ERBB2IP* ($P < 0.001$), and *CENTB1* ($P < 0.001$) in the jejunum and *SOCS1* ($P = 0.018$), *ERBB2IP* ($P = 0.033$) and *CENTB1* ($P < 0.001$) in the ileum of piglets with IUGR.

4. Discussion

The small intestine is the biggest immune organ closely related to immune and inflammatory reaction. Intestinal morphology reflects the gut health which can be assessed by VH, CD, VCR and VSA (Xun et al., 2015; Zou et al., 2019). In this experiment, VH, VCR and VSA were reduced, and CD was increased in the intestine of weaned piglets with IUGR in comparison with weaned piglets with

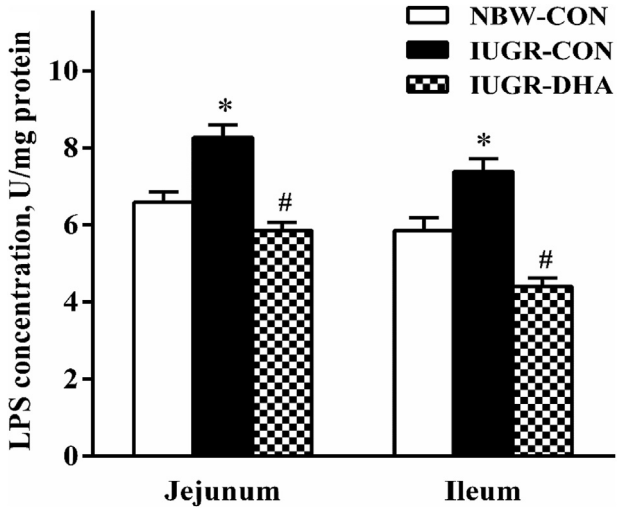

Fig. 5. Effect of dihydroartemisinin (DHA) on the intestinal lipopolysaccharide (LPS) concentration in weaned piglets with intrauterine growth retardation (IUGR). Results were showed as means ± SEM ($n = 8$). NBW-CON, normal body weight group fed a basal diet; IUGR-CON, IUGR group fed a basal diet; IUGR-DHA, IUGR group fed a diet supplemented with DHA at 80 mg/kg. *, a significant difference ($P < 0.05$) between NBW-CON group and IUGR-CON group; #, a significant difference ($P < 0.05$) between IUGR-DHA group and IUGR-CON group.

NBW, suggesting a decreased ability of intestinal absorption as well as a damaged intestinal integrity in piglets with IUGR. These results were consistent with previous studies on piglets with IUGR (Che et al., 2020; Dong et al., 2016; Su et al., 2018; Zhang et al., 2017). Dietary supplementation with DHA enhanced VH, VCR, and VSA, and decreased CD in piglets with IUGR, indicating that DHA could improve the intestinal morphology.

A previous study demonstrated that the impaired intestinal morphology may be related to the imbalance of cell apoptosis and proliferation (Li et al., 2018). In our study, piglets with IUGR enhanced the AI and reduced the PI of enterocytes when compared with weaned piglets with NBW. Similar results were found in IUGR neonatal piglets (Wang et al., 2012). The results were also similar to previous studies that IUGR increased cell apoptosis in the small intestine of rats (Baserga et al., 2004) and decreased enterocyte proliferation in newborn rabbits (Cellini and Buchmiller, 2006). However, the findings were dissimilar to a study reported by Li et al. (2018), who noted that IUGR increased the proportion of villus apoptosis cells and crypt proliferative cells in the ileum of IUGR weanling piglets. The reason may be attributed to a compensatory process in response to the excessive apoptosis in the villus. It is clear that caspase-3 is a frequently activated protease in mammalian cell apoptosis (Porter and Janicke, 1999). Proliferating cell nuclear antigen is an intranuclear polypeptide whose expression and synthesis are evaluated as the marker of cell proliferation (Connolly and Bogdanffy, 1993). In the present study, IUGR enhanced caspase-3 protein expression and reduced PCNA protein expression in the jejunum and ileum of weaned piglets. A previous research suggested that the expression of caspase-3 was increased and the expression of PCNA was decreased in the placentas of IUGR rats (Alqaryyan et al., 2016). The results were also similar to previous observations that the TUNEL staining and caspase-3 activity were increased in the kidney of IUGR rats (Pham et al., 2003). After dietary DHA supplementation, the protein expression of caspase-3 was decreased and PCNA was increased in piglets with IUGR. These results indicated that IUGR was linked with decreased cell proliferation and increased cell apoptosis in the small intestine and DHA inclusion could improve the excessive apoptosis in weaned piglets with IUGR.

It was reported that excessive intestinal epithelial cell apoptosis disrupted intestinal integrity and permitted the invasion of luminal antigens into the lamina propria, thereby leading to the inflammatory response and release of pro-inflammatory cytokines (Jozawa et al., 2019). The results of present study suggested that IUGR enhanced the concentrations of pro-inflammation cytokines IL-1β, IL-6, TNF-α in the jejunum and IL-1β, IL-6 in the ileum of piglets. In accordance with previous study, Huang et al. (2019) demonstrated that piglets with IUGR increased the concentrations of TNF-α and IL-6 at birth, which indicated that IUGR newborns were prone to inflammatory injury. Diets supplemented with DHA decreased the concentrations of pro-inflammation cytokines

Table 6
Effect of dihydroartemisinin (DHA) on intestinal mRNA expression of TLR4/NOD/NF-κB signaling pathway in weaned piglets with intrauterine growth retardation (IUGR).

Item	Treatment[1]			*P*-value	
	NBW-CON	IUGR-CON	IUGR-DHA	NBW-CON vs. IUGR-CON	IUGR-CON vs. IUGR-DHA
Jejunum					
TLR4	1.00 ± 0.05	1.41 ± 0.06*	0.64 ± 0.04#	<0.001	<0.001
MyD88	1.00 ± 0.04	1.36 ± 0.05*	1.20 ± 0.05	<0.001	0.076
IRAK1	1.00 ± 0.12	2.08 ± 0.08*	0.44 ± 0.07#	<0.001	<0.001
TRAF6	1.00 ± 0.05	0.87 ± 0.07	0.91 ± 0.05	0.272	0.883
NOD1	1.00 ± 0.07	1.55 ± 0.10*	1.03 ± 0.04#	<0.001	<0.001
NOD2	1.00 ± 0.06	0.92 ± 0.09	0.83 ± 0.06	0.690	0.636
RIPK2	1.00 ± 0.10	1.58 ± 0.11*	0.97 ± 0.04#	0.001	0.001
NF-κB p65	1.00 ± 0.09	1.75 ± 0.15*	0.76 ± 0.07#	0.001	<0.001
Ileum					
TLR4	1.00 ± 0.16	2.89 ± 0.19*	1.87 ± 0.15#	<0.001	0.002
MyD88	1.00 ± 0.12	0.97 ± 0.17	0.84 ± 0.18	0.993	0.823
IRAK1	1.00 ± 0.08	1.08 ± 0.07	0.72 ± 0.10	0.799	0.021
TRAF6	1.00 ± 0.10	0.71 ± 0.12	0.99 ± 0.16	0.280	0.299
NOD1	1.00 ± 0.14	1.99 ± 0.14*	1.40 ± 0.14#	<0.001	0.025
NOD2	1.00 ± 0.07	2.55 ± 0.16*	0.66 ± 0.09#	<0.001	<0.001
RIPK2	1.00 ± 0.15	2.42 ± 0.08*	1.17 ± 0.18#	<0.001	<0.001
NF-κB p65	1.00 ± 0.11	1.75 ± 0.15*	1.26 ± 0.07#	0.001	0.025

TLR4 = toll-like receptor 4; *MyD88* = myeloid differentiation factor 88; *IRAK1* = IL-1 receptor-associated kinase 1; *TRAF6* = TNF receptor-associated factor 6. *NOD* = nucleotide-binding oligomerization domain protein; *RIPK2* = receptor-interacting serine/threonine-protein kinase 2; *NF-κB p65* = nuclear factor-κB p65.
*, a significant difference ($P < 0.05$) between NBW-CON group and IUGR-CON group; #, a significant difference ($P < 0.05$) between IUGR-DHA group and IUGR-CON group.
[1] NBW-CON, normal body weight group fed a basal diet; IUGR-CON, IUGR group fed a basal diet; IUGR-DHA, IUGR group fed a DHA-supplemented diet at 80 mg/kg. Results were showed as means ± SEM ($n = 8$).

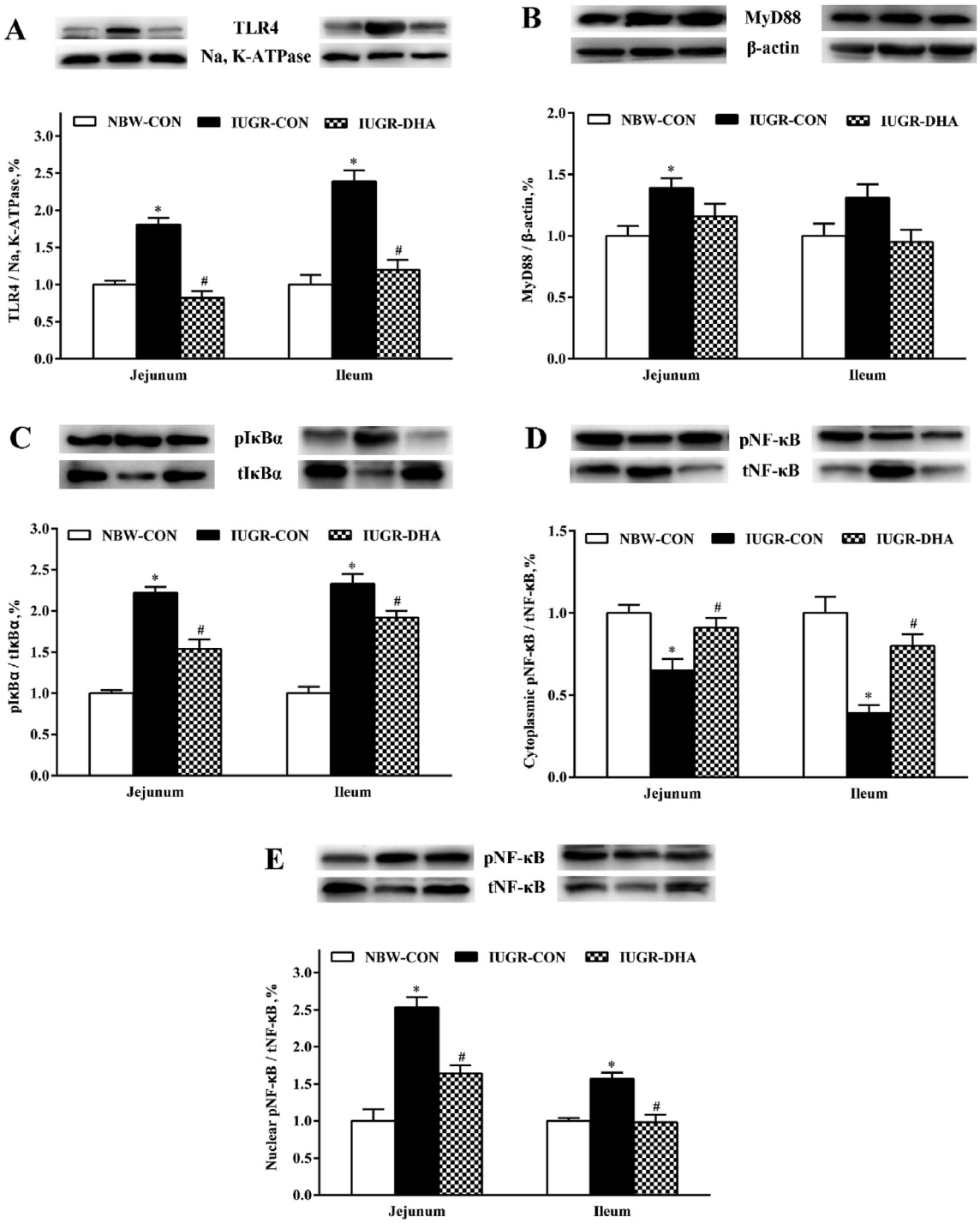

Fig. 6. Effect of dihydroartemisinin (DHA) on the protein expressions of TLR4 in the membrane (A), MyD88 in the cytoplasm (B), pIκBα in the cytoplasm (C), pNF-κB in the cytoplasm (D), and pNF-κB in the nucleus (E) of intestine in weaned piglets with intrauterine growth retardation (IUGR). Results were shown as means ± SEM ($n = 8$). NBW-CON, normal body weight group fed a basal diet; IUGR-CON, IUGR group fed a basal diet; IUGR-DHA, IUGR group fed a diet supplemented with DHA at 80 mg/kg. *, a significant difference ($P < 0.05$) between NBW-CON group and IUGR-CON group; #, a significant difference ($P < 0.05$) between IUGR-DHA group and IUGR-CON group. TLR4 = toll-like receptors 4; MyD88 = myeloid differentiation factor 88; tIκBα = total NF-κB inhibitor α; pIκBα = phosphorylated NF-κB inhibitor α; tNF-κB = total nuclear factor κB; pNF-κB = phosphorylated nuclear factor κB.

in piglets with IUGR. A previous study showed that dihydroartemisinin decreased the concentrations of IL-6 and IL-1β induced by TNF-α in endothelial cells (Yin et al., 2018). Research also demonstrated that dihydroartemisinin administration down-regulated the expressions of IL-1β and IL-6 in LPS-induced mice (Gao et al., 2020). The results indicated that DHA could attenuate the intestinal inflammatory response of piglets with IUGR by reducing the levels of pro-inflammation cytokines due to its anti-inflammatory activity.

To clearly illustrate the molecular mechanism of DHA supplementation on attenuating the intestinal inflammatory injury, we determined the function of TLR and NLR (Al-Sayeqh et al., 2010),

Table 7
Effect of dihydroartemisinin (DHA) on intestinal mRNA expressions of negative regulators of TLR4/NOD/NF-κB signaling pathway in weaned piglets with intrauterine growth retardation (IUGR).

Item	Treatment[1]			*P*-value	
	NBW-CON	IUGR-CON	IUGR-DHA	NBW-CON vs. IUGR-CON	IUGR-CON vs. IUGR-DHA
Jejunum					
RP105	1.00 ± 0.09	0.89 ± 0.07	0.86 ± 0.07	0.537	0.972
SOCS1	1.00 ± 0.04	1.02 ± 0.14	0.96 ± 0.18	0.991	0.955
Tollip	1.00 ± 0.05	0.57 ± 0.04*	0.86 ± 0.03#	<0.001	<0.001
ERBB2IP	1.00 ± 0.04	0.22 ± 0.03*	0.72 ± 0.05#	<0.001	<0.001
CENTB1	1.00 ± 0.05	0.56 ± 0.07*	1.13 ± 0.08#	0.001	<0.001
Ileum					
RP105	1.00 ± 0.08	1.05 ± 013	1.06 ± 0.17	0.969	0.998
SOCS1	1.00 ± 0.11	0.37 ± 0.07*	0.79 ± 0.10#	0.001	0.018
Tollip	1.00 ± 0.13	0.74 ± 0.13	1.02 ± 0.15	0.398	0.356
ERBB2IP	1.00 ± 0.08	0.28 ± 0.04*	0.61 ± 0.11#	<0.001	0.033
CENTB1	1.00 ± 0.03	0.31 ± 0.05*	0.82 ± 0.05#	<0.001	<0.001

RP105 = radioprotective 105; *SOCS1* = suppressor of cytokine signaling 1; *Tollip* = toll-interacting protein; *ERBB2IP* = Erbb2 interacting protein; *CENTB1* = centaurin β1.
*, a significant difference ($P < 0.05$) between NBW-CON group and IUGR-CON group; #, a significant difference ($P < 0.05$) between IUGR-DHA group and IUGR-CON group.
[1] NBW-CON, normal body weight group fed a basal diet; IUGR-CON, IUGR group fed a basal diet; IUGR-DHA, IUGR group fed a DHA-supplemented diet at 80 mg/kg. Results were showed as means ± SEM ($n = 8$).

which also play important roles in the dysregulated apoptosis (Subramanian et al., 2020). TLR4 is a best characterized member of TLR, which is a signaling receptor for recognizing LPS (Palsson-McDermott and O'Neill, 2004). LPS, the main composition of outer membrane of Gram-negative bacteria, is a potent activator that elicits inflammatory responses in mammalian cells (Rietschel et al., 1993). When the intestine is stimulated by LPS, TLR4/CD14/MD2 complex recruits and activates an adapter protein MyD88, which then recruits IRAK1 (Wesche et al., 1997). Afterwards the receptor complex interacts with the adapter molecule TRAF6 (Cao et al., 1996; Muzio et al., 1998) and subsequently activates the IκB kinase complex (IKKα and IKKβ) which directly phosphorylates IκB (Didonato et al., 1997; Scheidereit, 1998; Stancovski and Baltimore, 1997). The phosphorylation of IκB family eventually activates NF-κB and results in the subsequent translocation of NF-κB to the nucleus (Rothwarf and Karin, 1999). In addition, the intracellular NLR proteins are also involved in the activation of NF-κB pathway. Among NLRs, NOD1 and NOD2 identify dipeptideg-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP) respectively, which are produced by both Gram-positive and Gram-negative bacteria (Chamaillard et al., 2003; Girardin et al., 2003). Direct or indirect ligand recognition by NOD1 and NOD2 recruits RIPK2 to induce NF-κB signaling (Kanneganti et al., 2007). The activation of NF-κB leads to the synthesis and release of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α (Lawrence, 2009). Consequently, the pro-inflammatory cytokines elicit the inflammatory response and result in intestinal injury. In the current study, we firstly determined the intestinal LPS levels and found that IUGR increased the concentrations of LPS in the jejunum and ileum of weaned piglets. When the intestine is activated by LPS, the mRNA expressions of intestinal TLR4 (*TLR4*, *MyD88*, *IRAK1* in the jejunum, and *TLR4* in the ileum) and NOD signaling-related genes (*NOD1*, *RIPK2* in the jejunum and *NOD1*, *NOD2*, *RIPK2* in the ileum) and *NF-κB p65* were upregulated in the intestine of piglets with IUGR. The protein expressions of TLR4 and MyD88 in the jejunum and TLR4 in the ileum of piglets with IUGR were higher than those of piglets with NBW, which were consistent with the results of the related mRNA expressions in the present study. Weaned piglets with IUGR also increased the protein expressions of pIκBα and nuclear NF-κB and decreased cytoplasmic pNF-κB in the intestine. Similar results were found in the liver of IUGR rats (He et al., 2018). There are numerous studies about the mechanism of DHA in alleviating inflammation. However, the research on DHA suppressing intestinal inflammation via TLR4/NOD/NF-κB pathway was limited. A recent study reported that DHA attenuated the inflammation induced by lupus nephritis through TLR4 signaling pathway (Diao et al., 2019). Li et al. (2019) demonstrated that DHA derivative (9α, 12α-dihydroartemisinyl) bis (2′-chlorocinnmate) (DC32) inhibited inflammatory response in osteoarthritic synovium of rats via regulating Nrf2/NF-κB pathway. The study of Liu et al. (2017) also showed that dihydroartemisinin alleviated autoimmune thyroiditis of rats by inhibiting the CXCR3/PI3K/AKT/NF-κB signaling pathway. The present study showed that diet supplemented with DHA effectively reduced the related mRNA expressions of TLR4/NOD/NF-κB pathway, decreased the protein expressions of TLR4, pIκBα and nuclear NF-κB and improved cytoplasmic pNF-κB in the intestine of piglets with IUGR. Therefore, these data indicated that dietary supplementation with DHA could alleviate intestinal inflammatory response through TLR4/NOD/NF-κB signaling pathway in weaned piglets with IUGR.

It has been reported that TLR4/NOD signaling is also negatively modulated by multiple mechanisms (Wang et al., 2017). Research has shown that Tollip, RP105, and SOCS1 are considered to be the representative negative regulators of TLR4 signaling (Divanovic et al., 2005; Humbert-Claude et al., 2016; Kinjyo et al., 2002) and the typical negative regulators of NOD signaling are ERBB2IP and CENTB1 (Günthner et al., 2013; McDonald et al., 2005). In this experiment, IUGR exhibited lower mRNA expressions of jejunal *Tollip*, *ERBB2IP*, *CENTB1* and ileal *SOCS1*, *ERBB2IP*, *CENTB1* of weaned piglets. Dihydroartemisinin supplementation effectively upregulated the mRNA expressions of jejunal *Tollip*, *ERBB2IP*, *CENTB1* and ileal *SOCS1*, *ERBB2IP*, *CENTB1* of piglets with IUGR. Similar findings were observed in the intestine of pigs after LPS treatment (Wang et al., 2017). The results demonstrated that DHA inclusion increased the mRNA expressions of intestinal TLR4 and NOD negative regulators of piglets with IUGR, which were consistent with the reduced mRNA expressions of intestinal TLR4 and NOD signaling-related genes. Therefore, the inhibitory effects of DHA on TLR4 and NOD signaling may be attributed to the improvement of related gene expressions of their negative regulators.

5. Conclusions

The present results have shown that piglets with IUGR exhibited a high risk of intestinal inflammatory response. Dietary supplementation of DHA to weaned piglets with IUGR could improve intestinal morphology, regulate the proliferation and apoptosis of enterocytes, and attenuate intestinal inflammatory injury by

reducing the release of pro-inflammatory cytokines via the inhibition of TLR4/NOD/NF-κB signaling pathway. This study may provide a novel nutritional strategy for IUGR offspring to maintain intestinal health.

Author contributions

Yu Niu: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Yongwei Zhao:** Investigation. **Jintian He:** Conceptualization, Investigation. **Yang Yun:** Investigation. **Mingming Shen:** Investigation. **Zhending Gan:** Investigation. **Lili Zhang:** Project administration. **Tian Wang:** Resources, Writing – review & editing, Supervision, Funding acquisition.

Conflict of interest

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

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