Dietary Dihydroartemisinin Supplementation Alleviates Intestinal Inflammatory Injury Through TLR4/NODs/NF- κ B Signaling Pathway in Weaned Piglets with Intrauterine Growth Retardation

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Keywords: Intrauterine growth retardation, Weaned piglets, Dihydroartemisinin, Intestine injury, Inflammation, Morphology

Posted Date: October 2nd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-84327/v1>

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Version of Record: A version of this preprint was published at Animal Nutrition on May 1st, 2021. See the published version at <https://doi.org/10.1016/j.aninu.2020.12.009>.

Abstract

Background: Intrauterine growth retardation (IUGR) leads to impaired intestinal morphology and function. IUGR infants are at a risk for intestinal inflammatory diseases. Dihydroartemisinin (DHA) is a derivative of artemisinin, which possesses anti-inflammatory activity and immunomodulatory effect. However, little is known about the effects of DHA on IUGR piglets. Therefore, the present study was conducted to investigate whether dietary DHA supplementation could attenuate intestinal injury in IUGR weaned piglets.

Methods: Piglets with normal birth weight or IUGR were fed the basal diet or basal diet supplemented with 80 mg/kg DHA from 21 d to 49 days of age. At 49 days of age, eight piglets from each group with nearly similar body weight were sacrificed. The jejunal and ileal samples were collected for further analysis.

Results: IUGR impaired intestinal morphology, increased intestinal inflammatory response, raised enterocyte apoptosis and reduced enterocyte proliferation and activated TLR4/NODs/NF- κ B signaling pathway. DHA supplementation improved intestinal morphology, indicated by higher villus height, villus height to crypt depth ratio, villus surface area and lower villus width of IUGR piglets ($P < 0.05$). DHA inclusion exhibited higher apoptosis index and the expression of caspase-3, and lower proliferation index and the expression of proliferating cell nuclear antigen in the intestine of IUGR piglets ($P < 0.05$). Diet supplemented with DHA could attenuate intestinal inflammation, indicated by higher concentrations of intestinal inflammatory cytokines and lipopolysaccharides in IUGR piglets ($P < 0.05$). In addition, DHA down-regulated the related mRNA expressions of TLR4/NODs/NF- κ B signaling pathway and up-regulated mRNA expressions of TLR4 and NODs signaling negative regulators in the intestine of IUGR piglets ($P < 0.05$). Dietary DHA supplementation decreased the protein expressions of toll-like receptors 4, phosphorylated NF- κ B (pNF- κ B) inhibitor α , nuclear pNF- κ B, and increased the protein expression of cytoplasmic pNF- κ B in the intestine of IUGR piglets ($P < 0.05$).

Conclusions: Dietary DHA supplementation may have beneficial effects in improving intestinal morphology, regulating enterocyte proliferation and apoptosis, and alleviating intestinal inflammation through TLR4/NODs/NF- κ B signaling pathway in IUGR weaned piglets.

Background

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 during pregnancy [1]. Researches have suggested that approximately 5–10% of human newborns suffer from IUGR [2]. Pigs, as multi-fetal animals, exhibit high incidence of IUGR (15%-20%), which have been used as a model for human IUGR studies [3]. IUGR leads to increased risk for neonatal and long-term morbidities affecting multiple organ systems including the intestine [4, 5]. Infants with IUGR often display impaired intestinal morphology and function [4]. Study also demonstrated that infant with IUGR were at a risk for intestinal inflammatory diseases [6].

Dihydroartemisinin (DHA) is a kind of derivative of artemisinin, which is extracted from the traditional Chinese herb *Artemisia annua* L. [7]. DHA is mainly used to treat malaria for decades. Besides anti-malaria activity, DHA also possesses anti-inflammatory activity and immunomodulatory effect [8]. An increasing number of studies have shown that DHA attenuates inflammatory injury through suppressing the activation of nuclear factor- κ B (NF- κ B) pathway [9–11]. Transmembrane toll like receptors (TLRs) and nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) are the key protein families of pattern recognition receptors, which are involved in mediating inflammatory process and expressed in many tissues including the intestine [12]. TLR4 is a significant member of TLRs, which plays an important role in innate immunity and inflammation by sensing pathogen-associated molecular patterns, such as lipopolysaccharide (LPS) [13]. When stimulated by LPS, TLR4 with the accessory proteins causes the activation of NF- κ B via a series of signaling cascade reactions [14]. NOD1 and NOD2 are the better-characterized members of NLRs, which can also activate NF- κ B [15]. NF- κ B is a key transcription factor which regulates a large array of genes involved in the process of immune, inflammation and cell proliferation [16]. The activation of NF- κ B modulates downstream targets and stimulates the production of pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α , finally leading to tissue injury. However, no information is available about the effect and mechanism of DHA on intestinal inflammatory injury in IUGR piglets.

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 IUGR piglets via TLR4/NODs/NF- κ B signaling pathway. Therefore, the aim of this study is to investigate whether DHA could alleviate IUGR-induced intestinal injury in weaned piglets and to elaborate its mechanisms. This research also provides a reference for treatment of IUGR in humans.

Methods

Preparation of DHA

The DHA used in this experiment was purchased from DASF Biotechnology Co., Ltd (Nanjing, Jiangsu, China). The content of DHA was up to 99% as determined by High Performance Liquid Chromatography analysis.

Animals and experimental design

All experimental design and procedures were approved by Institutional Animal Care and Use Committee of Nanjing Agricultural University following the requirements of the Regulations for the Administration of Affairs Concerning Experimental Animals of China (NJAUCAST-2018-146). A total of twelve sows with similar birth order (third or fourth) were fed the same gestating diets that met the nutrient requirements of pregnant sows according to NRC (2012). At 114 days (SD 1) of gestation, the birth weight of each

neonatal piglet [Duroc × (Landrace × Yorkshire)] was recorded. Neonatal piglets with a birth weight (BW) close to the average BW of the total population (within 0.5 SD) were defined as normal birth weight (NBW), and those with a 2 SD lower BW were identified as IUGR [17, 18]. From each litter, one NBW piglet (1.56 ± 0.02 kg) and two IUGR piglets (0.99 ± 0.03 kg) were selected and marked. All the newborn piglets were suckled with their own sows until weaning at 21 days of age. In each litter, the NBW piglet and one of the IUGR piglets were divided into two groups fed the basal diet (NBW-CON and IUGR-CON group, respectively). The other IUGR piglet was assigned to the group fed the basal diet supplemented with 80 mg/kg DHA (IUGR-DHA group). The chemical composition of the diet was presented in Table 1, which was formulated to meet the nutrient requirements of piglets according to the NRC (2012). Piglets were housed individually in plastic floored pens (1 m × 0.6 m) at an ambient temperature of 25 to 28°C and given *ad libitum* access to feed and water throughout the whole experimental period.

Table 1
Composition and nutrient level of the basal diet (air-dry basis)

Ingredients	Ratio (%)	Calculated nutrient levels	Content
Corn	57.70	Digestible energy (MJ/kg)	14.04
Soybean meal (46%)	12.50	Crude protein (%)	18.31
Expanded corn	8.00	Lysine (%)	1.31
Full-fat soybean	8.00	Methionine (%)	0.40
Fermented soybean meal	4.00	Methionine + Cystine (%)	0.70
Whey powder	3.00	Threonine (%)	0.80
Fish meal (crude protein 67%)	3.00	Calcium (%)	0.85
Dicalcium phosphate	1.80	Total phosphorus (%)	0.72
Limestone	0.50		
L-lysine (78%)	0.30		
L-threonine	0.10		
DL-methionine	0.08		
Wheat middling	0.02		
Premix ¹	1		
Total	100		
¹ The premix provided the following per kg complete diet: vitamin A, 12000 IU; vitamin D ₃ , 3000 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 ·H ₂ O), 110 mg; Cu (CuSO ₄ ·5H ₂ O), 7 mg; Zn (ZnO), 110 mg; I (KIO ₃), 0.3 mg; Mn (MnSO ₄ ·H ₂ O), 5 mg; Se (Na ₂ SeO ₃), 0.3 mg.			

Sample collection

At 49 days of age, eight piglets from each group with nearly similar body weight (n = 8, four barrows and four gilts) were sacrificed by intramuscular injection of sodium pentobarbital (50 mg/kg BW). Blood sample was collected from jugular vein puncture in a nonheparinized tube. The serum was separated by centrifugation for 15 min at 3000 × g at 4°C and stored at -80°C until analysis. The small intestine without mesentery was removed from the abdominal cavity and divided into three segments: duodenum, jejunum and ileum according to previous study [19]. Segments measuring approximately 1 cm from the jejunum and ileum were collected and flushed gently with ice-cold phosphate-buffered saline (PBS, pH =

7.4). Then the samples were fixed in 4% fresh, cold paraformaldehyde solution for histological analysis. In addition, jejunal and ileal segments measuring about 20 cm were opened longitudinally and flushed gently with ice-cold PBS (pH = 7.4). The mucosa was scraped from luminal surface using a sterile glass microscope slide and stored at -80°C until analysis.

Intestinal morphology analysis

After fixation for 24 h, the jejunal and ileal samples were dehydrated using a graded series of ethanol (70-100%), cleared by xylene, and embedded in paraffin. Then the samples were cut at a thickness of 5 µm and stained with hematoxylin and eosin. Six slices for each sample were prepared and the images of intestinal morphology were acquired using an optical binocular microscope with a digital camera (Nikon H5500L, Tokyo, Japan). Villus height (VH) and crypt depth (CD) were determined using Image-Pro Plus software. VH/CD was calculated by villus height to crypt depth ratio and villus area (VSA) was calculated using the following equation:

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

Immunohistochemistry analysis

Villus cell apoptosis was determined by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) staining method. Briefly, the slides were deparaffinised in xylene, dehydrated with alcohol, and pretreated with protease K for antigen retrieval. Then the sections were incubated with TdT and dUTP (vol:vol = 1:9) according to the TUNEL kit (Roche Corporation, Basel, Switzerland). After washing with PBS (pH = 7.4), the slides were stained with DAPI dye and finally mounted with anti-fluorescein reagent. For each slide, the number of positive cells (stained cells) was counted from ten villi using a Nikon morphometric system (Nikon Corporation, Tokyo, Japan). Apoptosis index (AI) was defined as the ratio of the number of apoptotic TUNEL positive cells to total cell numbers multiplied by 100.

Ki-67 is a biomarker for proliferative activity and crypt cell proliferation is measured using Ki-67 immunohistochemistry staining [20]. Samples for intestinal morphology determination were used for immunohistochemistry analysis. The jejunal and ileal slices (5 µm thick) were deparaffinised in xylene, dehydrated with alcohol, and microwave-pretreated in 10 mM citrate buffer (pH = 6.0) for antigen retrieval. After washing with PBS (pH = 7.4), the tissue slices were incubated with 3% H₂O₂ in dark for 25 min to eliminate the interference of endogenous peroxidase and then blocked with normal goat serum for 30 min to inhibit the non-specific interactions of the tissue with the primary antibody. Then the slides were incubated with the primary antibody (rabbit polyclonal to Ki67, Abcam, Cambridge, UK; 1:500) for 2 h and with secondary antibody (goat anti-rabbit IgG, Abcam, Cambridge, UK; 1:1000) conjugated with horseradish peroxidase for 50 min at room temperature. After washing with PBS (pH = 7.4), the sections

were stained with diaminobenzidine (DAB) dye under the microscope to control the color-development time and then counterstained with hematoxylin for 3 min. Finally, the slides were dehydrated with ethanol and mounted with neutral balsam. For each slide, the number of positive cells (stained cells) was counted from ten crypts using a Nikon morphometric system (Nikon Corporation, Tokyo, Japan). The proliferation index (PI) was defined as the ratio of the number of Ki-67 positive cells to total cell numbers multiplied by 100.

Concentration of intestinal inflammatory cytokine analysis

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

Concentration of intestinal lipopolysaccharide analysis

The concentrations of lipopolysaccharide (LPS) 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).

Gene expression analysis

Total RNA was isolated from the frozen jejunal and ileal mucosa by using Trizol reagent (TaKaRa Biotechnology Co. Ltd, Dalian, Liaoning, China). The concentration of RNA was determined by using a spectrophotometer (NanoDrop 2000c, Thermo Scientific, Waltham, MA, USA) based on the absorption value at 260 nm or 280 nm. The quality of RNA was accessed by agarose gel electrophoresis. Then the 1 μ g of total RNA was reverse-transcribed into cDNA using the Perfect Real Time SYBR Premix Ex Taq kit (TaKaRa Biotechnology Co. Ltd, Dalian, China) according to the manufacturer's instructions. After that, quantitative real-time polymerase chain reaction (qRT-PCR) assays were conducted on an ABI StepOnePlus Real-Time PCR detection system (Applied Biosystems; Carlsbad, CA, USA) by using a SYBR Premix Ex Taq Kit (TakaRa Biotechnology Co. Ltd; Dalian, Liaoning, China). The primer sequences for the target and reference genes were presented in Table 2. In detail, the reaction system was 20 μ L in total, including 2 μ L of cDNA, 0.4 μ L of forward primers, 0.4 μ L of reverse primers, 0.4 μ L of ROX Reference Dye, 10 μ L of SYBR Premix Ex Taq and 6.8 μ L of double-distilled water (TaKaRa Biotechnology Co. Ltd., Dalian, Liaoning, China). The thermocycle used for qRT-PCR was 95 °C for 30 s for one cycle, 40 cycles of 5 s at 95 °C, and 30 s at 60 °C. Each sample was run in duplicate. The mRNA expression level of each gene was calculated with $2^{-\Delta\Delta C_t}$ method after normalization with the housekeeper gene β -actin.

Table 2
Primer sequences of target genes

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

Western blot analysis

Antibodies against caspase-3 (1:500), proliferating cell nuclear antigen (PCNA, 1:500), toll-like receptors 4 (TLR4, 1:500) were purchased from Abcam plc. (Cambridge, UK). Antibodies against myeloid differentiation factor 88 (MyD88, 1:1000), total nuclear factor κB (NF-κB, 1:1000), phosphorylated nuclear factor κB (pNF-κB, 1:1000), total NF-κB inhibitor α (IκBα, 1:1000) and phosphorylated NF-κB inhibitor α (pIκBα, 1:1000), β-actin (1:1000) and Na, K-ATPase (1:1000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The total, nuclear or cytoplasmic proteins of intestinal mucosal samples were extracted using assay kits according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). The protein concentration of each sample was measured using the BCA Protein Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). For western blot analysis, 60 μg protein from each sample was electrophoresed in sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. After blocking in Tris-Tween buffered saline (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 2 h at room temperature, the membranes were washed in TBST for three times and incubated with primary antibodies overnight at 4°C with gentle rocking. Then the membranes were washed for three times again and incubated with secondary antibodies (goat anti-rabbit IgG or goat anti-mouse IgG, 1:2000; Abcam, Cambridge, UK) at room temperature for 60 min. The blots were detected using enhanced chemiluminescence reagents (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) followed by autoradiography. Images of the membranes were snapped with a Luminescent Image Analyzer LAS-4000 system (Fujifilm Co. Ltd., Tokyo, Japan) and quantified by the Gel-Pro Analyzer 4.0 software (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

Data were analyzed using SPSS statistical software (Ver. 20.0 for windows, SPSS, Chicago, IL, USA). Statistical differences among treatment groups were determined by one-way analysis of variance (ANOVA) and Tukey's post hoc test was used for multiple comparisons. A probably level of $P < 0.05$ was considered as statistically significant. Values were presented as means ± SEM.

Results

Intestinal morphology

IUGR decreased ($P < 0.05$) VH, VCR and VSA and increased ($P < 0.05$) CD in both jejunum and ileum of piglets (Table 3). DHA administration effectively exhibited an increased ($P < 0.05$) VH, VCR and VSA and a decreased ($P < 0.05$) CD in the intestine of IUGR piglets.

Table 3

Effect of dietary dihydroartemisinin supplementation on intestinal morphology in weaned piglets with intrauterine growth retardation.

Items	Treatment ¹			P value ²	
	NBW-CON (NC)	IUGR-CON (IC)	IUGR-DHA (ID)	NC vs. IC	IC vs. ID
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	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	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

¹NBW-CON (NC) group, normal body weight weaned piglets fed with basal diet; IUGR-CON (IC) group, intrauterine growth retardation weaned piglets fed with basal diet; IUGR-DHA (ID) group, intrauterine growth retardation weaned piglets fed with 80 mg/kg dihydroartemisinin. Data were presented as mean ± SEM (n = 8). ²A value of * $P < 0.05$ was considered as statistically significant difference when compared with NBW-CON group; [#] $P < 0.05$ was considered as statistically significant difference when compared with IUGR-CON group. VH, villus height; VD, villus width; CD, crypt depth; VCR, villus height to crypt depth ratio; VSA, villus surface area.

Cell apoptosis and proliferation

IUGR piglets showed higher ($P < 0.05$) AI (Fig. 1) and lower ($P < 0.05$) PI (Fig. 2) in both jejunum and ileum than NBW piglets (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 ($P < 0.05$) the protein expression of caspase-3 (Fig. 3) and increased ($P < 0.05$) the protein expression of PCNA (Fig. 4) in jejunum and ileum of piglets. Diet supplemented with DHA effectively enhanced ($P < 0.05$) PI and level of PCNA, and reduced ($P < 0.05$) AI and level of caspase-3 in both jejunum and ileum of IUGR piglets.

Table 4

Effect of dietary dihydroartemisinin supplementation on enterocyte proliferation and apoptosis in weaned piglets with intrauterine growth retardation.

Items	Treatment ¹			P value ²	
	NBW-CON (NC)	IUGR-CON (IC)	IUGR-DHA (ID)	NC vs. IC	IC vs. ID
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
¹ NBW-CON (NC) group, normal body weight weaned piglets fed with basal diet; IUGR-CON (IC) group, intrauterine growth retardation weaned piglets fed with basal diet; IUGR-DHA (ID) group, intrauterine growth retardation weaned piglets fed with 80 mg/kg dihydroartemisinin. Data were presented as mean ± SEM (n = 8). ² A value of * $P < 0.05$ was considered as statistically significant difference when compared with NBW-CON group; [#] $P < 0.05$ was considered as statistically significant difference when compared with IUGR-CON group. AI, apoptosis index; PI, proliferation index.					

Concentrations of intestinal inflammatory cytokines

In the jejunum, the concentrations of inflammatory cytokines IL-1 β , IL-6 and TNF- α were increased ($P < 0.05$) in the IUGR-CON group compared with the NBW-CON group (Table 5). IUGR piglets fed the DHA diet significantly decreased ($P < 0.05$) the concentrations of IL-1 β and IL-6 compared with those fed the basal diet. In the ileum, the concentrations of IL-1 β and IL-6 in the IUGR-CON group were higher ($P < 0.05$) than

those of NBW-CON group. After treatment with DHA, IUGR piglets decreased ($P < 0.05$) the concentrations of IL-1 β , IL-6 and TNF- α .

Table 5
Effect of dietary dihydroartemisinin supplementation on the concentrations of intestinal inflammatory cytokines in weaned piglets with intrauterine growth retardation.

Items	Treatment ¹			P value ²	
	NBW-CON (NC)	IUGR-CON (IC)	IUGR-DHA (ID)	NC vs. IC	IC vs. ID
Jejunum					
IL-1 β (ng/g protein)	33.61 $\pm$ 3.21	54.08 $\pm$ 3.00*	40.44 $\pm$ 1.65 [#]	<0.001	0.008
IL-6 (ng/g protein)	64.73 $\pm$ 3.40	83.68 $\pm$ 2.96*	62.67 $\pm$ 3.63 [#]	0.005	0.002
TNF- α (ng/g protein)	17.93 $\pm$ 0.68	22.13 $\pm$ 1.13*	20.11 $\pm$ 1.12	0.024	0.350
Ileum					
IL-1 β (ng/g protein)	26.68 $\pm$ 1.86	33.66 $\pm$ 1.71*	26.59 $\pm$ 1.71 [#]	0.037	0.032
IL-6 (ng/g protein)	56.67 $\pm$ 3.05	72.24 $\pm$ 2.55*	60.91 $\pm$ 2.73 [#]	0.003	0.029
TNF- α (ng/g protein)	15.13 $\pm$ 1.29	16.76 $\pm$ 0.62	13.14 $\pm$ 0.63 [#]	0.433	0.032
¹ NBW-CON (NC) group, normal body weight weaned piglets fed with basal diet; IUGR-CON (IC) group, intrauterine growth retardation weaned piglets fed with basal diet; IUGR-DHA (ID) group, intrauterine growth retardation weaned piglets fed with 80 mg/kg dihydroartemisinin. Data were presented as mean $\pm$ SEM (n = 8). ² A value of * $P < 0.05$ was considered as statistically significant difference when compared with NBW-CON group; [#] $P < 0.05$ was considered as statistically significant difference when compared with IUGR-CON group. IL-1 β , interleukin 1 β ; IL-6, interleukin 6; TNF- α , tumor necrosis factor α .					

Concentrations of intestinal LPS

Piglets in the IUGR-CON group exhibited increased ($P < 0.05$) LPS concentration in comparison with those of NBW piglets in both jejunum and ileum (Fig. 5). Supplementation with DHA significantly reduced ($P < 0.05$) the concentrations of LPS in the intestine of IUGR piglets.

Intestinal mRNA expressions of TLR4/NODs/NF- κ B signaling pathway

As presented in Table 6, IUGR up-regulated ($P < 0.05$) the mRNA expressions of *TLR4*, *MyD88*, *IRAK1*, *NOD1*, *RIPK2*, and *NF- κ B p65* in the jejunum, and *TLR4*, *NOD1*, *NOD2*, *RIPK2*, and *NF- κ B p65* in the ileum of the IUGR-CON group in comparison with the NBW-CON group. Dietary DHA supplementation down-regulated ($P < 0.05$) the mRNA expressions of jejunal *TLR4*, *IRAK1*, *NOD1*, *RIPK2*, and *NF- κ B p65* and ileal *TLR4*, *NOD1*, *NOD2*, *RIPK2*, and *NF- κ B p65* of IUGR piglets.

Table 6

Effect of dietary dihydroartemisinin supplementation on intestinal mRNA abundance of TLR4/NODs/NF- κ B signaling pathway in weaned piglets with intrauterine growth retardation.

Items	Treatment ¹			P value ²	
	NBW-CON (NC)	IUGR-CON (IC)	IUGR-DHA (ID)	NC vs. IC	IC vs. ID
Jejunum					
<i>TLR4</i>	1.00±0.05	1.41±0.06*	0.64±0.04 [#]	<0.001	<0.001
<i>MyD88</i>	1.00±0.04	1.36±0.05*	1.20±0.05	<0.001	0.076
<i>IRAK1</i>	1.00±0.12	2.08±0.08*	0.44±0.07 [#]	<0.001	<0.001
<i>TRAF6</i>	1.00±0.05	0.87±0.07	0.91±0.05	0.272	0.883
<i>NOD1</i>	1.00±0.07	1.55±0.10*	1.03±0.04 [#]	<0.001	<0.001
<i>NOD2</i>	1.00±0.06	0.92±0.09	0.83±0.06	0.690	0.636
<i>RIPK2</i>	1.00±0.10	1.58±0.11*	0.97±0.04 [#]	0.001	0.001
<i>NF-κB p65</i>	1.00±0.09	1.75±0.15*	0.76±0.07 [#]	0.001	<0.001
Ileum					
<i>TLR4</i>	1.00±0.16	2.89±0.19*	1.87±0.15 [#]	<0.001	0.002
<i>MyD88</i>	1.00±0.12	0.97±0.17	0.84±0.18	0.993	0.823
<i>IRAK1</i>	1.00±0.08	1.08±0.07	0.72±0.10	0.799	0.021
<i>TRAF6</i>	1.00±0.10	0.71±0.12	0.99±0.16	0.280	0.299
<i>NOD1</i>	1.00±0.14	1.99±0.14*	1.40±0.14 [#]	<0.001	0.025
<i>NOD2</i>	1.00±0.07	2.55±0.16*	0.66±0.09 [#]	<0.001	<0.001
<i>RIPK2</i>	1.00±0.15	2.42±0.08*	1.17±0.18 [#]	<0.001	<0.001
<i>NF-κB p65</i>	1.00±0.11	1.75±0.15*	1.26±0.07 [#]	0.001	0.025

¹NBW-CON (NC) group, normal body weight weaned piglets fed with basal diet; IUGR-CON (IC) group, intrauterine growth retardation weaned piglets fed with basal diet; IUGR-DHA (ID) group, intrauterine growth retardation weaned piglets fed with 80 mg/kg dihydroartemisinin. Data were presented as mean $\pm$ SEM (n = 8). ²A value of * P < 0.05 was considered as statistically significant difference when compared with NBW-CON group; [#] P < 0.05 was considered as statistically significant difference when compared with IUGR-CON group. *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-

Intestinal protein expressions of TLR4/NODs/NF-κB signaling pathway

As shown in Fig. 6, IUGR piglets exhibited higher ($P < 0.05$) protein expressions of TLR4, pIκBα, nuclear pNF-κB and lower ($P < 0.05$) cytoplasmic pNF-κB levels in both jejunum and ileum than those of NBW piglets. DHA supplementation effectively improved ($P < 0.05$) the alternation of these protein expressions in IUGR piglets. The protein expression of MyD88 was also increased ($P < 0.05$) in the jejunum of IUGR-CON group compared to that of NBW-CON group. However, feeding the IUGR piglets with DHA diet had no significant effect on the MyD88 levels ($P > 0.05$).

Intestinal mRNA expressions of negative regulators of TLR4/NODs signaling pathway

As shown in Table 7, the mRNA expressions of jejunal *Tollip*, *ERBB2IP*, and *CENTB1* and ileal *SOCS1*, *ERBB2IP* and *CENTB1* were reduced ($P < 0.05$) in the IUGR-CON group when compared with the NBW-CON group. Diet supplemented with DHA increased ($P < 0.05$) the mRNA expressions of these genes in both jejunum and ileum of IUGR piglets.

Table 7

Effect of dietary dihydroartemisinin supplementation on intestinal mRNA abundance of negative regulators of TLR4/NODs signaling pathway in weaned piglets with intrauterine growth retardation.

Items	Treatment ¹			P value ²	
	NBW-CON (NC)	IUGR-CON (IC)	IUGR-DHA (ID)	NC vs. IC	IC vs. ID
Jejunum					
<i>RP105</i>	1.00±0.09	0.89±0.07	0.86±0.07	0.537	0.972
<i>SOCS1</i>	1.00±0.04	1.02±0.14	0.96±0.18	0.991	0.955
<i>Tollip</i>	1.00±0.05	0.57±0.04*	0.86±0.03 [#]	<0.001	<0.001
<i>ERBB2IP</i>	1.00±0.04	0.22±0.03*	0.72±0.05 [#]	<0.001	<0.001
<i>CENTB1</i>	1.00±0.05	0.56±0.07*	1.13±0.08 [#]	0.001	<0.001
Ileum					
<i>RP105</i>	1.00±0.08	1.05±0.13	1.06±0.17	0.969	0.998
<i>SOCS1</i>	1.00±0.11	0.37±0.07*	0.79±0.10 [#]	0.001	0.018
<i>Tollip</i>	1.00±0.13	0.74±0.13	1.02±0.15	0.398	0.356
<i>ERBB2IP</i>	1.00±0.08	0.28±0.04*	0.61±0.11 [#]	<0.001	0.033
<i>CENTB1</i>	1.00±0.03	0.31±0.05*	0.82±0.05 [#]	<0.001	<0.001
¹ NBW-CON (NC) group, normal body weight weaned piglets fed with basal diet; IUGR-CON (IC) group, intrauterine growth retardation weaned piglets fed with basal diet; IUGR-DHA (ID) group, intrauterine growth retardation weaned piglets fed with 80 mg/kg dihydroartemisinin. Data were presented as mean ± SEM (n = 8). ² A value of * <i>P</i> < 0.05 was considered as statistically significant difference when compared with NBW-CON group; [#] <i>P</i> < 0.05 was considered as statistically significant difference when compared with IUGR-CON group. <i>RP105</i> , radioprotective 105; <i>SOCS1</i> , suppressor of cytokine signaling 1; <i>Tollip</i> , toll-interacting protein; <i>ERBB2IP</i> , Erbb2 interacting protein; <i>CENTB1</i> , centaurin β1.					

Discussion

The intestine is the biggest immune organ involved in immune and inflammatory reaction. Intestinal integrity mainly depends on the intact intestinal morphology which reflects gut health in humans and animals. Intestinal morphology can be assessed by villus height, crypt depth, villus to crypt ratio and villus surface area [21, 22]. In the current study, VH, VCR and VSA were reduced and CD was increased in both jejunum and ileum of IUGR weaned piglets compared to the NBW weaned piglets, suggesting a

decreased ability of intestinal absorption as well as an impaired intestinal integrity in IUGR piglets. These results were consistent with previous studies on IUGR piglets [3, 23–25]. Dietary supplementation with DHA enhanced VH, VCR, and VSA and decreased CD in IUGR piglets, indicating that DHA could improve the intestinal morphology.

Previous study demonstrated that the impaired intestinal morphology may be associated with the imbalance of cell apoptosis and proliferation [26]. In the current study, IUGR piglets enhanced the AI and reduced the PI of enterocytes when compared with NBW weaned piglets. Similar results were found in IUGR neonatal piglets [27]. The results also agreed with previous studies that IUGR increased cell apoptosis in the small intestine of rats [28] and decreased enterocyte proliferation in newborn rabbits [29]. However, the findings were dissimilar to previous study reported by Li et al. [26], 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 [30]. PCNA is an intranuclear polypeptide whose expression and synthesis are evaluated as the marker of cell proliferation [31]. In the present study, IUGR enhanced the protein expression of caspase-3 and reduced the protein expression of PCNA in both jejunum and ileum of weaned piglets. Previous research suggested that the expression of caspase-3 was increased and the expression of PCNA was decreased in the placentas of IUGR rats [32]. The results were also similar to previous observations that the TUNEL staining and caspase-3 activity were increased in the kidney of IUGR rats [33]. After DHA treatment, the protein expression of caspase-3 was decreased and PCNA was increased in IUGR piglets. These results indicated that IUGR was linked with decreased cell proliferation and increased cell apoptosis in small intestine and DHA inclusion could improve the excessive apoptosis in IUGR weaned piglets.

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 [34]. The present study showed that IUGR increased the concentrations of pro-inflammation cytokines IL-1 β , IL-6, TNF- α in the jejunum and IL-1 β , IL-6 in the ileum of IUGR piglets. Consistent with previous study, Huang et al. [35] observed higher levels of TNF- α and IL-6 in IUGR piglets at birth, suggesting that IUGR newborns was prone to inflammatory injury. Diet supplemented with DHA decreased the concentrations of pro-inflammation cytokines. Previous study showed that DHA decreased the concentrations of IL-6 and IL-1 β induced by TNF- α in endothelial cells [7]. Research also demonstrated that DHA administration down-regulated the expressions of IL-1 β and IL-6 in LPS-induced mice [36]. The results indicated that DHA could attenuate the intestinal inflammatory response of IUGR piglets by reducing the levels of pro-inflammation cytokines due to its anti-inflammatory activity.

In order to clearly illustrate the molecular mechanism of DHA supplementation on attenuating the intestinal inflammatory response, we determined the function of TLRs and NLRs [37], which also play important roles in the dysregulated apoptosis [38]. TLR4 is a best characterized member of TLRs, which

is a signaling receptor for recognizing LPS [39]. LPS or endotoxin, the major component of outer membrane of Gram-negative bacteria, is a potent activator that elicits inflammatory responses in mammalian cells [40]. When the intestine is stimulated by LPS, TLR4/CD14/MD2 complex recruits and activates an adapter protein myeloid differentiation protein 88 (MyD88), which in turn recruits IL-1 receptor-associated kinase 1 (IRAK1) [41]. The receptor complex then interacts with the adapter molecule TNF receptor-associated factor 6 (TRAF6) [42, 43] and subsequently activates the NF- κ B inhibitor (I κ B) kinase complex (IKK α and IKK β) which directly phosphorylates I κ B [44–46]. The phosphorylation of I κ B family eventually activates nuclear factor- κ B (NF- κ B) and results in the subsequent translocation of NF- κ B to the nucleus [47]. In addition, the intracellular NLR proteins are also involved in the activation of NF- κ B pathway. Among NLRs, NOD1 and NOD2 recognize the dipeptidoglycyl-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP) respectively, which are produced by both Gram-positive and Gram-negative bacteria [48, 49]. Direct or indirect ligand recognition by NOD1 and NOD2 recruits receptor-interacting serine/threonine-protein kinase 2 (RIPK2) to induce NF- κ B signaling [50]. The activation of NF- κ B leads to the synthesis and release of pro-inflammatory mediators, including IL-1 β , IL-6 and TNF- α [51]. Consequently, the pro-inflammatory cytokines elicit the inflammatory response and result in intestinal injury. In the present study, we firstly investigated the intestinal LPS levels and found that IUGR increased the concentrations of LPS in both 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- κ B p65* were increased in IUGR piglets. The protein expressions of TLR4 and MyD88 in the jejunum and TLR4 in the ileum of IUGR piglets were higher than those of NBW piglets, which were consistent with the results of the related mRNA expressions. IUGR weaned piglets also increased the protein expressions of pI κ B and nuclear NF- κ B and decreased cytoplasmic pNF- κ B in the intestine. Similar results were found in the liver of IUGR rats [52]. There are numerous studies about the mechanism of DHA in alleviating inflammation. However, the research on DHA suppressing intestinal inflammation via TLR4/NODs/NF- κ B pathway was limited. Recent study reported that DHA attenuated the inflammation induced by Lupus Nephritis through TLR4 signaling pathway [53]. Jiang et al. [11] demonstrated that DHA derivative DC32 inhibited inflammatory response in osteoarthritic synovium of rats via regulating Nrf2/NF- κ B pathway. Study also showed that DHA alleviated autoimmune thyroiditis of rats by inhibiting the CXCR3/PI3K/AKT/NF- κ B signaling pathway [54]. In the present study, treatment with DHA effectively decreased the related mRNA expressions of TLR4/NODs/NF- κ B pathway, reduced the protein expressions of TLR4, pI κ B and nuclear NF- κ B and improved cytoplasmic pNF- κ B in both jejunum and ileum of IUGR piglets. Therefore, these data indicated that dietary DHA supplementation could alleviate intestinal inflammatory response through TLR4/NODs/NF- κ B signaling pathway in IUGR weaned piglets.

It has been reported that TLR4/NODs signaling is also negatively modulated by multiple mechanisms [55]. Numerous regulators of TLR4 and NODs signaling have been identified and characterized. Researches have shown that Tollip, RP105, and SOCS1 are considered to be the representative negative regulators of TLR4 signaling [56–58] and the typical negative regulators of NOD signaling are ERBB2IP

and *CENTB1* [59, 60]. In the current study, IUGR decreased the mRNA expressions of *Tollip*, *ERBB2IP*, *CENTB1* in the jejunum and *SOCS1*, *ERBB2IP*, *CENTB1* in the ileum of weaned piglets. Diet supplemented with DHA significantly increased the mRNA expressions of jejunal *Tollip*, *ERBB2IP*, *CENTB1* and ileal *SOCS1*, *ERBB2IP*, *CENTB1* of IUGR piglets. Similar findings were observed in the intestine of weaned pigs after LPS challenge [14]. The results demonstrated that DHA treatment up-regulated the mRNA expressions of TLR4 and NODs negative regulators in the intestine of IUGR piglets, which were in accordance with the decreased mRNA expressions of intestinal TLR4 and NODs signaling-related genes. Therefore, the inhibitory effects of DHA on TLR4 and NODs signaling might be also attributed to the improvement of related gene expressions of their negative regulators.

Conclusions

The results obtained in the present study have shown that IUGR piglets exhibited a high risk of intestinal inflammatory response. Dietary supplementation of DHA to IUGR weaned piglets could improve intestinal morphology, regulate the proliferation and apoptosis of enterocytes, and attenuate intestinal inflammatory injury by decreasing the release of pro-inflammatory cytokines via the inhibition of TLR4/NODs/NF- κ B signaling pathway. Our findings may be helpful in finding a novel nutritional strategy for IUGR infants to maintain intestinal health in the early life of humans.

Abbreviations

AI, apoptosis index; CD, crypt depth; *CENTB1*, centaurin β 1; DHA, dihydroartemisinin; *ERBB2IP*, *ErbB2* interacting protein; IL-1 β , interleukin 1 β ; IL-6, interleukin 6; LPS, lipopolysaccharide; IRAK1, IL-1 receptor-associated kinase 1; IUGR, intrauterine growth retardation; MyD88, myeloid differentiation factor 88; NBW, normal birth weight; NLRs, oligomerization domain (NOD)-like receptors; NOD, nucleotide-binding oligomerization domain protein; PCNA, proliferating cell nuclear antigen; *plkB α* , phosphorylated NF- κ B inhibitor α ; *pNF- κ B*, phosphorylated nuclear factor κ B; PI, proliferation index; RIPK2, receptor-interacting serine/threonine-protein kinase 2; RP105, radioprotective 105; *SOCS1*, suppressor of cytokine signaling 1; *tlkB α* , total NF- κ B inhibitor α ; TLRs, transmembrane toll like receptors; TLR4, toll-like receptor 4; TNF- α , tumor necrosis factor α ; *tNF- κ B*, total nuclear factor κ B; *Tollip*, toll-interacting protein; TRAF6, TNF receptor-associated factor 6; VCR, villus height to crypt depth ratio; VD, villus width; VH, villus height; VSA, villus surface area.

Declarations

Acknowledgements

We would like to thank Wen Xu, Jintian He and Yongwei Zhao for their great contribution and help in pig raising.

Authors' contributions

YN, LLZ and TW designed the study. YN, YWZ, JTH, YY, MMS and ZDG performed the study. YN analysed the data and wrote the manuscript. YN and JTH revised the manuscript. TW had had primary responsibility for the final content. All authors read and approved the final manuscript.

Funding

This research was supported by the National Natural Science Foundation of China (no. 31601948) and the Fundamental Research Funds for the Central Universities (no. KJQN201935).

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

All experimental design and procedures were approved by Institutional Animal Care and Use Committee of Nanjing Agricultural University following the requirements of the Regulations for the Administration of Affairs Concerning Experimental Animals of China (NJAUCAST-2018-14).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Representative micrographs of TUNEL staining carried out on paraformaldehyde-fixed sections from the jejunum [(A-C), 200 × magnification] and ileum [(D-F), 200 × magnification] of weaned piglets. The apoptotic cells were stained yellow or brown-yellow. NBW-CON group, normal body weight weaned piglets fed with basal diet; IUGR-CON group, intrauterine growth retardation weaned piglets fed with basal diet; IUGR-DHA group, intrauterine growth retardation weaned piglets fed with 80 mg/kg dihydroartemisinin.

Figure 2

Representative micrographs of Ki-67 staining carried out on paraformaldehyde-fixed sections from the jejunum [(A-C), 200 × magnification] and ileum [(D-E), 200 × magnification] of weaned piglets. The proliferative cells were stained yellow or brown-yellow. NBW-CON group, normal body weight weaned piglets fed with basal diet; IUGR-CON group, intrauterine growth retardation weaned piglets fed with basal diet; IUGR-DHA group, intrauterine growth retardation weaned piglets fed with 80 mg/kg dihydroartemisinin.

Figure 3

Effect of dietary dihydroartemisinin supplementation on the protein expression of caspase-3 in the jejunum and ileum of weaned piglets with intrauterine growth retardation. The bands were the representative Western blot images of caspase-3 (32 kDa) and β -actin (45 kDa). NBW-CON group, normal body weight weaned piglets fed with basal diet; IUGR-CON group, intrauterine growth retardation weaned piglets fed with basal diet; IUGR-DHA group, intrauterine growth retardation weaned piglets fed with 80 mg/kg dihydroartemisinin. Data were presented as mean $\pm$ SEM (n = 8). *A significant difference ($P < 0.05$) was observed when compared with NBW-CON group; #A significant difference ($P < 0.05$) was observed when compared with IUGR-CON group.

Figure 4

Effect of dietary dihydroartemisinin supplementation on the protein expression of PCNA in the jejunum and ileum of weaned piglets with intrauterine growth retardation. The bands were the representative Western blot images of PCNA (29 kDa) and β -actin (45 kDa). NBW-CON group, normal body weight weaned piglets fed with basal diet; IUGR-CON group, intrauterine growth retardation weaned piglets fed with basal diet; IUGR-DHA group, intrauterine growth retardation weaned piglets fed with 80 mg/kg dihydroartemisinin. Data were presented as mean $\pm$ SEM (n = 8). *A significant difference ($P < 0.05$) was observed when compared with NBW-CON group; #A significant difference ($P < 0.05$) was observed when compared with IUGR-CON group. PCNA, proliferating cell nuclear antigen.

Figure 5

Effect of dietary dihydroartemisinin supplementation on intestinal concentrations of lipopolysaccharide in weaned piglets with intrauterine growth retardation. NBW-CON group, normal body weight weaned piglets fed with basal diet; IUGR-CON group, intrauterine growth retardation weaned piglets fed with basal diet; IUGR-DHA group, intrauterine growth retardation weaned piglets fed with 80 mg/kg dihydroartemisinin. Data were presented as mean $\pm$ SEM (n = 8). *A significant difference ($P < 0.05$) was observed when compared with NBW-CON group; #A significant difference ($P < 0.05$) was observed when compared with IUGR-CON group. LPS, lipopolysaccharide.

Figure 6

Effect of dietary dihydroartemisinin supplementation on the protein expressions of TLR4 in the membrane (A), MyD88 in the cytoplasm (B), pIkBa 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. The bands were the representative Western blot images of TLR4 (96 kDa), MyD88 (33 kDa), pIkBa (39 kDa), tIkBa (40 kDa), tNF-κB (65 kDa), pNF-κB (65 kDa), β-actin (45 kDa), Na, K-ATPase (100 kDa). NBW-CON group, normal body weight weaned piglets fed with basal diet; IUGR-CON group, intrauterine growth retardation weaned piglets fed with basal diet; IUGR-DHA group, intrauterine growth retardation weaned piglets fed with 80 mg/kg dihydroartemisinin. Data were presented as mean ± SEM (n = 8). *A significant difference (P < 0.05) was observed when compared with NBW-CON group; #A significant difference (P < 0.05) was observed when compared with IUGR-CON group. TLR4, toll-like receptors 4; MyD88, myeloid differentiation factor 88; tIkBa, total NF-κB inhibitor α; pIkBa, phosphorylated NF-κB inhibitor α; tNF-κB, total nuclear factor κB; pNF-κB, phosphorylated nuclear factor κB.