Glutamine Ameliorates Mucosal Damage Caused by Immune Responses to Duck Plague Virus

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Abstract

The immune-releasing effects of L-glutamine (Gln) supplementation in duck plague virus (DPV)-infected ducklings were evaluated in 120 seven-day-old ducklings that were divided into 8 groups. The ducklings in control and DPV, 0.5Gln and DPV + 0.5Gln, 1.0Gln and DPV + 1.0Gln, and 2.0Gln and DPV + 2.0Gln received 0, 0.5, 1.0, and 2.0 g of Gln/kg feed/d by gastric perfusion, respectively. Then, the ducklings in control to 2.0Gln were injected with 0.2 mL of phosphate-buffered saline, while those in DPV to DPV + 2.0Gln were injected with DPV at 0.2 mL of 2000 $TCID_{50}$ (50% tissue culture infection dose) 30 minutes after gavage with Gln, sampled at 12 hours and days 1, 2, 4, and 6. Glutamine supplementation under physiological conditions enhanced immune function and toll-like receptor 4 (TLR4) expressions in a dose-dependent manner. An increase in Gln supplementation under DPV-infected conditions enhanced growth performance, decreased immunoglobulin (Ig) release in plasma and secretory IgA in the duodenum, ameliorated plasma cytokine levels, and suppressed overexpressions of the TLR4 pathway in the duodenum. The positive effects of Gln on the humoral immunity- and intestinal inflammation-related damage should be considered a mechanism by which immunonutrition can assist in the recovery from DPV infection.

Keywords

duck, duck plague virus, glutamine, humoral immunity, intestinal immunity, TLR4 pathway

Introduction

Duck viral enteritis, also known as duck plague, is prevalent worldwide and caused by duck plague virus (DPV), which is an enveloped, double-stranded DNA virus that causes high mortality and decreased egg production in wild and domestic waterfowl (ducks, geese, and swans), resulting in significant economic losses to the poultry industry.[1] This disease is characterized by extensive hemorrhaging and necrosis, especially at specific locations on the mucosal surface of the gastrointestinal tract.[2] Systemic and mucosal immune responses are important to resistance and clearance of viral infections.[3] Improvement of intestinal immune function is anticipated as a feasible approach to reduce damage to infected animals.

Amino acids with intestinal barrier functions, such as arginine, glutamate, and glutamine (Gln), have been shown to enhance immune function of chickens.[4] Glutamine is crucial for optimal immune responses in the intestine and is considered to be "conditionally essential" during inflammatory reactions in response to infection and injury.[5-8] Dietary Gln supplementation improves pregnancy outcome in mice

infected with type-2 porcine circovirus (PCV2).[9] Similar results were observed by Chen et al[10] that Gln starvation enhances PCV2 replication. Thus, we hypothesized that Gln supplementation may enhance host resistance to DPV infection. The present study was conducted with ducklings to test this novel hypothesis.

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Toll-like receptors (TLRs) are a family of pattern-recognition receptors that play key roles in the immune system. Myeloid differentiation primary response gene-88 (MyD88)/TLR signaling has emerged as a major pathway of pathogen recognition by the innate immune system. When engaged by a ligand, MyD88 transduces a signal, which is passed onward by TNF receptor-associated factor 6 (TRAF6), and nuclear factor-kappa-light-chain-enhancer of activated B-cells (NF-κB), which amplifies the signal and ultimately leads to the induction or suppression of genes that orchestrate the inflammatory response.[11-12] We hypothesized that Gln supplementation might altered TLR4 signaling pathway expressions to ameliorate DPV-induced intestinal mucosal damage.

Materials and Methods

Viruses

The DPV (CVCC AV1221) used in this study was provided by the China Institute of Veterinary Drugs Control (Beijing, China). Currently, the virus is stored in the Laboratory Animal and Comparative Medicine Unit, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China.

Animals and Experimental Protocol

Seven-day-old Jinding ducklings (body weight, 112-118 g) were obtained from Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. Glutamine was purchased from Amresco LLC (Solon, Ohio). One hundred and twenty ducklings were maintained in 8 isolation units in a biosecure animal facility and were provided with sterilized, deionized water and commercial duck diet ad libitum. The isolation units had a negative pressure differential relative to the corridor, a 12-hour:12-hour light:dark cycle, 10 to 15 air changes hourly, temperature of $24.2^{\circ}C \pm 1.1^{\circ}C$, and humidity of $50\% \pm 10\%$ and were used exclusively for this study. Ducklings, divided into 8 groups of 15 birds (10 males and 5 females) each, control and DPV, 0.5Gln and DPV + 0.5Gln, 1.0Gln and DPV + 1.0Gln, and 2.0Gln and DPV + 1.0Gln were individually supplied with 0, 0.5, 1.0, and 2.0 g of Gln/kg feed/d by gastric perfusion for 6 days, respectively. Ducklings in control to 2.0Gln were injected with 0.2 mL phosphate-buffered saline (PBS), while those in DPV to DPV + 2.0Gln were injected with DPV at a dose of 0.2 mL of 2000 $TCID_{50}$ (50% tissue culture infection dose) 30 minutes after Gln gavage. At 12 hours and days 1, 2, 4, and 6 after treatment, 3 ducklings (2 males and 1 female) from each group were weighted 30 minutes after gavage, and duodenum and plasma were collected for further analyses and killed under anesthesia through the intraperitoneal injection of sodium pentobarbital (0.5 mL/kg). Meanwhile, the feed of each group was weighted.

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The study protocol was approved by and carried out in full compliance with the animal welfare guidelines of the Animal Care and Use Committee of the Chinese Academy of Sciences (registry no 011063506).[13] All surgeries were performed under sodium pentobarbital anesthesia, and every effort was made to minimize suffering.

Analysis of Gln in Plasma and the Duodenum

Blood samples were collected in tubes and centrifuged at $4000g$ at $4^{\circ}C$ for 15 minutes to separate the plasma. All plasma samples were stored at $-70^{\circ}C$ until assayed. After thawing, Gln levels in the plasma and duodenal mucosa were determined using a commercial Gln assay (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer's protocol. Mean values are reported as micromoles of Gln per gram of tissue and as nanomoles of Gln per milligram of protein, respectively. Intestinal protein concentrations were determined as described elsewhere.[14]

Measurement of Immunoglobulins

Frozen duodenal tissue samples were weighed and homogenized in PBS containing 1% IGEPAL nondenaturing detergent (Sigma-Aldrich Corporation, St. Louis, Missouri), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and complete (ethylenediaminetetraacetic acid-free) protease inhibitor cocktail tablets. The homogenates were kept on ice for 30 minutes and then centrifuged at 10 000g at $4^{\circ}C$ for 10 minutes. Concentrations of immunoglobulin (IgG) and IgA in plasma and secretory IgA (s-IgA) in supernatants of mucosal tissue were measured using an automated biochemical analyzer (SYNCHRON CX PRO model; Beckman Coulter, Inc, Brea, California) with a commercially available kit (Biosino Bio-Technology & Science Inc, Beijing, China).

Enzyme-Linked Immunosorbent Assay Measurement of Plasma Cytokine Levels and TLR4 Pathway

Plasma interleukin 6 (IL-6) and IL-10 concentrations and duodenal tissue TLR4, MYD88, TRAF6, and NF-κB concentrations were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Jianglai Biotech, Shanghai, China) in accordance with the manufacturer's instructions. Optical densities were measured using a GENios microplate reader (Tecan Austria GmbH, Grödig, Austria) at a wavelength of 450 nm. Data were analyzed against the linear portion of a generated standard curve.

Total RNA Extraction and Real-Time Polymerase Chain Reaction Analysis

Total RNA was isolated from the organs of all ducklings using the E.Z.N.A. Total RNA kit (Omega Bio-tek, Inc, Doraville, Georgia) in accordance with the manufacturer's protocol. RNA

Table1. Primers Used for Quantitative Real-Time PCR.

Gene	GenBank Accession Number	Sequence	Fragment Length (bp)
GAPDH	AY436595.1	Forward primer: 5′-AGATGCTGGTGCTGAATACG-3′	104
		Reverse primer: 5′-CGGAGATGATGACACGCTTA-3′	
TLR4	JN048668.1	Forward primer: 5′-ACCCATTGTCACCAACATCA-3′	149
		Reverse primer: 5′-TTCCAGCCTTGTCTTCAACC-3′	
MyD88	KJ126990.1	Forward primer: 5′-GAAAGACCTCCAGTTTGTCCA-3′	165
		Reverse primer: 5′-GAAATGACGACCACCATCCT-3′	
TRAF6	XM005011329.1	Forward primer: 5′-ATTTGAGCCTGCCCTCTTCT-3′	111
		Reverse primer: 5′-TGTCTTACCAGCCGACCTTC-3′	
NF-κB	XM005025220.1	Forward primer: 5′-CCATCCACGACAGCAAGTC-3′	105
		Reverse primer: 5′-CGGAGATGATGACACGCTTA-3′	

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MyD88, myeloid differentiation primary response gene-88; NF-κB, nuclear factor-kappaB; PCR, polymerase chain reaction; TLR4, toll-like receptor 4; TRAF6, TNF receptor-associated factor 6.

concentrations were measured using a spectrophotometer at an absorbance of 260 nm. RNA integrity and quality were determined by agarose gel electrophoresis and confirmation that the A260 nm/A280 nm absorbance ratio was between 1.8 and 2.0.

Expression levels of TLR4, MyD88, TRAF6, NF-κB, and glyceraldehyde-3-phosphate dehydrogenase, as an internal control, were determined by real-time polymerase chain reaction (PCR) analysis using the iQ5 real-time PCR detection system (Bio-Rad Laboratories, Hercules, California). The first strand was synthesized from DNase-treated total RNA using random hexamers and murine Moloney leukemia virus reverse transcriptase with a high-capacity complementary DNA (cDNA) reverse transcription kit (Applied Biosystems China, Inc, Beijing, China) in accordance with the manufacturer's instructions. Primers were designed using Primer Express software (Applied Biosystems, Waltham, Massachusetts) showed in Table 1. Briefly, the PCR reactions consisted of 50 ng of first-strand cDNA and 500 nmol/L of primers in a final volume of 20 μL using SYBR Green IPCR core reagents included in the SYBR real-time PCR kit (TaKaRa Biotechnology Co Ltd, Dalian, China). The expression values were calculated as $2^{-\Delta Ct}$.[15]

Statistical Analyses

All statistical analyses were conducted using SPSS software 19.0 (IBM-SPSS, Inc, Chicago, Illinois). Because the data were normally distributed, all indices were analyzed using analysis of variance and Duncan multiple range tests. A probability (P) value of <.05 was considered statistically significant.

Results

Growth Performance

The mean feed consumption of each duckling is shown in Figure 1. The differences between control to 2.0Gln and DPV to DPV + 2.0Gln gradually increased with time growth. Except 1.0Gln, 2.0Gln, DPV + 0.5Gln, and DPV + 1.0Gln at days 3 to 4, feed consumption increased in a Gln dose-dependent manner in ducklings with or without DPV infection ($P < .05$) in 1 to 2
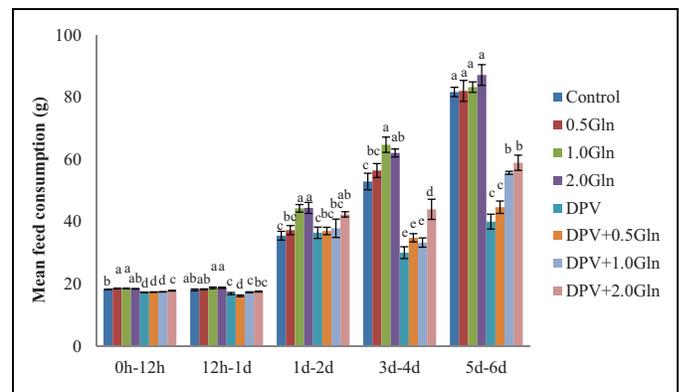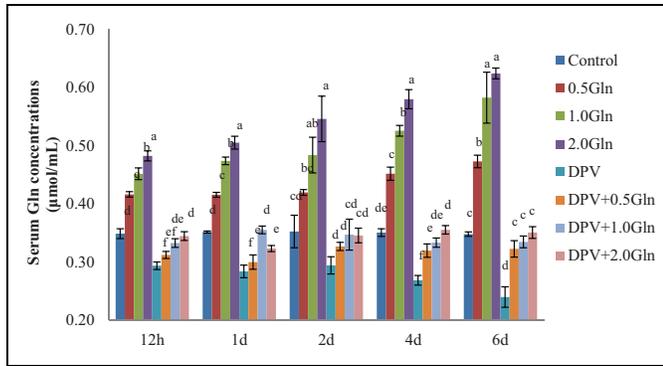

Figure 1. Mean feed consumption per duckling during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine.
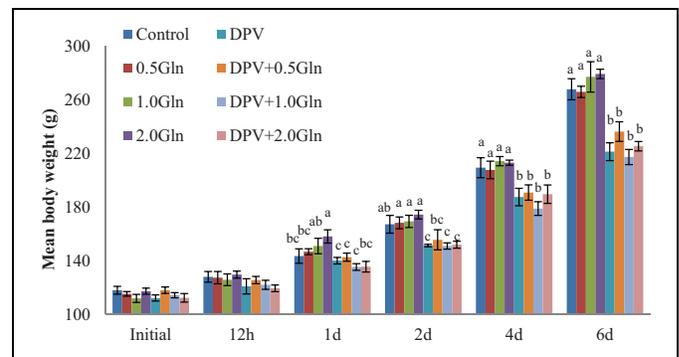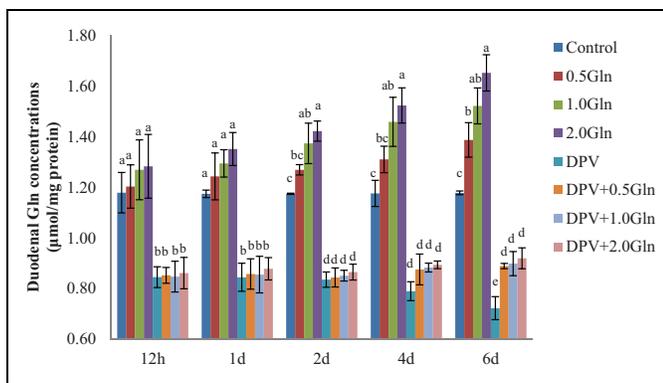

Figure 2. Mean body weight per duckling during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine.

days, 3 to 4 days, and 5 to 6 days. In control, 0.5Gln, 1.0Gln, and 2.0Gln, mean feed consumption sharply raised with Gln supplementations. Otherwise, mean feed consumption of DPV, DPV + 0.5Gln, and DPV + 1.0Gln decreased in 3 to 4 days compared with 1 to 2 days and then increased in 5 to 6 days.

The mean body weight of each duckling was presented in Figure 2. The average weights of the animals in the 8 groups

Figure 3. Plasma Gln concentrations (μmol/mL) during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine.
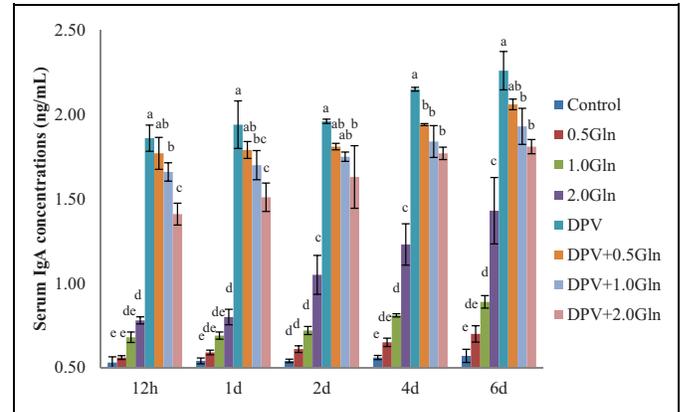

Figure 4. Duodenal Gln concentrations during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine.

Figure 5. Plasma IgA concentrations during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine; IgA, immunoglobulin A.
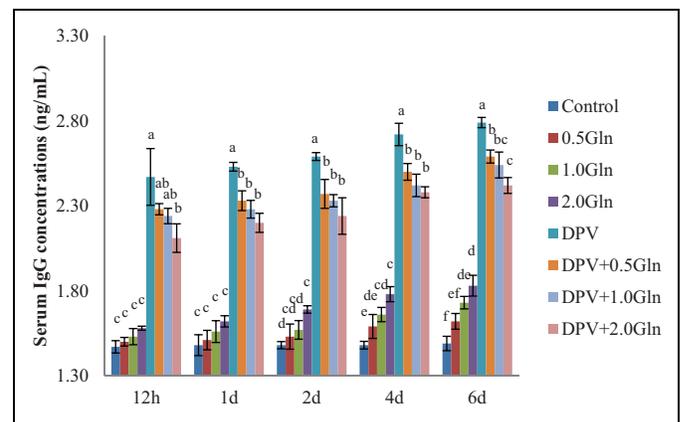
Figure 6. Plasma IgG concentrations during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine; IgG, immunoglobulin G.

were similar at the beginning and 12 hours ($P > .05$). Mean body weight was significantly greater in 1.0Gln and 2.0Gln than in DPV to DPV + 1.0Gln at day 1 ($P < .05$). Similarly, mean body weight was significantly greater in 0.5Gln to 2.0Gln than in DPV, DPV + 1.0Gln, and DPV + 2.0Gln at day 2 ($P < .05$). At days 4 and 6, mean body weight was significantly greater in control to 2.0Gln than in DPV to DPV + 2.0Gln ($P < .05$).

Concentrations of Gln in Plasma and Duodenum

As shown in Figure 3, plasma Gln concentrations significantly increased in a dose-dependent manner in control to 2.0Gln at 12 hours and days 1, 4, and 6 ($P < .05$). Plasma Gln concentrations were significantly greater in 0.5Gln to 2.0Gln than DPV to DPV + 2.0Gln ($P < .05$), except 0.5Gln, DPV + 1.0Gln, and DPV + 2.0Gln at day 2. In DPV, plasma Gln concentrations increased at day 2 and then decreased at days 4 and 6.

As shown in Figure 4, duodenal Gln concentrations were significantly greater in control to 2.0Gln than DPV to DPV + 2.0Gln ($P < .05$). Duodenal Gln concentrations increased in a dose-dependent manner in control to 2.0Gln at days 2, 4, and 6. From 12 hours to 4 days, duodenal Gln concentrations had no

significant difference in DPV to DPV + 2.0Gln ($P > .05$). Duodenal Gln concentrations of DPV decreased significantly than DPV + 0.5Gln to DPV + 2.0Gln at day 6 ($P < .05$).

Changes in Ig Subsets in Plasma and the Duodenum

Concentrations of IgA and IgG in plasma and s-IgA in the duodenum are shown in Figures 5 to 7, respectively. Plasma IgA concentrations in DPV-infected groups were significantly greater than those of control to 2.0Gln with dose-dependent manner ($P < .05$). Plasma IgA concentrations in 2.0Gln were significantly greater than control, 0.5Gln, and 1.0Gln at days 2, 4, and 6 ($P < .05$) and were significantly greater than control at each time points ($P < .05$). Plasma IgA concentrations in DPV were significantly greater than DPV + 1.0Gln and DPV + 2.0Gln at 12 hours and days 1, 4, and 6 ($P < .05$).
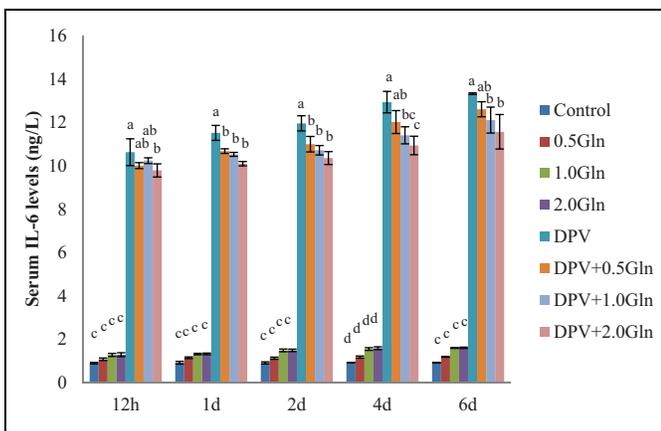
Plasma IgG concentrations in DPV-infected groups were significantly greater than those of control to 2.0Gln with dose-dependent manner ($P < .05$). Plasma IgG concentrations in 2.0Gln were significantly greater than control at days 2, 4, and
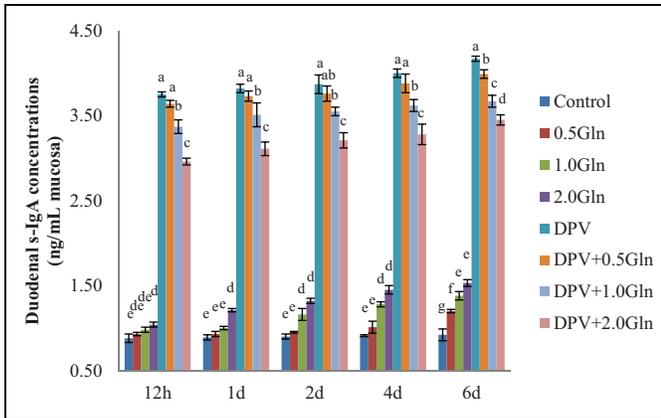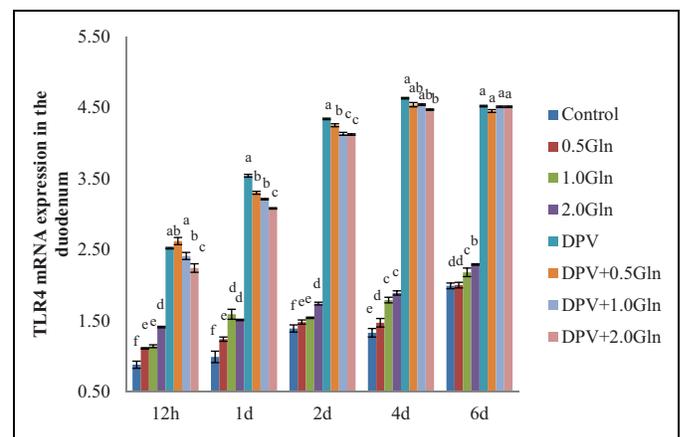
Figure 7. Duodenal s-IgA concentrations during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine; s-IgA, secretory immunoglobulin A.
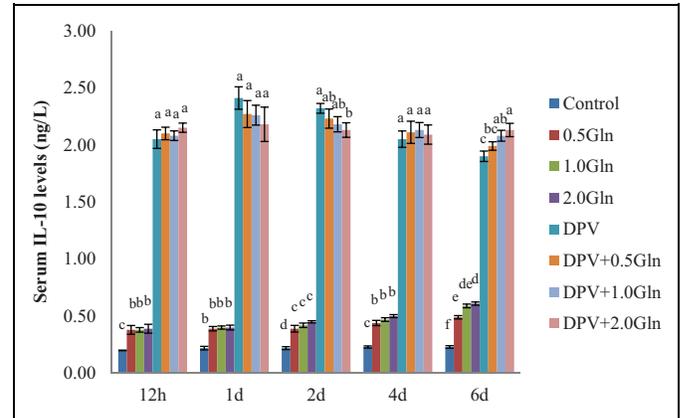
Figure 9. Plasma IL-10 levels during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine; IL-10, interleukin 10.

Figure 8. Plasma IL-6 levels during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine; IL-6, interleukin 6.

Figure 10. TLR4 mRNA expression in the duodenum during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine; mRNA, messenger RNA; TLR4, toll-like receptor 4.

6 ($P < .05$). Plasma IgG concentrations in DPV were significantly greater than DPV + 0.5Gln, DPV + 1.0Gln, and DPV + 2.0Gln at days 1, 2, 4, and 6 ($P < .05$).

Plasma duodenum s-IgA concentrations in DPV-infected groups were significantly greater than those of control to 2.0Gln with dose-dependent manner ($P < .05$). Plasma duodenum s-IgA concentrations in 2.0Gln were significantly greater than control at each time points ($P < .05$). Plasma duodenum s-IgA concentrations in DPV and DPV + 0.5Gln were significantly greater than DPV + 1.0Gln and DPV + 2.0Gln at 12 hours and days 1, 4, and 6 ($P < .05$).

Cytokine Levels

Plasma cytokine (IL-6 and IL-10) concentrations at 12 hours and days 1, 2, 4, and 6 are shown in Figures 8 and 9, respectively. Plasma IL-6 and IL-10 levels in DPV-infected ducklings were almost 7 and 4 times greater than in noninfected ducklings ($P < .05$).

As shown in Figure 8, plasma IL-6 levels had no significant difference in control to 2.0Gln at each time points ($P > .05$).

Plasma IL-6 levels in DPV + 2.0Gln decreased significantly than DPV ($P < .05$) at each time points.

As shown in Figure 9, plasma IL-10 concentrations in 0.5Gln, 1.0Gln, and 2.0Gln were significantly greater than control at each time points ($P < .05$), and plasma IL-10 concentrations in 2.0Gln were significantly greater than 0.5Gln at day 6 ($P < .05$). Plasma IL-10 concentrations had no significant difference in control to 2.0Gln at 12 hours and days 1 and 4 ($P > .05$). Plasma IL-10 concentrations in DPV were significantly greater than DPV + 2.0Gln at day 2 ($P < .05$), whereas plasma IL-10 concentrations in DPV + 2.0Gln were significantly greater than DPV and DPV + 0.5Gln at day 6 ($P < .05$).

Messenger RNA Expressions Analysis

Messenger RNAs expressions of TLR4, MyD88, TRAF6, and NF-κB were observed in the duodenum at 12 hours and days 1, 2, 4, and 6, as shown in Figures 10 to 13, respectively. Messenger RNA expression levels of TLR4, MyD88, TRAF6, and
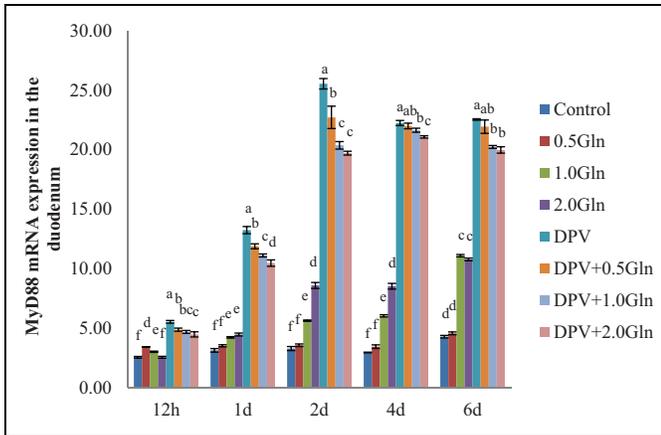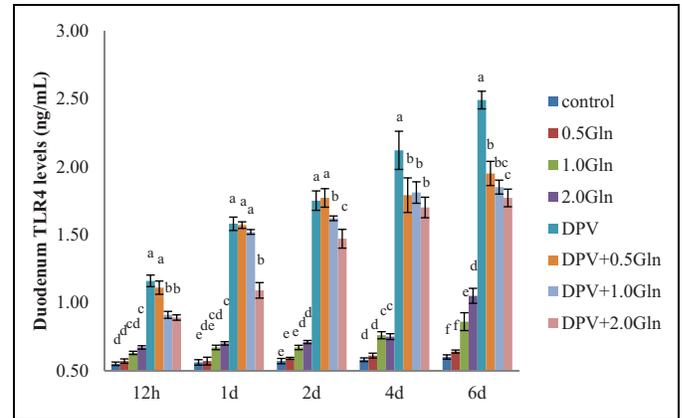
Figure 11. MyD88 mRNA expression in the duodenum during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine; mRNA, messenger RNA.
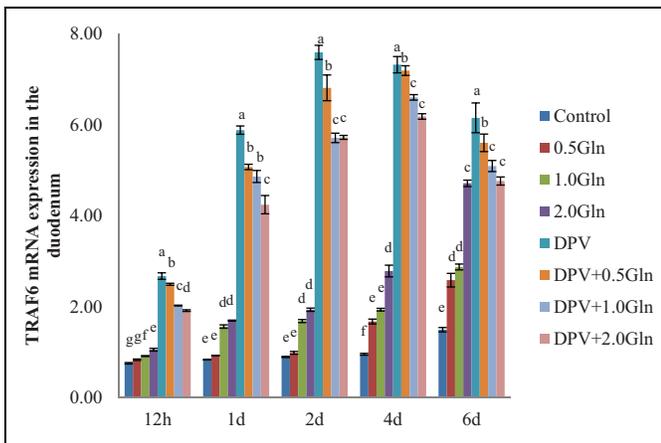
Figure 12. TRAF6 mRNA expression in the duodenum during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine; mRNA, messenger RNA; TRAF6, TNF receptor-associated factor 6.
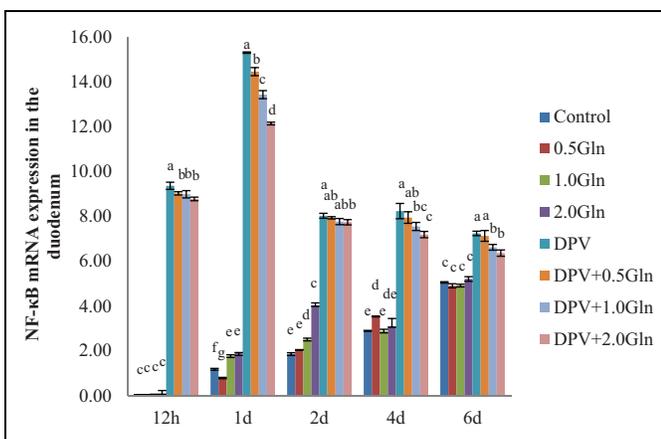
Figure 13. NF-κB mRNA expression in the duodenum during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine; mRNA, messenger RNA; NF-κB, nuclear factor-kappaB.

Figure 14. Duodenum TLR4 levels during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine; TLR4, toll-like receptor 4.

NF-κB were significantly greater in DPV-infected ducklings than in those not infected ($P < .05$).

As shown in Figure 10, TLR4 mRNA expressions increased in a dose-dependent manner in control, 0.5Gln, 1.0Gln, and 2.0Gln, and mRNA expressions of 2.0Gln were significantly higher than control and 0.5Gln at each time points ($P < .05$). Toll-like receptor 4 mRNA expressions of DPV + 2.0Gln were significantly lower than control at 12 hours and days 1, 2, and 4 ($P < .05$).

As shown in Figure 11, MyD88 mRNA expressions increased in a dose-dependent manner in control, 0.5Gln, 1.0Gln, and 2.0Gln at days 1, 2, and 4. The MyD88 mRNA expressions of 1.0Gln and 2.0Gln were significantly higher than control and 0.5Gln at days 1, 2, 4, and 6 ($P < .05$). The MyD88 mRNA expressions decreased in a dose-dependent manner in DPV-injected groups. The MyD88 mRNA expressions of DPV were significantly higher than DPV + 1.0Gln and DPV + 2.0Gln at each time points ($P < .05$).

As shown in Figure 12, TRAF6 mRNA expressions increased in control, 0.5Gln, 1.0Gln, and 2.0Gln, whereas decreased in DPV, DPV + 0.5Gln, DPV + 1.0Gln, and DPV + 2.0Gln in a dose-dependent manner. The TRAF6 mRNA expressions of 2.0Gln were significantly higher than control and 0.5Gln at 12 hours and days 1, 2, 4, and 6 ($P < .05$). The TRAF6 mRNA expressions of DPV and DPV + 0.5Gln were significantly higher than DPV + 1.0Gln and DPV + 2.0Gln at each time points ($P < .05$).

As shown in Figure 13, NF-κB mRNA expressions decreased in DPV, DPV + 0.5Gln, DPV + 1.0Gln, and DPV + 2.0Gln in a dose-dependent manner. The NF-κB mRNA expressions of 1.0Gln and 2.0Gln were significantly higher than control and 0.5Gln at days 1 and 2 ($P < .05$). The NF-κB mRNA expressions of DPV were significantly higher than DPV + 1.0Gln and DPV + 2.0Gln at 12 hours and days 1, 4, and 6 ($P < .05$).

## Concentrations of TLR4, MyD88, TRAF6, and NF-κB in Duodenum

As shown in Figures 14 to 17, the concentrations of TLR4, MyD88, TRAF6, and NF-κB in infected groups were
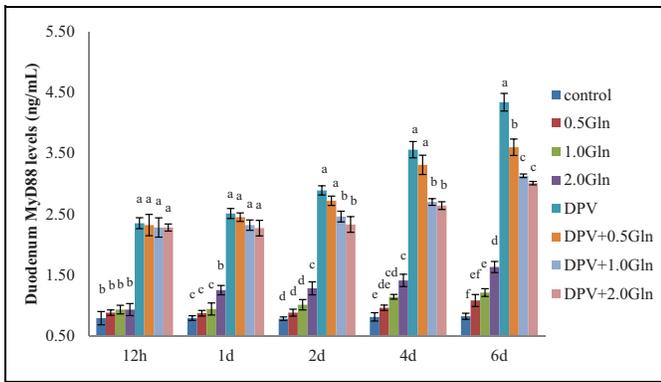
Figure 15. Duodenum MyD88 levels during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine; MyD88, myeloid differentiation in primary response gene-88.
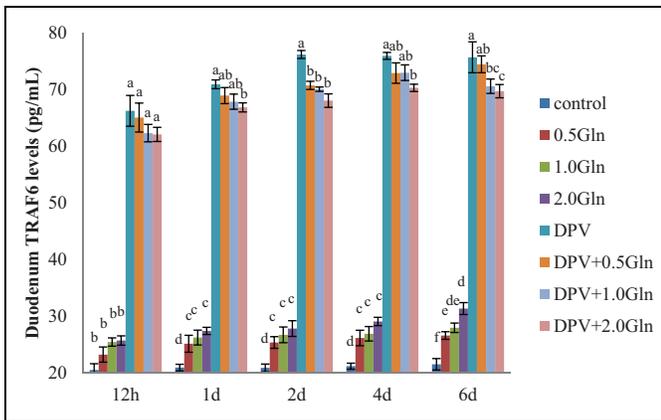
Figure 16. Duodenum TRAF6 levels during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine; TRAF6, TNF receptor-associated factor 6.
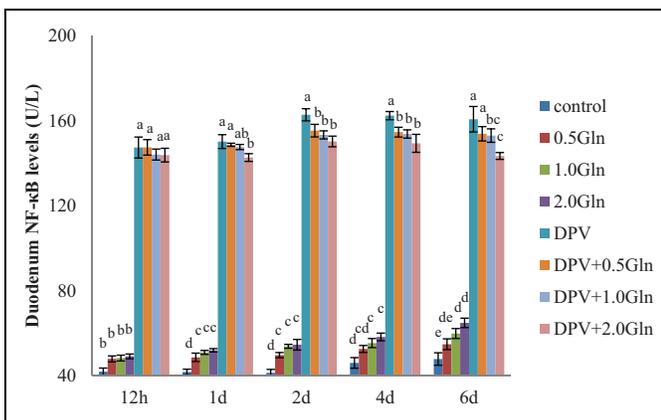
Figure 17. Duodenum NF-κB levels during 6 days of ducklings with and without Gln or DPV injection. DPV indicates Duck plague virus; Gln, glutamine; NF-κB, nuclear factor-kappaB.

significantly higher than noninfected groups at each time point ($P < .05$). All concentrations increased in control, 0.5Gln, 1.0Gln, and 2.0Gln, whereas decreased in DPV, DPV

+ 0.5Gln, DPV + 1.0Gln, and DPV + 2.0Gln with increase dosage of Gln.

Toll-like receptor 4 levels of 2.0Gln were significantly higher than control and 0.5Gln at 12 hours and day 1, and in 1.0Gln and 2.0Gln were significantly higher than control and 0.5Gln at days 2, 4, and 6 ($P < .05$). Toll-like receptor 4 levels of DPV were significantly higher than DPV + 2.0Gln at each time points ($P < .05$).

The MyD88 levels of 2.0Gln were significantly higher than control at each time points ($P < .05$). The MyD88 levels of DPV and DPV + 0.5Gln were significantly higher than DPV + 1.0Gln and DPV + 2.0Gln at days 2, 4, and 6 ($P < .05$).

The TRAF6 levels of control were significantly lower than 0.5Gln, 1.0Gln, and 2.0Gln at days 1, 2, 4, and 6 ($P < .05$). The TRAF6 levels of DPV were significantly higher than DPV + 2.0Gln at days 1, 2, 4, and 6 ($P < .05$).

The NF-κB levels of 1.0Gln and 2.0Gln were significantly higher than control at days 1, 2, 4, and 6 ($P < .05$). The NF-κB levels of DPV were significantly higher than DPV + 2.0Gln at each time points ($P < .05$).

Discussion

Duck plague virus infection has been reported in duck-producing regions worldwide. Naturally occurring DPV infections have been observed in ducks ranging from 7 days of age to mature breeders.[16] Lethal DPV infection can result in 100% mortality in duck flocks.[17] At present, inoculation of ducklings with live attenuated DPV vaccines is the primary approach to prevent and control lethal DPV infections in ducks.[2-3] However, this method has had limited success for various reasons under practical conditions.[18] Thus, nutritional supplementation to strengthen the intestinal immune responses of the host[19] appears to be the best available choice of prophylactic adjuvant therapy against DPV infection. Glutamine, the most abundant amino acid in blood,[20] is a nonessential amino acid that is beneficial to the prevention of infectious morbidity and mortality in seriously ill patients.[11] Glutamine plays a significant role in intestinal immune function[6] and is mainly absorbed in the proximal region of the small intestine, resulting in little entry, if any, into the colon.[21] Therefore, the duodenum was chosen for analysis in the present research. Previous studies of Gln metabolism and immune responses have primarily focused on the chicken[22] and mammals.[23] However, less is known about the functions of Gln supplementation in intestinal immunity of waterfowl, particularly in DPV infection. In the present study, Gln supplementation was found to alter growth performances, enhance intestinal immune function, and partially regulate TLR4 signaling pathway.

The results of this study showed that an increase in Gln dosage resulted in an increase in mean feed consumption and mean body weight gains of ducklings with or without DPV infection. Similar results were reported by Bartell and Batal,[24] who showed that under physiological conditions, 1% Gln improved growth performance of chickens. Moreover, Yi et al[25] reported superior body weight gains of broilers

supplemented with 1% Gln as compared to controls. In contrast, Le Bacquer et al[26] found that 1.5% Gln supplementation did not affect feed intake of piglets weaned at 21 days of age, and Yi et al[27] indicated that 2.0% Gln supplementation did not affect growth performance during an 11-day feeding regimen of piglets weaned at 17 days of age. This disparity may be due to different species or experimental conditions.

Duck plague virus infection suppresses growth performance of ducklings. Fasina et al[28] reported greater body weights and body weight gains of chicks with *Salmonella* cecal infection in the 1% Gln group than in the no Gln or bacterium group, no Gln and challenged with bacterium group, and 1% Gln and challenged with bacterium group, indicating that Gln supplementation can enhance growth performance in healthy and diseased chicks in a dose-dependent manner.

Illness, injury, and low birth weight can lead to a significant decrease of Gln both in the plasma and intestine[29] which was in accordance with the data of the DPV infection groups presented in Figures 3 and 4. Shu et al[30] reported that the Gln contents in blood and tissues can decrease significantly during ischemia–reperfusion injury. In this study, we found that Gln content decreased during body and intestinal inflammation in response to DPV infection. Likewise, Bertrand et al[31] suggested that the beneficial effects of Gln pretreatment were limited by chemotherapy or surgery-induced intestinal injury. In the current study, increasing Gln supplementation ameliorated Gln concentration loss both in the plasma and duodenum of DPV-infected ducklings.

Sera of immune competent donors contain antibodies of the IgG, IgA, and IgM classes. Antibodies of the IgG subclasses are most abundant in animals, accounting for about 75% of total serum antibody levels.[32] Serum IgA and IgG play critical roles in humoral immune responses against specific antigens.[3] Glutamine is a distinct nutrient under physiological conditions and plays multiple roles in the maintenance of normal immunological function under both stressful and pathological conditions.[9] In support, Sturgill et al[33] reported an increase in IgG and IgE production by purified B-cells and peripheral mononuclear cells when cultured with Gln in vitro. The results of the present study revealed significantly greater IgA and IgG concentrations in the plasma of ducklings in 2.0Gln than in control, indicating that Gln supplementation enhances humoral immunity under physiological conditions. Duck plague virus infection is known to disrupt the immune response. Serum IgG levels were higher than those of serum IgA,[3] both in ducklings with or without DPV infection, as presented in Figure 5. This finding suggests that IgG is the primary antibody produced by immune cells against DPV infection, as reported elsewhere.[3] Our results revealed that the systemic antibody response was strongly triggered by DPV infection, and plasma IgA and IgG concentrations significantly increased accordingly, indicating that not only IgG but also IgA might contribute to the protection of ducklings against DPV infection.

Mucosal immunity is an important part of the humoral immune response, and s-IgA is an effector molecule of mucosal immunity. As a first line of defense against invading pathogens in the gastrointestinal tract,[34] s-IgA is the most prominent antibody present at the mucosal surfaces.[32] Our results showed that Gln supplementation enhanced s-IgA content in a dose-dependent manner under physiological conditions. The birds fed diets supplemented with 1% Gln had higher IgA concentrations in the intestines and thus may have been more resistant to infection.[24] Hsu et al[35] showed that dietary provision of 2% Gln was essential for the maintenance of gut-associated lymphoid tissues and for s-IgA synthesis by the small intestine. Production of s-IgA appears to be a limiting factor in mucosal immunity, as a reduction in s-IgA was experimentally associated with functional deficits in established immunity against various viruses and bacteria.[36] Indeed, a deficiency or the inability to produce IgA results in frequent intestinal infections.[37] Our results demonstrated that intestinal s-IgA content from ducklings after infection with DPV was significantly greater than from ducklings in the control group. Duck plague virus infection is known to dysregulate Ig production and impede the humoral and intestinal immune responses of ducklings. By increasing Gln supplementation, s-IgA concentrations were decreased in the DPV-infected groups to protect the mucosa, which was because the results of previous studies showing that abnormal regulation of s-IgA production due to pathological invasion will impair the mucosal immune system.[38] These results also suggested that humoral as well as mucosal immunity is involved in protection against DPV challenge.[3]

Toll-like receptors, when activated by bacterial ligands, produce intracellular signals to upregulate the expression of various cytokines in the intestine, including pro-(IL-1β, IL-6, and IL-8) and anti-inflammatory cytokines (IL-10), in humans.[11] Other studies have demonstrated that the function of humoral immunity and the intestinal barrier is likely regulated by a network of multiple cytokines, including ILs, interferons (IFNs), and tumor necrosis factor alpha (TNF-α).[39-40] Interleukin 6 is an important mediator in the early phase of infection[41] and plays a very complex role in immune responses.[8] A previous study showed that Gln supplementation attenuated pro-inflammatory cytokine release, protected against organ damage, and decreased mortality.[41-42] Glutamine suppresses the production of inflammatory and suppressor cytokines,[9] which was similar to the data of DPV infection groups in our study, and improves the potentiation and function of immunocompetent cells,[9] which may be the reason for the tendency of increased IL-6 production in response to Gln supplementation of ducklings in control to 2.0Gln. Therefore, an increase in Gln supply can enhance the immune response by upregulating IL-6 production under physiological conditions. In contrast, increased Gln supplementation decreased the inflammatory reaction produced by DPV, in accordance with the observations reported by Ren et al, who suggested that a reduced serum IL-6 level may be indicative of a reduced PCV2 load in Gln-supplemented mice.[8] Glutamine administration attenuates the inflammatory reaction in nonhepatic organs by regulating IL-6 levels.[41] Xu et al[40] reported that treatment with Gln significantly decreased IL-6 levels after hypobaric hypoxia

stimulation, whereas serum levels of TNF-α, IL-6, and IFN-γ were increased. This result suggests that Gln may improve the permeability of the intestinal mucosa to protect the intestine. The primary function of IL-10 is to inhibit many functions of immune cells (ie, natural killer cells, T cells, macrophages, and dendritic cells) and to reduce the production of inflammatory cytokines.[43] Glutamine is a major energy substrate for lymphocytes, macrophages, and other immune cells and thus plays an important role in immune function.[9] In this study, we observed that Gln supplementation enhanced IL-10 levels at each time point in noninfected ducklings and enhanced IL-10 levels at 12 hours and on days 4 and 6 in infected ducklings. A possible explanation for these observations is that dietary supplementation with functional amino acids can enhance the responses of both innate and adaptive immune systems.[5,9]

Toll-like receptor signaling has been shown to function in several pathways that generally mediate tissue injury. Toll-like receptor 4 is thought to be involved in the first immune barrier of the gastrointestinal tract.[40] Recent studies have shown that TLR2 and TLR4 are overexpressed in intestinal epithelial cells, which has been correlated with mucosal damage.[11] Therefore, TLRs deserve careful consideration as possible mediators of the inflammatory reaction induced by DPV infection. The observed increase in mRNA expression and concentrations after DPV infection between control and DPV + 0.5Gln, which was similar to the increased expression of TLRs in intestinal tissues during experimental necrotizing enterocolitis, which is a well-known pediatric intestinal inflammatory disease, supports this conclusion.[44] In accordance with the decreased expression of TLR4, the expression of MyD88/TRAF6/NF-κB was also decreased. The decreased expression of downstream genes was associated with the subsequent decreased expression of upstream genes. Until ligands of TLRs were identified, all responses to TLR ligands were considered to be identical and entirely dependent on MyD88.[45] The findings of Inoue et al[46] indicate that TRAF6 is involved in the MyD88-mediated immune response. The TRAF6 is a highly conserved downstream signaling protein of the TLR4 pathway and can activate NF-κB, enabling it to enter the nucleus and activate transcription of select genes.[47] The NF-κB, the final effector molecule of the TLR4 signaling pathway, is a key transcription factor in the regulation of the inflammatory reaction.[30] Cytokine expression induced by all TLRs, with the exception of TLR3, is dependent on TRAF6,[46] which were associated with increased cytokine production in our research. Glutamine is considered an essential amino acid during certain disease conditions and is beneficial to ameliorate inflammatory reactions.[41,48] The positive effect of Gln on intestinal immune function may be considered a mechanism by which immunonutrition is beneficial in the recovery of oncological patients receiving chemotherapy.[11] The upregulation of TLR4 in response to DPV infection activates the TLR4/MyD88/NF-κB signaling pathway. We found that with an increase of Gln supplementations in infected groups, the levels of TLR4/MyD88/TRAF6/NF-κB were decreased, thereby preventing NF-κB activation and subsequently decreasing secretion of inflammatory cytokines.[30] Although intestinal mucosal mRNA expression levels and concentrations were significantly upregulated after DPV infection, as compared between DPV + 0.5Gln and DPV + 2.0Gln, these levels and concentrations were significantly lowered by Gln supplementation. Thus, Gln acts a pleiotropic transcription factor, regardless of DPV infection, as reported by Pasparakis,[33,49] suggesting that Gln plays important roles in intestinal immune regulation and the activation of TLR4 signaling, which was consistent with the changes in plasma IL-6 levels observed in the present study. In addition, Gln metabolism plays multiple roles in the regulation of intestinal gene expression.[29]

In summary, our results demonstrated that Gln supplementation conveys beneficial effects both under physiological conditions and during challenge by DPV infection and were effective to improve immune function and prevent damage to the small bowel mucosal.[50] These finding suggest that inflammatory bowel disease maybe ameliorated, at least in part, by Gln-supplemented nutritional support. Also, not only humoral immunity but also mucosal immunity is involved in Gln-derived protection against DPV challenge. The data presented here provide new insights into the immune mechanisms of Gln attenuation of DPV infection, which could confer protective immunity against DPV challenge in Jinding ducks.

Declaration of Conflicting Interests

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