Dietary leonurine hydrochloride supplementation attenuates lipopolysaccharide challenge-induced intestinal inflammation and barrier dysfunction by inhibiting the NF- κ B/MAPK signaling pathway in broilers

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ABSTRACT: This study was performed to evaluate the beneficial effects of dietary leonurine hydrochloride (LH) supplementation on intestinal morphology and barrier integrity and further illuminate its underlying antioxidant and immunomodulatory mechanisms in lipopolysaccharide (LPS)-treated broilers. A total of 120 1-d-old male broilers (Ross 308) were assigned to 4 treatment groups with 6 replicates of 5 birds per cage. The experiment was designed in a 2 $\times$ 2 factorial arrangement with LH (0 or 120 mg/kg) and LPS (injection of saline or 1.5 mg/kg body weight) as treatments. On days 14, 16, 18, and 20 of the trial, broilers were intraperitoneally injected with LPS or physiological saline. Compared with the control group, LPS-challenged broilers showed impaired growth performance ($P < 0.05$) from day 15 to day 21 of the trial, increased serum diamine oxidase (DAO) and D-lactic acid (D-LA) levels coupled with reduced glutathione (GSH) content and total superoxide dismutase (T-SOD) activity (duodenal and jejunal mucosa), reduced malondialdehyde (MDA) content (duodenal, jejunal, and ileal mucosa), and compromised morphological structure of the duodenum and jejunum. Additionally, LPS challenge increased ($P < 0.05$) the mRNA expression of proinflammatory cytokine genes and reduced tight junction

(TJ) protein expression in the jejunum. However, dietary LH prevented LPS-induced reductions in average daily gain (ADG) and average daily feed intake (ADFI) in broilers. It also alleviated LPS challenge-induced increases in serum DAO levels, MDA content (duodenal and jejunal mucosa), and jejunal crypt depth ($P < 0.05$) but reduced villus height, GSH content (jejunal mucosa), and T-SOD activity (duodenal and jejunal mucosa) ($P < 0.05$). Additionally, LH supplementation significantly downregulated the mRNA expression of nuclear factor (NF)- κ B, cyclooxygenase-2 (COX-2), and proinflammatory cytokines (TNF- α , IL-1 β , and IL-6) and upregulated the mRNA expression of zonula occludens-1 (ZO-1) and Occludin in the jejunal mucosa induced by LPS ($P < 0.05$). On the other hand, LH administration prevented LPS-induced activation of the p38, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) mitogen-activated protein kinases (MAPKs) and attenuated I κ B alpha (I κ B α) phosphorylation and nuclear translocation of NF- κ B (p65) in the jejunal mucosa. In conclusion, dietary LH supplementation attenuates intestinal mucosal disruption mainly by accelerating the expression of TJ proteins and inhibiting activation of the NF- κ B/MAPK signaling pathway.

Keywords: barrier dysfunction, broiler, intestinal inflammation, leonurine hydrochloride, lipopolysaccharide

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INTRODUCTION

Intestinal mucosal barrier function not only prevents the invasion of exogenous pathogenic bacteria, toxins, and other harmful substances but also plays a critical role in maintaining intestinal homeostasis and physical health. Many types of bacteria and toxins are parasitic in the intestines of humans and animals. Under normal physiological conditions, endotoxins produced by intestinal bacterial metabolism can be absorbed into the portal vein through the intestinal wall and cleared by the phagocytosis of Kupffer cells adhering to the hepatic sinus. However, when the integrity of the intestinal barrier is destroyed, leading to increased intestinal permeability and causing water and plasma proteins to leak into the intestinal lumen, intestinal bacteria and/or microbial by-products enter the bloodstream, which contributes to the development of sepsis, shock, multiple organ dysfunction syndrome, multiple organ failure, and other diseases (Deitch et al., 2006; Gatt et al., 2007). Therefore, maintaining the integrity of the mucosal barrier is critical for intestinal homeostasis (Hecht, 1999; Otte et al., 2009).

Bacterial lipopolysaccharide (LPS), a membrane glycolipid, is an active component of the outer coats of gram-negative bacteria. Previous studies have shown that intraperitoneal or intravenous injection of LPS can cause a variety of changes in the intestinal mucosal barrier, such as villous atrophy, alteration of intestinal flora, impaired intestinal barrier integrity, intestinal inflammation, pathogen infection, and so on (Zhu et al., 2015; Chen et al., 2018). It has been established that inflammation plays an important role in the pathogenesis of LPS-induced barrier dysfunction (Wu et al., 2013). A large number of studies have demonstrated that LPS can induce an intestinal inflammatory response by activating the nuclear factor (NF)- κ B and mitogen-activated protein kinase (MAPK) signaling pathways and promoting the expression of proinflammatory cytokine genes, which are involved in the body's immune and antioxidant systems (Binion et al., 2008; Song et al., 2014). Thus, suppressing these inflammatory pathways is a reasonable strategy to alleviate the effects of LPS, including intestinal inflammation and barrier dysfunction. Dietary supplementation of plant-derived compounds with anti-inflammatory and antioxidant effects can provide a viable and practical way to alleviate the harmful consequences of intestinal dysfunction caused by endotoxins.

Leonurus sibiricus (also known as motherwort), a member of the Labiatae family, is a type of traditional herbal medicine that is found in China, Japan, North Korea, and other Asian countries (Kong et al., 1976). In ancient times, motherwort was widely used for many years to treat various disorders, such as cardiovascular, digestive, menstrual, and other gynecological disorders, due to its relatively low toxicity (Bensky et al., 2004; Liu et al., 2010). Leonurine hydrochloride (LH) [3,5-dimethoxy-4-hydroxy-benzoic acid (4-guanidino)-1-butyl ester hydrochloride monohydrate; Fig. 1], a specific unique compound found only in *L. sibiricus*, has been shown to possess various biological activities, including vasodilatory, antiapoptotic, antioxidant, and anti-inflammatory effects (Song et al., 2014; Xu et al., 2014). Several studies have suggested that the beneficial effects of LH in various disease challenge models are due to its inhibition of the NF- κ B and MAPK signaling pathways, reducing the expression of proinflammatory cytokine genes and increasing the activity of antioxidant enzymes (Li et al., 2011; Xu et al., 2014). In addition, previous studies from our laboratory have shown that supplementation with 120 mg/kg LH can alleviate the negative effects of LPS challenge by increasing the activity of antioxidant enzymes and reducing the levels of proinflammatory cytokines in broilers. This result prompted us to further investigate the possible therapeutic potential of LH in an intestinal inflammation model with broiler chickens. However, an understanding of the effects of LH treatment on intestinal inflammation and barrier function in chickens is lacking. Hence, based on previous studies (Yang et al., 2018), this study is a further exploration of the possible protective effects of LH supplementation on LPS-induced intestinal inflammation in broiler chickens and a deeper exploration of potential molecular mechanisms. Our results will provide insights useful for the future application of LH against intestinal damage in broilers.

MATERIALS AND METHODS

Reagents

Leonurine hydrochloride was purchased from AnHui New Star Pharmaceutical Development

Figure 1. Chemical structure of leonurine hydrochloride.

Co., Ltd (HeFei, P. R. China). The purity of the LH was over 98%. Lipopolysaccharide from *Escherichia coli* (*E. coli*) serotype O55:B5 (L2880) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The specific primary antibodies used in this study included rabbit anti-Occludin, rabbit anti-c-Jun N-terminal kinase (JNK), and rabbit anti-phospho I κ B α (I κ B α) from Abcam (Rockford, IL, USA); rabbit anti-ZO-1, rabbit anti-phospho-JNK, and rabbit anti-NF- κ B p65 from Thermo Scientific (Rockford, IL, USA); rabbit anti- β -actin, rabbit anti-phospho-p38, rabbit anti-p38, rabbit anti-extracellular signal-regulated kinase (ERK), rabbit anti-phospho-ERK, rabbit anti-NF- κ B phospho-p65, and rabbit anti-I κ B α from Cell Signaling Technology (Beverly, MA, USA); and HRP-conjugated goat anti-rabbit IgG.

Animals and Management

All experimental procedures involved in this study were approved by the Animal Care and Use Committee of Shihezi University. A total of 120 1-d-old male Ross 308 broilers with similar hatching weights obtained from a local commercial hatchery were used in this study. Broilers were

randomly allocated to 4 treatments containing 6 replicates (pens) of 5 birds per replicate in each group. The 4 treatment groups were as follows: 1) control group (broilers given a basal diet and administered sterile saline); 2) LPS-challenged group (broilers given a basal diet and receiving intraperitoneal administration of LPS); 3) LH group (broilers given a basal diet supplemented with 120 mg/kg LH and administered sterile saline); and 4) LH + LPS group (broilers given a basal diet supplemented with 120 mg/kg LH and receiving intraperitoneal administration of LPS). All birds were housed in a temperature- and light-controlled room with continuous light and ad libitum access to mash feed and water. Meanwhile, the brooding temperature was maintained at 32 to 35 °C for 7 d and then gradually decreased by 1 °C every 2 d until a final temperature of 22 °C was reached at 21 d. In addition, all birds were vaccinated according to a routine immunization program. All birds were inoculated with an inactivated infectious bursal disease vaccine on days 14 and 21 and with a Newcastle disease vaccine on day 7. Antimicrobial and anticoccidial drugs were not added to the basal diet, which was formulated per the National Research Council to meet the nutritional requirements of broilers (Table 1). The experiment consisted of a 2 $\times$ 2 factorial experimental design with LH treatments (0 or 120 mg/kg diet) and challenge status injection of saline (9 g/liter wt/vol) or LPS (1.5 mg/kg body weight) as the main factors tested. Lipopolysaccharide from *E. coli* was dissolved in sterile saline at a concentration of 500 mg/mL. At days 16, 18, and 20 of age, the broilers received an intraperitoneal injection of LPS solution or an equal amount of sterile saline. The dosage and administration of LPS adopted in this study referred to available findings (Kamboh et al., 2016; Zheng et al., 2016; Zhang et al., 2017a).

Sample Collection

On days 0, 14, and 21 of the experiment, the birds were weighed after a 12 h feed withdrawal. The average daily feed intake (ADFI), average daily gain (ADG), and feed conversion ratio (FCR, $\text{FCR} = \text{ADFI}/\text{ADG}$) were calculated by using body weight and feed intake, which were recorded by replicate (cages) during different periods.

One bird per replicate was randomly selected for sample collection at 21 d. Blood samples were individually collected, with 3 to 5 mL of blood collected from the wing vein in serum tubes. After sitting for up to 30 min at room temperature, the

Table 1. Diet composition and calculated analysis of the standard diet

Item	Starter (day 1 to day 14)	Grower (day 15 to day 28)
Ingredients, %		
Corn, yellow	52.7	59.2
Soybean meal	40.2	33.9
Soybean oil	3.0	3.0
Dicalcium phosphate	1.87	1.76
Limestone	1.16	1.24
Common salt	0.4	0.3
Vitamin premix ¹	0.25	0.25
Mineral premix ²	0.25	0.25
DL-Methionine	0.17	0.10
Nutrient composition		
ME, kcal/kg	3,000	3,030
CP, %	21.57	18.94
Methionine, %	0.49	0.36
Lysine, %	1.18	1.01
Calcium, %	0.94	0.85
Nonphytate P, %	0.43	0.34

¹Supplied per kilogram of diet: vitamin A (retinyl acetate), 15,000 IU; vitamin D3, 5,000 IU; vitamin E (DL- α -tocopheryl acetate), 80 mg; vitamin K, 5 mg; thiamin, 3 mg; riboflavin, 10 mg; pyridoxine, 5 mg; vitamin B12, 0.02 mg; niacin, 70 mg; folic acid, 2 mg; biotin, 0.4 mg; pantothenic acid, 20 mg.

²Supplied per kilogram of diet: manganese, 80 mg; zinc, 75 mg; iron, 70 mg; copper, 10 mg; iodine, 0.35 mg; selenium, 0.2 mg.

samples were processed by centrifugation (15 min at $1,788 \times g$) and stored at -20°C until further analysis.

After decapitation, the broiler abdominal cavity was opened, and the whole gastrointestinal tract was rapidly removed. The small intestine was immediately separated from the mesentery and connective tissue and placed on a chilled stainless steel tray. Segments of approximately 2 cm of mid-duodenum, mid-jejunum, and mid-ileum were rinsed with ice-cold phosphate-buffered saline (PBS) to remove digesta. Next, the 2-cm segments were fixed in 4% paraformaldehyde solution for histological examination. The remaining small intestine segments were opened longitudinally and washed with ice-cold PBS to remove digesta. The jejunal and ileal mucosa was then scratched carefully using a sterile glass microscope slide and collected into sterile frozen tubes, flash-frozen in liquid N_2 and preserved at -80°C until further analysis.

Intestinal Morphology

The frozen small intestinal samples fixed in 4% paraformaldehyde were dehydrated in increasing concentrations of alcohol, embedded in paraffin, sectioned into 5- μm -thick sections, and stained with hematoxylin and eosin. The crypt depth and villus height of 10 well-oriented villi per segment were measured using an image processing and analysis system (Nikon Eclipse 80i, Nikon Co., Tokyo, Japan). Crypt depth was measured as the vertical distance from the villus-crypt junction to the lower limit of the crypt. Villus length was defined as the vertical distance from the villus tip to the villus-crypt junction. The mean villus length and mean crypt depth were calculated to obtain the villus height-to-crypt depth ratio (VCR).

Assessment of the Antioxidant System

Approximately 0.3 g each of duodenal, jejunal, and ileal mucosa were homogenized with 9 mL of 0.9% sodium chloride solution (wt/vol, 1:9) on ice and then centrifuged at $3,000 \times g$ at 4°C for 15 min to obtain the supernatants. The supernatants were analyzed for total protein content and enzyme activities and stored at -80°C until further analysis. The protein content was measured by the Coomassie blue protein-binding method using crystalline bovine serum albumin as a standard. Total superoxide dismutase (T-SOD) activity and reduced glutathione (GSH) and malondialdehyde (MDA) content in the supernatants of the

duodenal, jejunal, and ileal mucosa homogenates were determined using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, P. R. China) via an automated spectrophotometric analyser (Cobas FARA II, Roche, Palo Alto, CA, USA). All procedures were performed per the manufacturer's instructions. The biochemical data were normalized against homogenate protein content.

Diamine Oxidase and D-Lactate in Serum

The concentration of serum D-lactic acid (D-LA) was determined using a D-LA colorimetric assay kit (BioVision Inc., Milpitas, CA, USA) according to the manufacturer's protocols. The diamine oxidase (DAO) activity in the serum was determined using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, P. R. China).

Quantitative Real-Time PCR Analysis

The mRNA expression of NF- κB , zonula occludens-1 (ZO-1), Occludin, tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , IL-6, and cyclooxygenase-2 (COX-2) in the broilers' jejunal mucosa was quantified by quantitative real-time PCR. β -Actin was used as the reference gene to normalize the gene expression data. The primer information for all genes is listed in Table 2. All gene primers were designed using chicken sequences from GenBank. The specific method is the same as that used in a previous study (Yang et al., 2018).

Western Blotting Analysis

Approximately 50 mg of frozen jejunal mucosa (5 randomly selected samples per group) was homogenized in 1 mL of RIPA Lysis Buffer (Thermo Scientific, Rockford, IL, USA) containing protease inhibitors (Roche Applied Science, Penzberg, Germany) and centrifuged ($12,000 \times g$ for 15 min at 4°C) to collect the supernatants. Total protein concentration was quantified using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL, USA). Equal protein amounts of boiled samples (30 μg per lane) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Veenendaal, The Netherlands). The membranes were blocked in 5% skim milk solution for 2 h at room temperature (20°C) with gentle

Table 2. Sequences for real-time PCR primers

Gene ¹	Accession number	Forward primers (5'-3')	Reverse primers (5'-3')	Product size (bp)
<i>β-Actin</i>	NM_205518.1	ACCGCAAATGCTTCTAAACC	ATAAAGCCATGCCAATCTCG	100 bp
<i>NF-κB</i>	NM_205134.1	GCACAACGCCTCTTCACATA	GGCTCAAAGTTCTCAACGTG	100 bp
<i>ZO-1</i>	XM_413773.4	AAGTGTTTCGGGTTGTGGAC	GCTGTCTTTGGAAGCGTGTA	160 bp
<i>Occludin</i>	NM_205128.1	TCATCGCCTCCATCGTCTAC	TCTTACTGCGCGTCTTCTGG	240 bp
<i>TNF-α</i>	NM_204267	TGTGTATGTGCAGCAACCCGTAGT	GGCATTGCAATTTGGACAGAAGT	229 bp
<i>IL-1β</i>	HQ329098.1	TCTTCTACCGCCTGGACACG	TAGGTGGCGATGTTGACCTG	145 bp
<i>IL-6</i>	HM_179640.1	AAATCCCTCCTCGCCAATCT	CCCTCACGGTCTTCTCCATAAA	106 bp
<i>COX-2</i>	M64990.1	TCCACCAACAGTGAAGGACA	GGACCAAGCCAAACACCTC	101 bp

¹NF-κB, nuclear factor kappaB; ZO-1, zonula occludens-1; IL-1β, interleukin 1 beta; IL-6, interleukin 6; TNF-α, tumor necrosis factor-alpha; COX-2, cyclooxygenase-2.

shaking and then incubated overnight at 4 °C with appropriate primary antibodies against β-actin, Occludin, ZO-1, p-p65, p65, p-IkBα, IkBα, p-p38, p38, p-ERK, ERK, p-JNK, and JNK. Following extensive washing in PBST, the membranes were incubated with the appropriate HRP-conjugated secondary antibody for 2 h at room temperature. Finally, the membranes were washed in PBST, and chemiluminescence signals were visualized using a chemiluminescence method (ECL Western Blotting Detection Kit, Thermo Fisher Scientific Inc., Boston, MA, USA) according to the manufacturer's instructions. The intensity of the bands was digitally analyzed using ImageJ 1.47.

Statistical Analyses

All data were analyzed by 2-way ANOVA (a 2 × 2 factorial arrangement) using the general linear model procedure, and the model included the main effects of dietary LH treatment and LPS challenge as well as their interaction. If a significant treatment effect of their interaction was observed, the significance between the treatment differences was identified separately by Duncan's multiple range test. The level of significance was set at probability values less than 0.05 for all analyses.

RESULTS

Growth Performance

Data on growth performance are shown in Table 3. During LPS stimulation (days 15 to 21), the LPS-challenged group showed significantly decreased ADG and ADFI values of broilers ($P < 0.05$) compared to those of the saline-challenged groups. Dietary supplementation with LH significantly increased ADG and ADFI values compared with those of the unsupplemented group ($P < 0.05$). The interaction between dietary LH and LPS challenge

was significant for ADFI and ADG ($P < 0.05$), but no significant interaction was observed for FCR ($P > 0.05$).

Intestinal Morphology

The intestinal permeability and morphology data of the broilers are shown in Table 4. Compared with saline-treated chickens, the LPS-challenged groups showed significantly decreased villus length and VCR in the duodenum and jejunum ($P > 0.05$) but significantly increased crypt depth in the jejunum ($P > 0.05$). Interestingly, dietary supplementation of LH increased villus height ($P > 0.05$) but significantly reduced crypt depth in the jejunum compared with those in the unsupplemented group ($P > 0.05$). No significant impact on the morphometry of the ileum ($P > 0.05$) was observed among broilers fed LH or challenged with LPS. A significant interaction between LH and LPS challenge was observed for villus length and crypt depth, but no significant interaction was observed for VCR in the jejunum ($P > 0.05$).

Oxidative Capacity

The small intestinal mucosal antioxidant capacity and lipid peroxidation data from the broilers are shown in Table 5. Dietary supplementation of LH significantly increased ($P < 0.05$) GSH content (jejunal mucosa) and T-SOD activity (duodenal and jejunal mucosa) but decreased MDA content (duodenal, jejunal, and ileal mucosa) compared with those in the unsupplemented group ($P < 0.05$). Compared with the control group, LPS challenge significantly increased ($P < 0.05$) the MDA content (duodenal, jejunal, and ileal mucosa) but decreased ($P < 0.05$) the GSH content and T-SOD activity (duodenal and jejunal mucosa) of broilers. Dietary LH treatment and LPS challenge did not significantly impact GSH and T-SOD in the ileum ($P > 0.05$). Moreover, significant

Table 3. Effects of leonurine hydrochloride on growth performance of broilers challenged with lipopolysaccharide

Item ¹	Basal diet		LH diet ²		SEM ³	P-value		
	Saline	LPS	Saline	LPS		LH	LPS	Interaction
1 to 14 d								
ADG, g/bird	25.29	24.43	24.57	24.44	0.16	0.175	-	-
ADFI, g/bird	42.35	42.21	41.73	41.55	0.226	0.562	-	-
FCR, g/g	1.57	1.61	1.59	1.6	0.011	0.625	-	-
15 to 21 d								
ADG, g/bird	43.98 ^a	39.61 ^b	44.73 ^a	42.57 ^a	0.549	0.038	0.001	0.025
ADFI, g/bird	71.44 ^a	64.29 ^b	72.14 ^a	70.40 ^a	0.861	0.016	0.002	0.049
FCR, g/g	1.63	1.63	1.61	1.66	0.018	0.87	0.557	0.624

^{a,b}Means with no common superscript within each row are significantly ($P < 0.05$) different.

¹ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion rate.

²LH, basal diet supplemented with leonurine hydrochloride; LPS, lipopolysaccharide.

³SEM, standard error of means.

Table 4. Effects of leonurine hydrochloride on small intestinal morphology of broilers challenged with lipopolysaccharide at 21 d of age

Item ¹	Basal diet		LH diet ²		SEM ³	P-value		
	LPS(−)	LPS(+)	LPS(−)	LPS(+)		LH	LPS	Interaction
Duodenal								
Villus length, μm	861	756	851	784	2.73	0.275	<0.001	0.54
Crypt depth, μm	129	136	126	132	0.92	0.437	0.171	0.958
VCR	6.11	5.56	5.99	5.94	0.43	0.515	<0.001	0.261
Jejunum								
Villus length, μm	635 ^a	556 ^b	645 ^a	600 ^a	2.14	0.001	<0.001	0.015
Crypt depth, μm	120 ^b	128 ^a	122 ^b	123 ^b	0.86	0.034	<0.001	0.003
VCR	5.29	4.33	5.27	4.86	0.281	0.114	<0.001	0.201
Ileum								
Villus length, μm	460	454	463	455	2.31	0.318	0.105	0.575
Crypt depth, μm	92	94	93	93	0.44	0.916	0.598	0.598
VCR	4.99	4.83	4.97	4.90	0.23	0.601	0.214	0.547

^{a,b}Means with no common superscript within a column are significantly ($P < 0.05$) different.

¹VCR, villus height-to-crypt depth ratio.

²LH, basal diet supplemented with leonurine hydrochloride; LPS, lipopolysaccharide.

³SEM, standard error of means.

interactions between LH and LPS were observed for the MDA content (duodenal and jejunal mucosa), GSH content (jejunal mucosa), and T-SOD activity (duodenal and jejunal mucosa) of broilers ($P < 0.05$).

Diamine Oxidase and D-Lactate in Serum

On day 21, the LPS-challenged groups had higher ($P < 0.05$) DAO and D-LA levels in serum than the unchallenged groups (Table 6). Dietary supplementation with LH significantly reduced ($P < 0.05$) serum concentrations of DAO and D-LA compared with those in the unsupplemented group. The interaction between dietary LH treatment and LPS challenge significantly impacted DAO in the serum ($P < 0.05$).

Gene Expression of Tight Junction Proteins

On day 21, when the broilers were challenged with LPS, dietary supplementation with LH significantly increased the mRNA expression of ZO-1 and Occludin in the jejunal mucosa compared with that in the unsupplemented group ($P < 0.05$; Fig. 2). When fed the basal diet, the broilers in the LPS-challenged group exhibited reduced expression levels of ZO-1 and Occludin in the jejunal mucosa compared with those in the unchallenged group ($P < 0.05$).

Inflammation-Related Gene Expression

As shown in Fig. 2, LPS treatment increased the mRNA expression of TNF- α , IL-1 β , IL-6, and COX-2

Table 5. Effects of leonurine hydrochloride on small intestinal mucosaoxidative capacity of broilers challenged with lipopolysaccharide at 21 d of age

Item ¹	Basal diet		LH diet ²		SEM ³	<i>P</i> -value		
	LPS(−)	LPS(+)	LPS(−)	LPS(+)		LH	LPS	Interaction
Duodenal mucosa								
MDA, nmol/mg protein	8.62 ^b	10.73 ^a	7.21 ^c	9.19 ^b	0.712	0.049	0.011	0.031
GSH, μmol/g protein	193.31	174.65	207.77	191.09	10.331	0.052	0.041	0.354
T-SOD, U/mg protein	337.21 ^b	270.31 ^c	371.20 ^a	321.21 ^b	13.221	0.012	0.037	0.045
Jejunal mucosa								
MDA, nmol/mg protein	6.42 ^b	9.21 ^a	5.04 ^c	6.98 ^b	0.419	0.001	<0.001	0.016
GSH, μmol/g protein	225.51 ^b	183.68 ^c	262.82 ^a	219.91 ^b	11.309	0.028	<0.001	0.037
T-SOD, U/mg protein	298.99 ^b	266.68 ^c	331.33 ^a	301.25 ^b	12.032	0.008	0.041	0.048
Ileal mucosa								
MDA, nmol/mg protein	7.89	9.41	7.01	8.31	0.419	0.044	0.037	0.387
GSH, μmol/g protein	161.77	157.91	172.36	159.62	5.981	0.436	0.275	0.565
T-SOD, U/mg protein	317.38	306.74	329.21	321.35	14.821	0.213	0.653	0.871

^{a-c}Means with no common superscript within a column are significantly ($P < 0.05$) different.

¹MDA, malondialdehyde; GSH, glutathione; T-SOD, total superoxide dismutase.

²LH, basal diet supplemented with leonurine hydrochloride; LPS, lipopolysaccharide.

³SEM, standard error of means.

Table 6. Effects of dietary leonurine hydrochloride on serum diamine oxidase activity and D-lactic acid concentration of broilers challenged with lipopolysaccharide

Item ¹	Basal diet		LH diet ²		SEM ³	P-value		
	LPS(-)	LPS(+)	LPS(-)	LPS(+)		LH	LPS	Interaction
DAO, U/L	1.12 ^c	1.68 ^a	1.14 ^c	1.32 ^b	0.006	0.031	0.028	0.046
D-Lactic acid, mmol/L	6.88	9.40	6.73	7.83	0.243	0.037	0.046	0.243

^{a,b}Means with no common superscript within a column are significantly ($P < 0.05$) different.

¹DAO, diamine oxidase.

²LH, basal diet supplemented with leonurine hydrochloride; LPS, lipopolysaccharide.

³SEM, standard error of means.

in the jejunal mucosa compared with that in the group of chickens that received a saline injection. When the broilers were challenged with LPS, dietary supplementation with LH significantly downregulated the mRNA expression of NF- κ B, TNF- α , IL-1 β , IL-6, and COX-2 in the jejunal mucosa compared with that in the unsupplemented group ($P < 0.05$; Fig. 2).

Western Blotting Analysis

Protein expression of tight junction proteins. As shown in Fig. 3, when the broilers were challenged with LPS, dietary supplementation with LH resulted in significantly higher protein expression levels of ZO-1 and Occludin in the jejunal mucosa compared with those in the unsupplemented group ($P < 0.05$; Fig. 3). When fed the basal diet, the broilers in the LPS-challenged group exhibited reduced protein expression levels of ZO-1 and Occludin

in the jejunal mucosa compared with those in the unchallenged group ($P < 0.05$).

Protein expression of the NF- κ B signaling pathway. As shown in Fig. 4, when the broilers were challenged with LPS, dietary supplementation with LH resulted in significantly reduced protein expression levels of phosphorylated p65 and I κ B α in the jejunal mucosa compared with those in the unsupplemented group ($P < 0.05$; Fig. 4). When fed the basal diet, the broilers in the LPS-challenged group exhibited increased protein expression levels of phosphorylated p65 in the jejunal mucosa compared with those in the unchallenged group ($P < 0.05$).

Protein expression of MAPK signaling pathways. As shown in Fig. 5, when the broilers were challenged with LPS, dietary supplementation with LH

resulted in significantly reduced protein expression levels of phosphorylated p38, ERK, and JNK in the jejunal mucosa compared with those in the unsupplemented group ($P < 0.05$; Fig. 5). When fed the basal diet, the broilers in the LPS-challenged group exhibited increased protein expression levels of phosphorylated p38, ERK, and JNK in the jejunal mucosa compared with those in the unchallenged group ($P < 0.05$).

DISCUSSION

Increasing evidence from epidemiological and animal experiments suggests that environmental factors, including stress and diet, can disrupt

Figure 2. Expression of NF-κB, ZO-1, occludin, TNF-α, IL-1β, IL-6, and COX-2 in the jejunum mucosa of broilers fed diets with or without 120 mg/kg leonurine hydrochloride after intraperitoneal administration of lipopolysaccharide or saline on day 21. ^{a-c}Mean values with different letters differed significantly ($P < 0.05$).

the epithelial barrier and contribute to the pathogenesis of gastrointestinal inflammatory diseases (Lambert, 2009; Wu et al., 2013). In broilers, a great deal of research has shown that intraperitoneal or intravenous LPS can suppress growth performance, which may be attributable to intestinal dysfunction caused by bacterial challenge (Gao et al., 2013; Wang et al., 2016). In our study, we also confirmed the negative effects of LPS challenge on the growth performance of broilers, evidenced by reduced ADG and ADFI. Moreover, our data illustrated that dietary supplementation with LH significantly alleviated the LPS-induced declines in ADG and ADFI in broilers, which suggested that LH might be able to ameliorate the adverse effects of LPS challenge on growth performance in chickens.

Intestinal integrity is essential for the prevention of pathogenic microorganism invasion in farm animals, especially broiler chickens, which are susceptible to disease due to genetic selection for rapid growth. Intestinal inflammation is a serious and common disease in broiler chickens that can result in decreased production or even cause sudden death (Mathlouthi et al., 2002; Lucke et al., 2018). Villus height and crypt depth can be considered measurements of the capacity of a bird to absorb nutrients from its feed. High villus and crypt ratios are usually associated with excellent gut health and high capacity for digestion and absorption (García et al., 2007). In addition, a number of studies have demonstrated that LPS challenge can cause damage

Figure 3. Expression of ZO-1 and Occludin proteins in the jejunal mucosa of broilers fed diets with or without 120 mg/kg leonurine hydrochloride after intraperitoneal administration of lipopolysaccharide or saline on day 21. (A) Representative western blots and bar diagrams showing densitometric analyses of (B) the ZO-1/β-actin ratio and (C) the Occludin/β-actin ratio. ^{a,b}Mean values with different letters differed significantly ($P < 0.05$).

Figure 4. Expression of NF- κ B signaling proteins in the jejunal mucosa of broilers fed diets with or without 120 mg/kg leonurine hydrochloride after intraperitoneal administration of lipopolysaccharide or saline on day 21. (A) Representative western blots and bar diagrams showing densitometric analyses of (B) NF- κ B (p-p65)/NF- κ B (p65) ratio and (C) p-I κ B α /I κ B α ratio. ^{a,b}Mean values with different letters differed significantly ($P < 0.05$).

Figure 5. Expression of MAPKs signaling proteins in the jejunal mucosa of broilers fed diets with or without 120 mg/kg leonurine hydrochloride after intraperitoneal administration of lipopolysaccharide or saline on day 21. (A) Representative western blots and bar diagrams showing densitometric analyses of (B) p-p38/p38 ratio, (C) p-JNK(1/2)/JNK(1/2) ratio, and (D) p-ERK(1/2)/ERK(1/2) ratio. ^{a,b}Mean values with different letters differed significantly ($P < 0.05$).

to the structure and function of the intestinal barrier (Dickinson et al., 1999; Jiang et al., 2015), and we have reconfirmed its harmful consequences on the intestinal barrier structure of broilers in this study, as evidenced by decreased villus height and VCR and increased crypt depth of the duodenum and jejunum. Previous studies have shown that dietary supplementation of plant-derived compounds with immunomodulatory and antioxidant effects can mitigate the adverse effects of LPS challenge on intestinal morphology (Rosillo et al., 2011). Our results show similar findings: dietary supplementation with LH significantly increased villus height and VCR in the duodenum and jejunum, demonstrating that LH can prevent many adverse effects on the morphology of the small intestine caused by intraperitoneal injection of LPS. However, the reason that LH causes these effects is still unclear, and further research is needed.

Oxidative stress is considered one of the major pathogenic factors leading to inflammatory diseases and is associated with gastrointestinal health in broilers (Wu et al., 2015). In addition, the activity of antioxidant enzymes in the intestinal mucosa reflects the antioxidant state of the intestine due to direct contact between the mucosal surface and intestinal contents (Scocco et al., 2017). The front line of defense for antioxidant defense systems includes total SOD and GSH. Superoxide dismutase is responsible for catalyzing the dismutation of O_2^- into O_2 followed by conversion into H_2O_2 (Aluwong et al., 2013). Glutathione, a biomarker of inflammation and oxidative stress, is the most abundant endogenous antioxidant synthesized by rate-limiting enzymes (Tian et al., 2017). Malondialdehyde is the major lipid peroxidation product used to evaluate the degree of lipid oxidation damage (Jiang et al., 2015). Numerous studies have demonstrated that LPS disrupts the redox balance by reducing the activity of antioxidant enzymes while increasing lipid peroxidation products in the small intestinal mucosa (Wu et al., 2015; Scocco et al., 2017). Our results also confirmed that LPS challenge significantly reduced T-SOD activity and GSH content while increasing the level of MDA in the duodenum and jejunum. Nutrition plays an essential role in protecting the integrity and function of the intestinal mucosal barrier by maintaining redox balance (Aluwong et al., 2013). Studies have shown that antioxidants in the diet can help the small intestine resist free radical attacks (Zhu et al., 2012; Zhang et al., 2013). Several researchers have found that LH is a natural product that has a strong ability to scavenge free radicals and inhibit lipid peroxidation of

various oxidative systems in cells or tissues (Zhang et al., 2012; Qi et al., 2017). This finding also supports our observation that LH can reduce oxidative damage to the jejunal and ileal mucosa after LPS challenge, which is beneficial to the rapid recovery of intestinal function. At the same time, our results revealed that LH has a more protective effect on the ileum and jejunum than on the duodenum, which may be related to absorptive, enzymatic, or other metabolic differences. We believe that LH achieves its antioxidant potential by blocking the lipid peroxidation process and enhancing the antioxidant defense system to interfere with the production of free radicals. The production of excess oxygen free radicals is the main trigger for the activation of inflammatory pathways.

Serum DAO and D-LA activity were employed as markers for monitoring the extent of intestinal permeability and mucosal damage (Wu et al., 2013). Diamine oxidase is a highly active intracellular enzyme produced by intestinal epithelia, and it exists only in the intestinal mucosa and ciliated cells (Chen et al., 2017), whereas D-LA is a product of intestinal bacterial fermentation (Nieto et al., 2000). Both molecules are released directly into the blood circulation when the intestinal mucosa is destroyed (Quiros and Nusrat, 2014). In this study, after LPS challenge, DAO and D-LA levels in the sera of broilers were significantly increased at 21 d, which indirectly indicated that LPS challenge might seriously impair the structure and barrier function of the intestinal mucous. Our results showed that LPS challenge can damage the intestinal morphology of broilers at 21 d and significantly increase DAO and D-LA levels in the serum, which was consistent with previous studies and demonstrated the successful construction of the intestinal inflammation model through the injection of LPS into broilers (Gadde et al., 2017; Chen et al., 2018). Our data showed that the addition of 120 mg/kg LH to the diet improved the permeability of the intestinal mucosa in our LPS-induced broiler enteritis model.

Tight junctions (TJs) are pivotal components of the intestinal mucosal barrier and play a significant role in the regulation of intestinal permeability by maintaining intestinal barrier integrity and ensuring normal barrier function (Quiros and Nusrat, 2014). The cytoplasmic plaque protein ZO-1 and the transmembrane protein Occludin are important components of TJs (Yang et al., 2017). In the present study, LPS challenge destroyed jejunal TJs, characterized by disordered mRNA expression and protein levels of ZO-1 and Occludin in the jejunal mucosa. The results were consistent with

those of previous studies (Wang et al., 2014; Zhu et al., 2015). Decreased expression of TJ proteins is related to the destruction of the jejunum mucosal barrier and increased intestinal permeability caused by LPS challenge, and this destruction allows many noxious compounds to enter the systemic blood circulation by transmission through the intestinal barrier. The increase in serum DAO and D-LA levels also supports this view. On the other hand, LH markedly increased the expression of TJ-related genes and proteins in LPS broilers and thus contributed to the restoration of intestinal function in broilers after LPS challenge. A previous study demonstrated that LH can significantly increase the protein expression levels of ZO-1 and Occludin in the brain to protect against blood-brain barrier breakdown in a transient middle cerebral artery occlusion rat model (Zhang et al., 2017b).

In addition, we demonstrated that dietary LH supplementation dramatically improved ileal villus structure, decreased intestinal permeability, and maintained the intestinal mucosa oxidation balance in LPS-challenged birds. These results further support the idea that LH can alleviate the intestinal barrier dysfunction induced by LPS. However, the possible causes of these changes are unclear because there is limited data on the effects of LH on intestinal barrier function in animals. Therefore, further molecular studies are required to elucidate the relationship between LH and intestinal barrier function in broilers under LPS challenge.

Emerging evidence indicates that the release of proinflammatory cytokines such as TNF- α , IL-1 β , and IL-6 affects not only the expression of TJ proteins (Alsadi et al., 2008) but also the activation and perpetuation of the inflammatory response in the intestinal mucosa (Zhou et al., 2014). Several studies have indicated that NF- κ B plays a pivotal role in the development of intestinal inflammation by synthesizing and releasing proinflammatory cytokines (TNF- α , IL-1 β , and IL-6) and inducible proinflammatory enzymes (COX-2) (Ren et al., 2015; Medicherla et al., 2016). Under resting conditions, the NF- κ B and I κ B proteins form a complex trimer that exists in the cytoplasm in an inactive form. Upon stimulation, I κ B kinase (IKK) mediates the phosphorylation and ubiquitination of the I κ B protein, leading to its subsequent degradation, and nuclear translocation of NF- κ B, triggering the transcription of the corresponding target gene (Zhang et al., 2017c). To further explore the underlying molecular mechanisms of LH in the inflammatory response, we showed that LPS challenge significantly upregulated the levels of proinflammatory

factors (TNF- α , IL-1 β , IL-6) and a proinflammatory enzyme (COX-2) mRNA in the jejunum, but dietary LH supplementation promoted the recovery of these cytokines and enzyme to normal levels. In addition, our results further showed that LH inhibited NF- κ B (p65) translocation and prevented the phosphorylation and degradation of I κ B α in the jejunal mucosa of LPS-induced intestinal inflammation. At the same time, our results are partly supported by Yin and Lei (2018), who found that LH visibly inhibited NF- κ B activity in a murine osteoarthritis model by suppressing I κ B α phosphorylation and degradation. Jia et al. (2017) reported that LH significantly reduced the mRNA expression of proinflammatory cytokines (IL-1 β , IL-6, and TNF- α) and inhibited the phosphorylation and degradation of NF- κ B alpha (I κ B α), thus preventing the nuclear translocation of the NF- κ B p65 subunit and thereby reducing neuroinflammation in mice. Furthermore, Liu et al. (2012) found that leonurine can regulate the activation of NF- κ B in LPS-induced inflammatory responses of human umbilical vein endothelial cells.

Mitogen-activated protein kinase also plays an important role in autoimmune and inflammation-related diseases such as inflammatory bowel disease (Kyriakis and Avruch, 2012). It is well established that MAPKs require activation via phosphorylation of any of the 3 extracellular signal-regulated protein kinases (p38, ERK1/2, JNK MAPKs), and subsequently, signals are transduced from the cell surface to the nucleus. Recent studies have demonstrated that MAPK can regulate the levels of proinflammatory cytokines (TNF- α and IL-6) and upregulate the expression of proinflammatory enzymes (COX-2 and INOS) in a murine model of Crohn's disease (Rosillo et al., 2011). The MAPK signaling pathway is considered a key upstream factor for the activation of the NF- κ B signaling complex (Kaminska, 2005; Li et al., 2017). In this study, we found that LH significantly inhibited the LPS-induced phosphorylation of NF- κ B and MAPK signaling molecules, including p65, I κ B α , p38, ERK, and JNK, in the jejunal mucosa of broilers. Our results were similar to those of recent investigations showing that LH can alleviate inflammatory stress in LPS-induced mouse mastitis by regulating the MAPK/NF- κ B signaling pathways (Song et al., 2014). Consistent with our data, a recent study showed that LH can regulate the activation of the NF- κ B and MAPK signaling pathways in synovial inflammation and joint destruction in mice (Li et al., 2017). Taken together, these results present convincing evidence to support

our view that LH prevents LPS-induced intestinal barrier injury by inhibiting NF- κ B nuclear translocation and MAPK phosphorylation.

In summary, we report that dietary supplementation with LH at 120 mg/kg could alleviate the harmful consequences of LPS-induced increased intestinal permeability, decreased intestinal integrity, and weakening of the immune barrier in broilers. Furthermore, our study clarifies that LH anti-inflammatory and antioxidant activity may be associated with the suppression of the NF- κ B and MAPK signaling pathways and the subsequent inhibition of proinflammatory cytokine and proinflammatory enzyme expression. Therefore, our findings encourage the use of LH as a complementary and alternative supplement for the prevention of intestinal inflammatory disease.

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