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

--Manuscript Draft--

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Abstract:	The aim of present study was to evaluate whether diet supplemented with dihydroartemisinin (DHA) could alleviate intestinal inflammatory injury in weaned piglets with intrauterine growth retardation (IUGR). Twelve normal birth weight (NBW) piglets and 24 IUGR piglets were fed the basal diet (NBW-CON and IUCR-CON groups) or basal diet supplemented with DHA at 80 mg/kg (IUGR-DHA group) from 21 to 49 days of age. At 49 days 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 TLR4/NODs/NF-κB signaling pathway. DHA inclusion ameliorated intestinal morphology, indicated by increased villus height, villus height to crypt depth ratio, villus surface area and decreased villus width of IUGR piglets ($P < 0.05$). DHA supplementation exhibited higher apoptosis index and caspase-3 expression, and lower proliferation index and proliferating cell nuclear antigen expression in the intestine of IUGR piglets than NBW piglets ($P < 0.05$). DHA supplementation attenuated intestinal inflammation of IUGR piglets, 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/NODs/NF-κB signaling pathway and up-regulated mRNA expressions of negative regulators of TLR4 and NODs signaling pathway in the intestine of IUGR piglets ($P < 0.05$). 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 of 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/NODs/NF-κB signaling pathway in IUGR weaned piglets.
Suggested Reviewers:	Qiujue Wu wuqiujue@163.com She is an expert on animal nutrition.

	Xiaoli Wan 641280919@qq.com She has published lots of manuscripts on animal nutrition.
Opposed Reviewers:	

Cover letter

Dear editor,

Thank you very much for your consideration of our manuscript and your suggestions are very important to improve the quality of our manuscript. We greatly agree with your suggestion for scientific paper writing. We have made changes in the revised paper with yellow highlighting, according to your comments.

Thank you again for your suggestions and advices. We hope that the changes have been made in the revision would meet your requests. If you have any more questions, please do not hesitate to contact us. We sincerely hope this paper could be published in Animal Nutrition.

Yours sincerely,

Yu Niu, Tian Wang

Respected reviewer:

Thank you very much for your consideration of our manuscript and your suggestions are very important to improve the quality of our manuscript. We greatly agree with your suggestion for scientific paper writing. We have made changes in the revised paper with yellow highlighting, according to your comments. The responses are as follows:

1. line 40, were collected?

Re: Thank you for your comments. We are so sorry that we forget to write the word “collected”. We have added “collected” in this sentence, line 40.

2. line 71, change "were" to "was".

Re: Thank you for your comments. We have changed “were” to “was” as you suggested, line 71.

3. line 97, is the past tense more appropriate? change "is" to "was".

Re: Thank you for your comments. We have changed “is” to “was” as you suggested, line 97.

4. line 98, change "can" to "could".

Re: Thank you for your comments. We have changed “can” to “could” as you suggested, line 98.

5. line 89, immunity

Re: Thank you for your comments. We have changed “immune” to “immunity” as you suggested, line 89.

6. line 99, change "provides" to "may provide".

Re: Thank you for your comments. We have changed “provides” to “may provide” as

you suggested, line 99.

7. line 102, the content of DHA?

Re: Thank you for your comments. The concentration of DHA was up to 99% as determined by high performance liquid chromatography (HPLC) analysis. We have added the content of DHA in the “Preparation of DHA” section, line 105-107.

8. line 115, 121, the end of the experiment is 50 days of age or 49 days of age? Please confirm!

Re: Thank you for your comments. We have confirmed that the end of the experiment is 49 days of age. We have changed “50 days of age” to “49 days of age”, line 119-120.

9. line 126, change "Jejunal" to "jejunal".

Re: Thank you for your comments. We have changed “Jejunal” to “jejunal” as you suggested, line 130.

10. line 138, what is "VW" in the equation?

Re: Thank you for your comments. The meaning of “VW” is villus width. We have added the explanation of “VW” in the “Materials and Methods”, line 139.

11. line 268-270, I would like to know whether there are other reports focused on the effect of DHA on IUGR, especially pig intestinal morphology?

Re: Thank you for your comments. In the previous study, our group investigated the effects of dietary DHA supplementation on growth, intestinal digestive function and nutrient transporters in IUGR weaned piglets. But there is no study about the effect of DHA on intestinal injury in IUGR piglets, especially pig intestinal morphology. So this is the first study to focus on the effect of DHA on the intestinal morphology of IUGR

weaned piglets.

12. line 275, agreed? Change it to "were similar".

Re: Thank you for your comments. We have changed “agreed” to “were similar” as you suggested, line 290.

13. line 285, Which segment of small intestine? The jejunum or ileum? Please describe the details.

Re: Thank you for your comments. The segment of small intestine is jejunum and ileum. We have changed “intestine” to “jejunum and ileum” as you suggested, line 301.

14. line 289, change "After DHA treatment" to "After dietary DHA supplementation".

Re: Thank you for your comments. We have changed “After DHA treatment” to “After dietary DHA supplementation” as you suggested, line 305.

15. line 303, this sentence describe the changes of "proinflammatory factors" in which group?

Re: Thank you for your comments. Diet supplemented with DHA decreased the concentrations of pro-inflammation cytokines in IUGR piglets. We have added “in IUGR piglets” as you suggested, line 319.

16. line 324, intracellular.

Re: Thank you for your comments. We have changed “intracellulars” to “intracellular” as you suggested, line 340.

17. line 342, add "in the present study".

Re: Thank you for your comments. We have added “in the present study” as you suggested, line 357.

18. line 353, change "treatment with DHA" to "diet supplemented with DHA".

Re: Thank you for your comments. We have changed “treatment with DHA” to “diet supplemented with DHA” as you suggested, line 368.

19. line 354, change "reduced" to "decreased".

Re: Thank you for your comments. We have changed “reduced” to “decreased” as you suggested, line 369.

20. line 355, change "intestinal" to "intestine".

Re: Thank you for your comments. We have changed “intestinal” to “intestine” as you suggested, line 371.

Respected reviewer:

Thank you very much for your consideration of our manuscript and your suggestions are very important to improve the quality of our manuscript. We greatly agree with your suggestion for scientific paper writing. We have made changes in the revised paper with yellow highlighting, according to your comments. The responses are as follows:

1. line 40. You mean "The jejunal and ileal samples were collected for further analysis?" Please added collected in this sentence.

Re: Thank you for your comments. We are so sorry that we forget to write the word “collected”. We have added “collected” in this sentence, line 40.

2. line 44 and 49. Change "higher" to "increased".

Re: Thank you for your comments. We have changed “higher” to “increased” as you suggested, line 44 and 49.

3. line 46. The expression about the comparative form is "higher…than". So please rewrite this sentence.

Re: Thank you for your comments. We have rewritten this sentence as you suggested, line 48.

4. line 101. This section is titled 'Preparation of DHA', yet all you say is that it was purchased from the DASF Biotechnology Co., Ltd. You should explain the purity of DHA and discuss how it was added to the diets of the pigs.

Re: Thank you for your comments. DHA ($C_{15}H_{24}O_5$, MW, 284.35), a derivative of artemisinin, is one of the largest groups of sesquiterpene lactones. DHA 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 proper proportion. The concentration of DHA was up to 99% as determined by high performance liquid chromatography (HPLC) analysis. We have revised the the section of “Preparation of DHA” as you suggested, line 102-107.

5. line 111. Please state the sex of the selected piglets.

Re: Thank you for your comments. The sex of the selected piglets were half male and female. We have added the sex of the selected piglets (n = 12, half male and half female) as you suggested, line 115-116.

6. line 112. Change "letter" to "litter".

Re: Thank you for your comments. We have changed “letter” to “litter” as you suggested, line 117.

7. line 115. The end of the experiment is "50 days of age" or "49 days of age"? You mentioned "49 days of age" in the Abstract. Please confirm and revise.

Re: Thank you for your comments. The end of the experiment is “49 days of age”. We have confirmed and revised it as you suggested, line 119-120.

8. line 138. What does "VW" mean? Please explain the abbreviation when it appears for the first time.

Re: Thank you for your comments. VW means villus width in the present study. We have explained the abbreviation as you suggested, line 139.

9. line 169 and 172. Please briefly describe the procedure of the ELISA method and provide the source of the kit.

Re: Thank you for your comments. We have added the procedure of the ELISA method

and provide the source of the kit as you suggested, line 174-176, and 179-181.

10. line 228 and 257. Delete "of".

Re: Thank you for your comments. We have deleted “of” as you suggested, line 243 and 272.

11. line 348-352. The contents you described in these two sentences are inconsistent with the related references in the Reference section. The studies about "DHA derivative DC32" is investigated by Li et al, not Jiang et al. Please confirm and revise.

Re: Thank you for your comments. We have confirmed and revised these two sentences and the related references in the “Reference” section as you suggested, line 364, 367 and 506-508.

12. line 255 and 256. Please write the full names of these genes because they appear for the first time in this manuscript.

Re: Thank you for your comments. We have write the full names of these genes in the “Materials and Methods” section and deleted the full names of the related genes in the “Discussion” section, line 192-199, 334, 335 and 345.

13. line 278. What are the reasons for the different results? Since it is mentioned that there are different results, I think it is necessary to speculate the reasons.

Re: Thank you for your comments. Li et al. (2018) noted that IUGR increased the proportion of villus apoptosis cells and crypt proliferative cells in the ileum of IUGR weanling piglets, which was dissimilar to our results. The reason may be attributed to a compensatory process in response to the excessive apoptosis in the villus. We have added the possible reasons as you suggested, line 295-296.

14. line 335. Which segment of the intestine do you mean in the sentence "in the intestine of weaned piglets". Please specify it.

Re: Thank you for your comments. We have changed “intestine” to “jejunum and ileum” as you suggested, line 350-351.

15. Figure 1 and 2. Please explain the meaning of NBW-CON, IUGR-CON and IUGR-DHA in the Figure captions.

Re: Thank you for your comments. We have added the explanations of the NBW-CON, IUGR-CON and IUGR-DHA in the figure captions of Figure 1, 2, 3, 4, 5 and 6, line 584-586, 591-593, 596-599, 604-607, 612-615.

Dietary dihydroartemisinin supplementation alleviates intestinal inflammatory injury through TLR4/NODs/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 diet supplemented with dihydroartemisinin (DHA) could alleviate intestinal inflammatory injury in weaned piglets with intrauterine growth retardation (IUGR). Twelve normal birth weight (NBW) piglets and 24 IUGR piglets were fed the basal diet (NBW-CON and IUCR-CON groups) or basal diet supplemented with DHA at 80 mg/kg (IUGR-DHA group) from 21 to 49 days of age. At 49 days 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 TLR4/NODs/NF-κB signaling pathway. DHA inclusion ameliorated intestinal morphology, indicated by increased villus height, villus height to crypt depth ratio, villus surface area and decreased villus width of IUGR piglets ($P < 0.05$). DHA supplementation exhibited higher apoptosis index and caspase-3 expression, and lower proliferation index and proliferating cell nuclear antigen expression in the intestine of IUGR piglets than NBW piglets ($P < 0.05$). DHA supplementation attenuated intestinal inflammation of IUGR piglets, 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/NODs/NF-κB signaling pathway and up-regulated mRNA expressions of negative regulators of TLR4 and NODs signaling pathway in the intestine of IUGR piglets ($P < 0.05$). 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 of 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/NODs/NF-κB signaling pathway in IUGR weaned piglets.

Keywords: intrauterine growth retardation, piglet, dihydroartemisinin, intestine injury, inflammation, morphology

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 during pregnancy (Wu et al. 2006). As multi-fetal animals, pigs exhibit an incidence of IUGR as high as 15%-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). DHA 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 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 (Sanderson and Allan, 2007). 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) (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 NLRs 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 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-κB signaling pathway. Therefore, the aim of this study was to estimate whether DHA could attenuate intestinal injury in IUGR weaned piglets 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 DHA

DHA ($C_{15}H_{24}O_5$, MW, 284.35), a derivative of artemisinin, is one of the largest groups of sesquiterpene lactones. DHA 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 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 animal protocols (NJAU-CAST-2018-146). At 114 days (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 NBW piglet (1.56 ± 0.02 kg) and two IUGR piglets (0.99 ± 0.03 kg) were marked by different tags. The criteria for the selection of IUGR and NBW piglets in this experiment was similar with previous studies (Wang et al., 2005). All the newborn piglets (n = 12, half male and half female) were suckled with their own sows until weaning at 21 days of age. In each litter, one NBW weaned piglet and one IUGR weaned piglet received the basal diet (NBW-CON and IUGR-CON groups), and the other IUGR weaned piglet received the basal diet supplemented with DHA at 80 mg/kg (IUGR-DHA group) until 49 days 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°C to 28°C and relative humidity ranging from 50% to 70%. All piglets have free access to feed and water.

2.3. Sample collection

At the 49 days of age, 8 piglets with similar body weight from each group (half male and half female) were killed with intravenous sodium pentobarbital (50 mg/kg BW). Blood sample was collected from jugular vein puncture in a nonheparinized tube

and centrifuged at 3000 × g for 15 min at 4°C and then stored at −80°C until analysis. The small intestine without 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.

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 field of vision was 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 TdT-mediated 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 and dUTP (vol:vol = 1:9) according to the TUNEL kit (Roche Corporation, Basel, Switzerland). Finally, the

slides were stained with DAPI dye and finally mounted with anti-fluorescein reagent. 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 total cell numbers multiplied by 100.

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; 1:1000) conjugated with horseradish peroxidase for 50 min at room temperature. Subsequently, the slices 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 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 lipopolysaccharide (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 by 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) by using a SYBR Premix Ex Taq Kit (TakaRa Biotechnology Co. Ltd; Dalian, Liaoning, China). The primer sequences for toll-like receptor 4 (*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), 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 (tIκBα, 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 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, 1:2000; 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 determine the statistical differences among treatment groups. A level of $P < 0.05$ indicated that the difference was statistically significant. Results were presented as means ± SEM.

3. Results

3.1. Intestinal morphology

IUGR decreased VH, VCR and VSA and increased CD in the intestine of piglets ($P < 0.05$) (Table 3). DHA administration effectively exhibited higher VH, VCR and VSA and lower CD in the intestine of IUGR piglets ($P < 0.05$).

3.2. Cell proliferation and apoptosis

IUGR piglets showed higher AI (Fig. 1) and lower PI (Fig. 2) in the jejunum and ileum than NBW piglets ($P < 0.05$) (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 (Fig. 3) and increased the protein

expression of PCNA (Fig. 4) in jejunum and ileum of piglets ($P < 0.05$). Diet supplemented with DHA effectively enhanced PI and level of PCNA, and reduced AI and level of caspase-3 in both jejunum and ileum of IUGR piglets ($P < 0.05$).

3.3. Concentrations of intestinal inflammatory cytokines

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

3.4. Concentrations of intestinal LPS

IUGR piglets exhibited increased concentration of intestinal LPS in comparison with that of NBW piglets ($P < 0.05$) (Fig. 5). Dietary supplementation with DHA significantly reduced the concentrations of LPS in the intestine of IUGR piglets ($P < 0.05$).

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

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

and *NF-κB p65* of IUGR piglets ($P < 0.05$).

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

As presented in Fig. 6, IUGR piglets increased the protein expressions of TLR4, pIκBα, nuclear pNF-κB and decreased cytoplasmic pNF-κB levels in both jejunum and ileum than those of NBW piglets ($P < 0.05$). Diet supplemented with DHA effectively improved the alternation of these protein expressions in IUGR piglets ($P < 0.05$). The protein expression of jejunal MyD88 was also increased in the IUGR-CON group compared to that of NBW-CON group ($P < 0.05$).

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

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

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 IUGR weaned piglets in comparison with NBW weaned piglets, suggesting a decreased ability of intestinal absorption as well as a damaged intestinal integrity in IUGR piglets.

These results were consistent with previous studies on IUGR piglets (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 IUGR piglets, indicating that DHA could improve the intestinal morphology.

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, 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 (Wang et al., 2012). The results were also similar with 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 previous 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). PCNA 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. 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 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 (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 IUGR piglets. In accordance with previous study, Huang et al. (2019) demonstrated that IUGR piglets increased the concentrations of TNF-α and IL-6 at birth, which indicated that IUGR newborns was prone to inflammatory injury. Diet supplemented with DHA decreased the concentrations of pro-inflammation cytokines in IUGR piglets. Previous study showed that DHA decreased the concentrations of IL-6 and IL-1β induced by TNF-α in endothelial cells (Yin et al., 2018). Research also demonstrated that DHA 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 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 injury, we determined the function of TLRs and NLRs (Al-Sayeqh et al., 2010), which also play important roles in the dysregulated apoptosis (Subramanian et al., 2020). TLR4 is a best characterized member of TLRs, 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 (Gao 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 ielum) 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 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 in the present study. IUGR weaned piglets 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/NODs/NF-κB pathway was limited. 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 DC32 inhibited inflammatory response in osteoarthritic synovium of rats via regulating Nrf2/NF-κB pathway. Study also showed that DHA alleviated autoimmune thyroiditis of rats by inhibiting the CXCR3/PI3K/AKT/NF-κB signaling pathway (Liu et al., 2017). The present study showed that diet supplemented with DHA effectively reduced the related mRNA expressions of TLR4/NODs/NF-κB pathway, decreased the protein

371 expressions of TLR4, pIκBα and nuclear NF-κB and improved cytoplasmic pNF-κB in
372 the intestine of IUGR piglets. Therefore, these data indicated that dietary DHA
373 supplementation could alleviate intestinal inflammatory response through
374 TLR4/NODs/NF-κB signaling pathway in IUGR weaned piglets.

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

391 **5. Conclusions**

392 The present results 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 reducing the release of pro-inflammatory cytokines via the inhibition of TLR4/NODs/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 and 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 and editing, Supervision, Funding acquisition.

Conflict of interest

The authors declare that there is no conflict of interest.

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Figures:

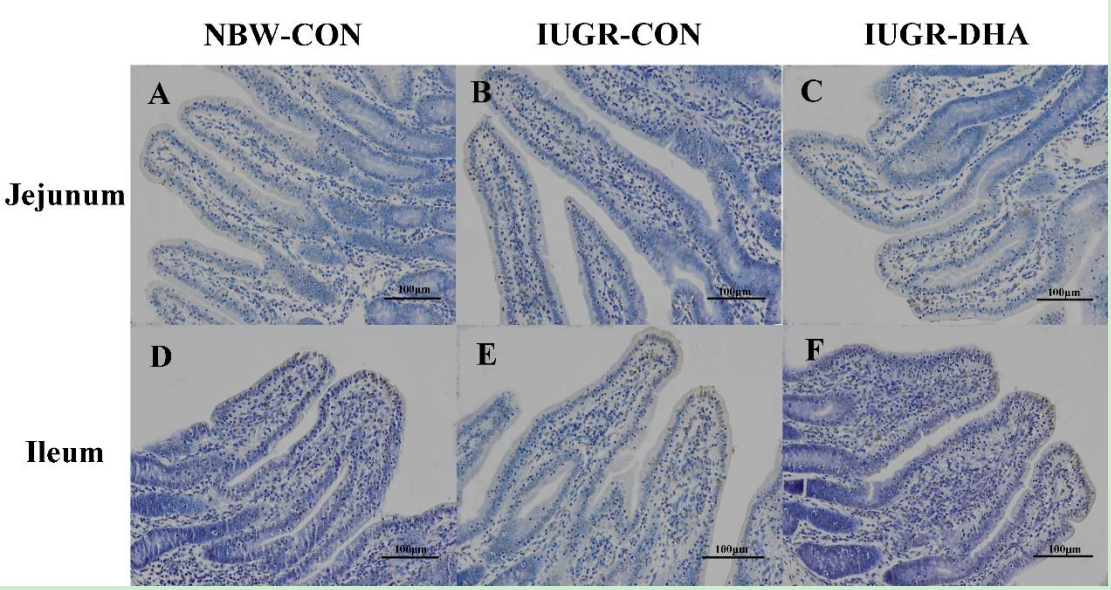
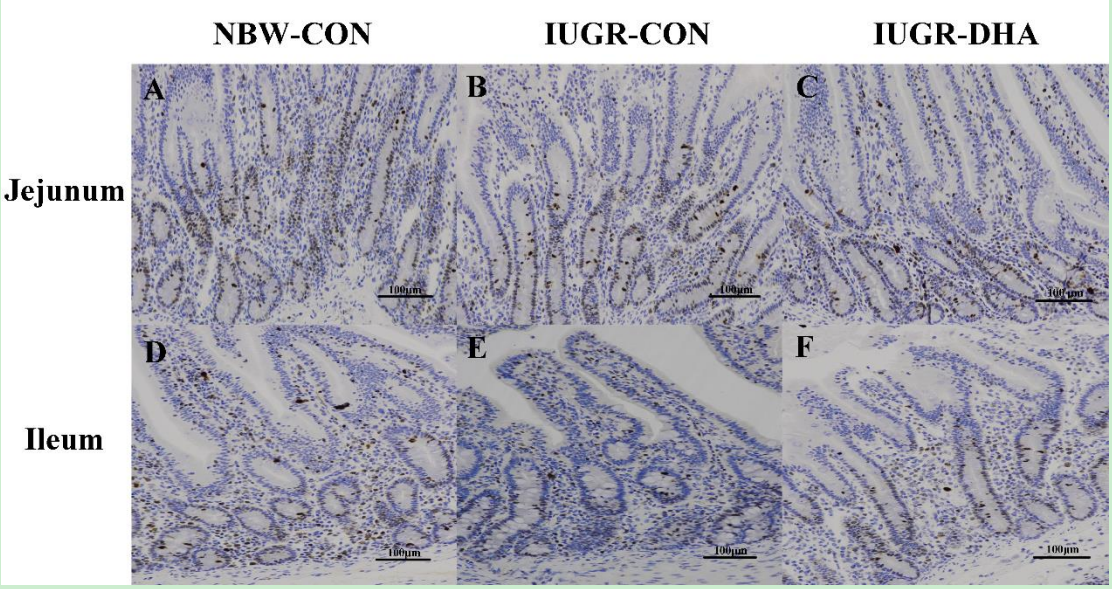
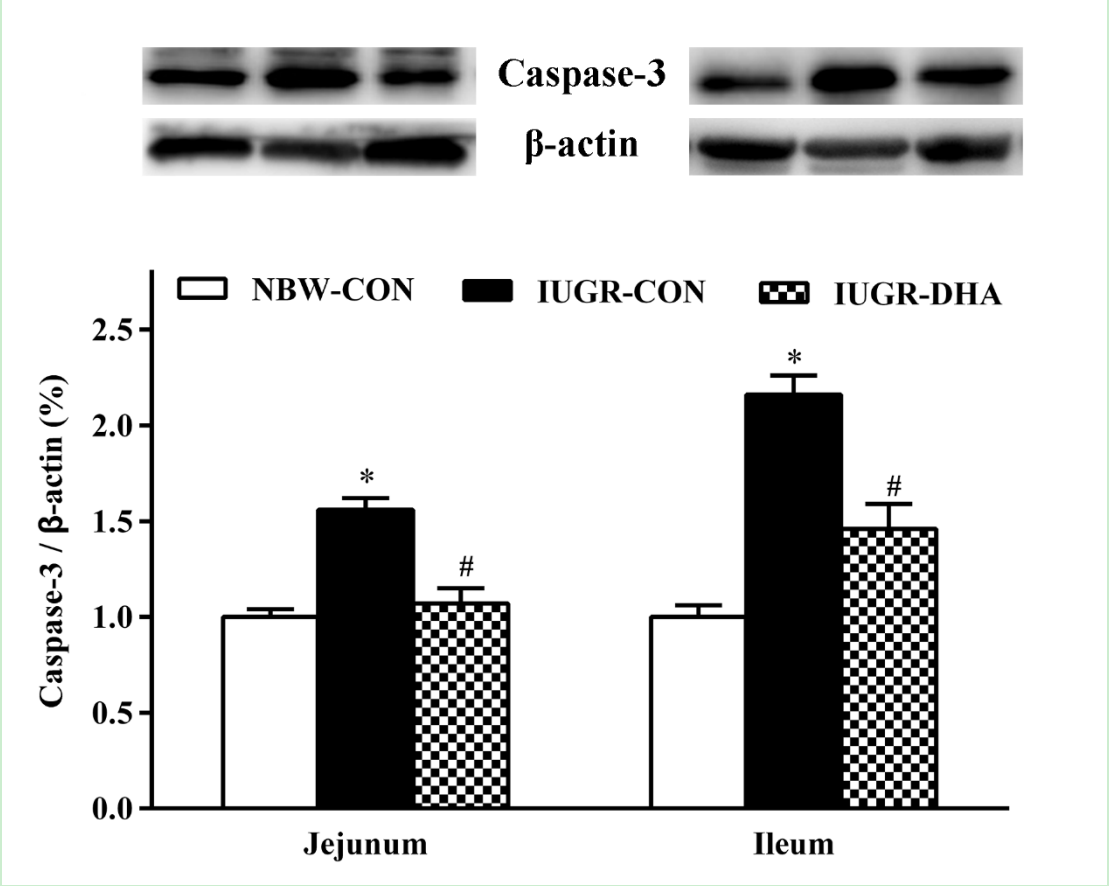


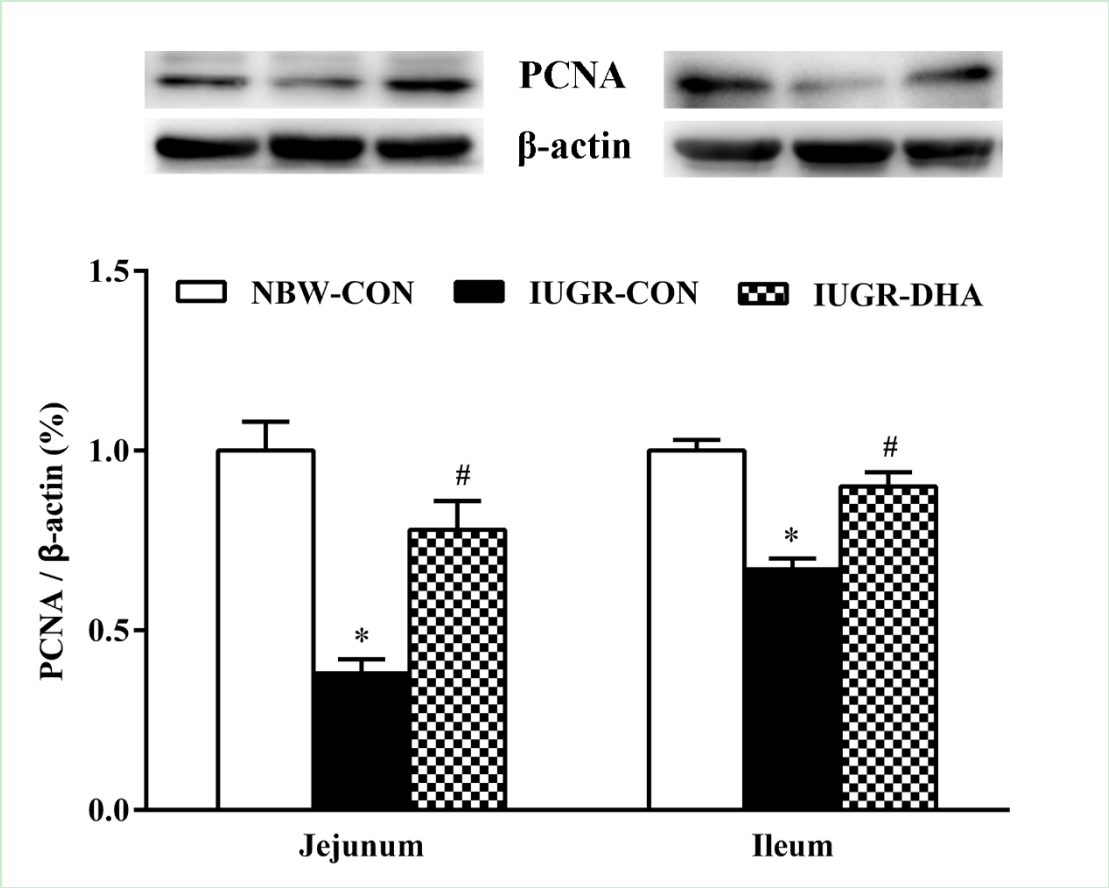
Fig. 1 Effects of dihydroartemisinin on TUNEL-positive cells in the jejunum [(A-C), TUNEL immunohistochemical staining, × 200, scale = 100 μm] and ileum [(D-F), TUNEL immunohistochemical staining, × 200, scale = 100 μm] of intrauterine growth retardation weaned piglets. The apoptotic cells were stained yellow or brown-yellow. NBW-CON, normal body weight group given a basal diet; IUGR-CON, intrauterine growth retardation group given a basal diet; IUGR-DHA, intrauterine growth retardation group given a dihydroartemisinin supplemented diet at a level of 80 mg/kg.



588 **Fig. 2** Effects of dihydroartemisinin on Ki-67 positive cells in the jejunum [(A-C), Ki-
589 67 immunohistochemical staining, × 200, scale = 100 μm] and ileum [(D-F), Ki-67
590 immunohistochemical staining, × 200, scale = 100 μm] of intrauterine growth
591 retardation weaned piglets. The proliferative cells were stained yellow or brown-yellow.
592 NBW-CON, normal body weight group given a basal diet; IUGR-CON, intrauterine
593 growth retardation group given a basal diet; IUGR-DHA, intrauterine growth
594 retardation group given a dihydroartemisinin supplemented diet at a level of 80 mg/kg.



595 **Fig. 3** Effect of dihydroartemisinin on the protein expression of caspase-3 in the
596 intestine of intrauterine growth retardation weaned piglets. Results were showed as
597 mean ± SEM (n = 8). NBW-CON, normal body weight group given a basal diet; IUGR-
598 CON, intrauterine growth retardation group given a basal diet; IUGR-DHA,
599 intrauterine growth retardation group given a dihydroartemisinin supplemented diet at
600 a level of 80 mg/kg. * A significant difference ($P < 0.05$) between NBW-CON group and
601 IUGR-CON group; # A significant difference ($P < 0.05$) between IUGR-DHA group
602 and IUGR-CON group.



603 **Fig. 4** Effect of dihydroartemisinin on the protein expression of PCNA in the intestine
604 of intrauterine growth retardation weaned piglets. Results were showed as mean ± SEM
605 (n = 8). NBW-CON, normal body weight group given a basal diet; IUGR-CON,
606 intrauterine growth retardation group given a basal diet; IUGR-DHA, intrauterine
607 growth retardation group given a dihydroartemisinin supplemented diet at a level of 80
608 mg/kg. * A significant difference ($P < 0.05$) between NBW-CON group and IUGR-
609 CON group; # A significant difference ($P < 0.05$) between IUGR-DHA group and
610 IUGR-CON group.

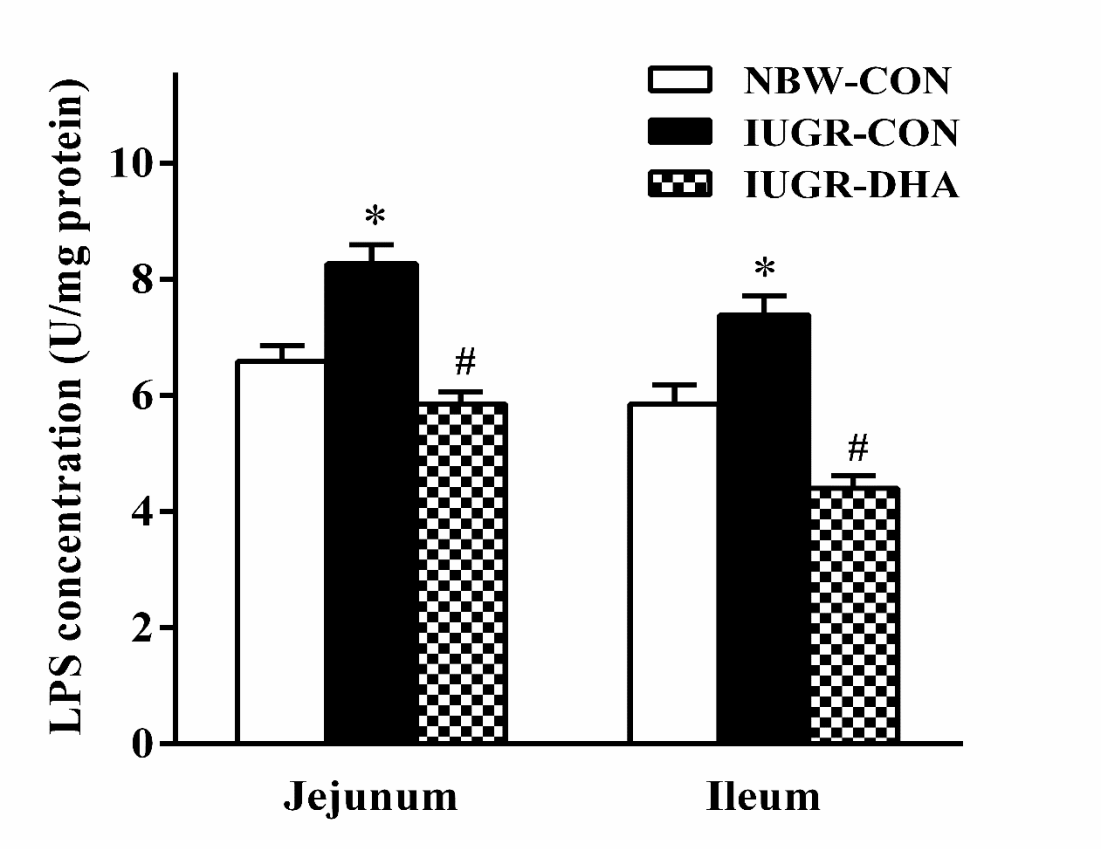
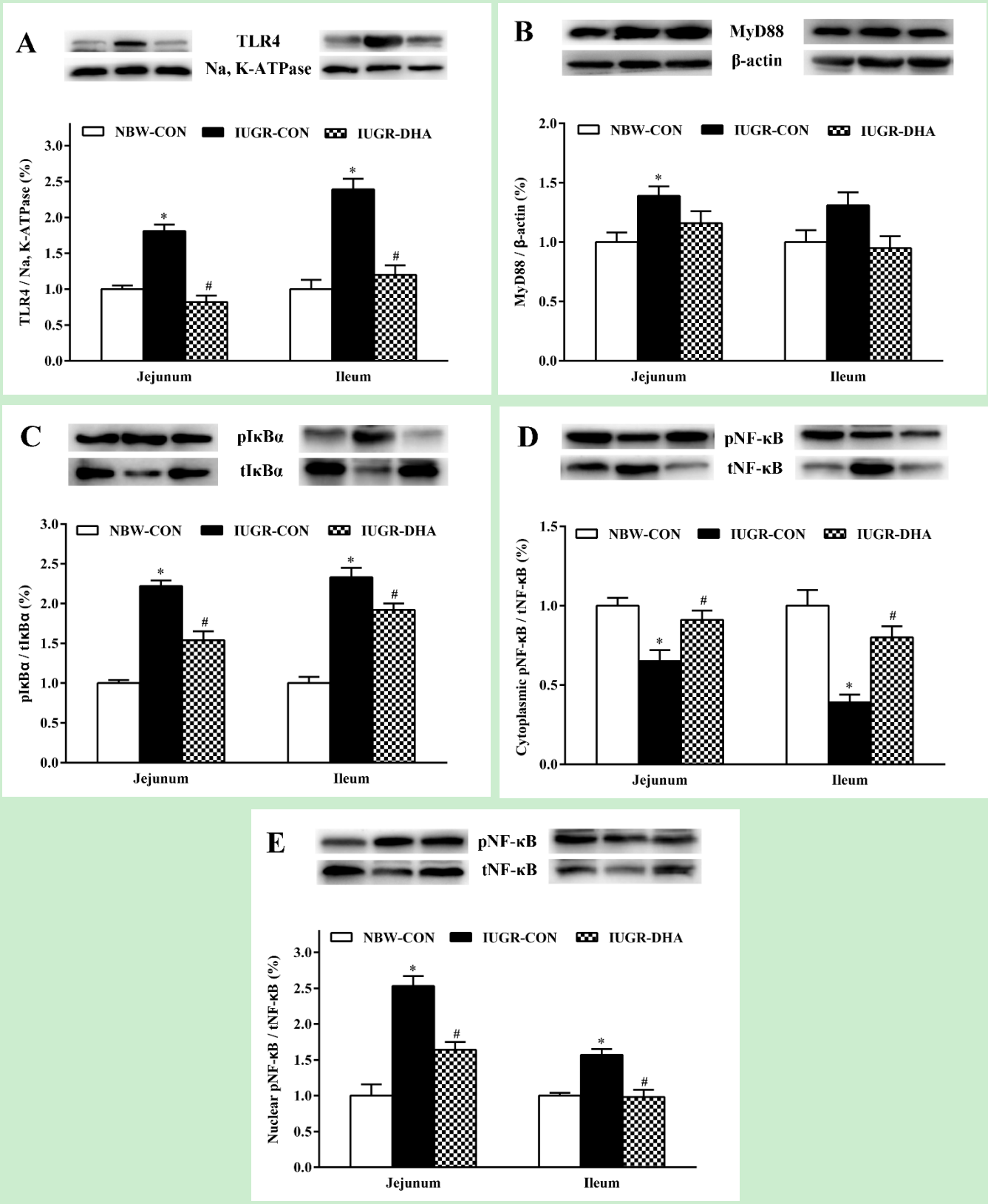


Fig. 5 Effect of dihydroartemisinin on the intestinal lipopolysaccharide concentration in weaned piglets with intrauterine growth retardation. Results were showed as mean ± SEM (n = 8). NBW-CON, normal body weight group given a basal diet; IUGR-CON, intrauterine growth retardation group given a basal diet; IUGR-DHA, intrauterine growth retardation group given a dihydroartemisinin supplemented diet at a level of 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. LPS, lipopolysaccharide.



619 **Fig. 6** Effect of dihydroartemisinin on the protein expressions of TLR4 in the membrane
620 (A), MyD88 in the cytoplasm (B), pIκBα in the cytoplasm (C), pNF-κB in the
621 cytoplasm (D) and pNF-κB in the nucleus (E) of intestine in intrauterine growth
622 retardation weaned piglets. Results were showed as mean ± SEM (n = 8). NBW-CON,
623 normal body weight group given a basal diet; IUGR-CON, intrauterine growth

retardation group given a basal diet; IUGR-DHA, intrauterine growth retardation group given a dihydroartemisinin supplemented diet at a level of 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.

631 **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		

632 [1]The premix provided the following per kg complete diet: vitamin A, 12000 IU; vitamin
633 D_3, 3000 IU; α-tocopherol, 50 mg; vitamin K_3, 4 mg; vitamin B_1, 4 mg; vitamin B_2, 10
634 mg; vitamin B_6, 7 mg; vitamin B_{12}, 0.05 mg; niacin, 30 mg; pantothenic acid, 15 mg;
635 folic acid, 0.3 mg; biotin, 0.08 mg; choline chloride, 500 mg; Fe ($FeSO_4 \cdot H_2O$), 110 mg;
636 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
637 mg; Se (Na_2SeO_3), 0.3 mg.

638 **Table 2** Primer sequences of target genes

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

639 *TLR4*, toll-like receptor 4; *MyD88*, myeloid differentiation factor 88; *IRAK1*, IL-1 receptor-
640 associated kinase 1; *TRAF6*, TNF receptor-associated factor 6. *NOD*, nucleotide-binding
641 oligomerization domain protein; *RIPK2*, receptor-interacting serine/threonine-protein kinase
642 2; *NF-κB* p65, nuclear factor-κB p65; *RP105*, radioprotective 105; *SOCS1*, suppressor of
643 cytokine signaling 1; *Tollip*, toll-interacting protein; *ERBB2IP*, Erbb2 interacting protein;
644 *CENTB1*, centaurin β1.

Table 3 Effect of dihydroartemisinin on intestinal morphology in intrauterine growth retardation weaned piglets.

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 (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

[1]NBW-CON (NC), normal body weight group given a basal diet; IUGR-CON (IC), intrauterine growth retardation group given a basal diet; IUGR-DHA (ID), intrauterine growth retardation group given a dihydroartemisinin supplemented diet at a level of 80 mg/kg. Results were showed as mean ± SEM (n = 8).

[2] * 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. VH, villus height; VW, villus width; CD, crypt depth; VCR, villus height to crypt depth ratio; VSA, villus surface area.

Table 4 Effect of dihydroartemisinin on enterocyte proliferation and apoptosis in intrauterine growth retardation weaned piglets.

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), normal body weight group given a basal diet; IUGR-CON (IC), intrauterine growth retardation group given a basal diet; IUGR-DHA (ID), intrauterine growth retardation group given a dihydroartemisinin supplemented diet at a level of 80 mg/kg. Results were showed as mean ± SEM (n = 8).

[2] * 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. AI, apoptosis index; PI, proliferation index.

664 **Table 5** Effect of dihydroartemisinin on the concentrations of intestinal inflammatory
665 cytokines in intrauterine growth retardation weaned piglets.

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

666 [1]NBW-CON (NC), normal body weight group given a basal diet; IUGR-CON (IC),
667 intrauterine growth retardation group given a basal diet; IUGR-DHA (ID), intrauterine
668 growth retardation group given a dihydroartemisinin supplemented diet at a level of 80
669 mg/kg. Results were showed as mean ± SEM (n = 8).
670 [2] * A significant difference ($P < 0.05$) between NBW-CON group and IUGR-CON group;
671 # A significant difference ($P < 0.05$) between IUGR-DHA group and IUGR-CON group.
672 IL-1β, interleukin 1β; IL-6, interleukin 6; TNF-α, tumor necrosis factor α.

673 **Table 6** Effect of dihydroartemisinin on intestinal mRNA expression of
674 TLR4/NODs/NF-κB signaling pathway in intrauterine growth retardation weaned
675 piglets.

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

676 [1]NBW-CON (NC), normal body weight group given a basal diet; IUGR-CON (IC),
677 intrauterine growth retardation group given a basal diet; IUGR-DHA (ID), intrauterine
678 growth retardation group given a dihydroartemisinin supplemented diet at a level of 80
679 mg/kg. Results were showed as mean ± SEM (n = 8).
680 [2] * A significant difference ($P < 0.05$) between NBW-CON group and IUGR-CON group;
681 # A significant difference ($P < 0.05$) between IUGR-DHA group and IUGR-CON group.
682 *TLR4*, toll-like receptor 4; *MyD88*, myeloid differentiation factor 88; *IRAK1*, IL-1
683 receptor-associated kinase 1; *TRAF6*, TNF receptor-associated factor 6. *NOD*,
684 nucleotide-binding oligomerization domain protein; *RIPK2*, receptor-interacting
685 serine/threonine-protein kinase 2; *NF-κB p65*, nuclear factor-κB p65.

686 **Table 7** Effect of dihydroartemisinin on intestinal mRNA expressions of negative
687 regulators of TLR4/NODs signaling pathway in intrauterine growth retardation weaned
688 piglets.

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

689 [1]NBW-CON (NC), normal body weight group given a basal diet; IUGR-CON (IC),
690 intrauterine growth retardation group given a basal diet; IUGR-DHA (ID), intrauterine
691 growth retardation group given a dihydroartemisinin supplemented diet at a level of 80
692 mg/kg. Results were showed as mean ± SEM (n = 8).
693 [2*] A significant difference ($P < 0.05$) between NBW-CON group and IUGR-CON group;
694 [#] A significant difference ($P < 0.05$) between IUGR-DHA group and IUGR-CON group.
695 *RP105*, radioprotective 105; *SOCS1*, suppressor of cytokine signaling 1; *Tollip*, toll-
696 interacting protein; *ERBB2IP*, Erbb2 interacting protein; *CENTB1*, centaurin β1.

Conflict of interest

The authors declare that there is no conflict of interest.

Author Statement:

Yu Niu: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing-Original Draft, Writing-review and 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 and editing, Supervision, Funding acquisition.