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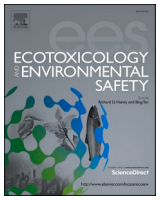

Zinc glycinate alleviates LPS-induced inflammation and intestinal barrier disruption in chicken embryos by regulating zinc homeostasis and TLR4/NF-κB pathway

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ABSTRACT

The effect of an immune challenge induced by a lipopolysaccharide (LPS) exposure on systemic zinc homeostasis and the modulation of zinc glycinate (Zn-Gly) was investigated using a chicken embryo model. 160 Arbor Acres broiler fertilized eggs were randomly divided into 4 groups: CON (control group, injected with saline), LPS (LPS group, injected with 32 μg of LPS saline solution), Zn-Gly (zinc glycinate group, injected with 80 μg of zinc glycinate saline solution) and Zn-Gly+LPS (zinc glycinate and LPS group, injected with the same content of zinc glycinate and LPS saline solution). Each treatment consisted of eight replicates of five eggs each. An in ovo feeding procedure was performed at 17.5 embryonic day and samples were collected after 12 hours. The results showed that Zn-Gly attenuated the effects of LPS challenge-induced upregulation of pro-inflammatory factor interleukin 1β (IL-1β) level (P =0.003). The LPS challenge mediated zinc transporter proteins and metallothionein (MT) to regulate systemic zinc homeostasis, with increased expression of the jejunum zinc export gene zinc transporter protein 1 (*ZnT-1*) and elevated expression of the import genes divalent metal transporter 1 (*DMT1*), Zrt- and Irt-like protein 3 (*Zip3*), *Zip8* and *Zip14* ($P < 0.05$). A similar trend could be observed for the zinc transporter genes in the liver, which for *ZnT-1* mitigated by Zn-Gly supplementation (P =0.01). Liver *MT* gene expression was downregulated in response to the LPS challenge (P =0.004). These alterations caused by LPS resulted in decreased serum and liver zinc levels and increased small intestinal, muscle and tibial zinc levels. Zn-Gly reversed the elevated expression of the liver zinc finger protein *A20* induced by the LPS challenge (P =0.025), while Zn-Gly reduced the gene expression of the pro-inflammatory factors *IL-1β* and *IL-6*, decreased toll-like receptor 4 (*TLR4*) and nuclear factor kappa-B p65 (*NF-κB p65*) ($P < 0.05$). Zn-Gly also alleviated the LPS-induced downregulation of the intestinal barrier gene Claudin-1. Thus, LPS exposure prompted the mobilization of zinc transporter proteins and MT to perform the remodeling of systemic zinc homeostasis, Zn-Gly participated in the regulation of zinc homeostasis and inhibited the production of pro-inflammatory factors through the TLR4/NF-κB pathway, attenuating the inflammatory response and intestinal barrier damage caused by an immune challenge.

1. Introduction

Intensive poultry farming facilitates the efficient production of animal protein, yet has nonetheless led to increased challenges for birds, including susceptibility to pathogenic microorganisms, environmental pollution, and factors related to food which has resulted in immune

Abbreviations: ALP, alkaline phosphatase; CAT, catalase; Cu,Zn-SOD, copper-zinc superoxide dismutase; DMT1, divalent metal transporter 1; IL-1β, interleukin 1β; IL-6, interleukin 6; IFN-γ, interferon-γ; INOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MDA, malondialdehyde; MT, metallothionein; MTF-1, metal transcription factor-1; NF-κB, nuclear factor kappa-B; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; TLR, toll-like receptor; T-AOC, total antioxidant capacity; ZO-1, zonula occludens 1 ; Zip, Zrt- and Irt-like protein; Zn-Gly, zinc glycinate; ZnT, zinc transporter protein.

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challenges and threats of oxidative stress (Van Hoeck et al., 2020, Zhang et al., 2023). Under these circumstances, the immune system of poultry play a crucial role in combating bacterial infections, particularly in the global context of increasing concerns regarding antimicrobial resistance and the prohibition of antibiotic additives in feed (Hood-Pishchany et al., 2020, Palmer et al., 2020). Therefore, the application of feed additives to alleviate immune stress in poultry and regulate innate immune responses to inflammation has become an effective strategy (Das et al., 2021).

As an important component of many enzymes in animals, zinc is known to influence a variety of physiological processes (Liu et al., 2023). As such, it is commonly added to feeds to improve animal growth and performance, which is related to its function in improving intestinal barrier function and reducing inflammatory responses (Shimizu et al., 2020, Nguyen et al., 2021). Zinc amino acid chelates have for instance been widely used in recent years due to their higher biological availability (Farhadi Javid et al., 2021). The widespread application of zinc is due to a number of physiological functions in its participation. The three components, zinc bound to metallothionein (MT) with low affinity, zinc bound to organelles and cytoplasmic free zinc, constitute the zinc pool after zinc absorption by tissue cells and removal of the fraction bound to metalloenzymes as structural components or cofactors (Nolin et al., 2019, Endo et al., 2020). Zinc homeostasis is a complex physiological homeostasis regulated by a family of MT, Zrt- and Irt-like proteins (Zips) and a family of zinc transporter proteins (ZnTs) (Betrie et al., 2021). A previous study showed that zinc homeostasis in broilers under heat stress was regulated by zinc transporter proteins and metallothionein. (Xiao et al., 2022). However, the effects and mechanisms of immune challenges on zinc metabolism in birds remain elusive.

As a major component of Gram-negative bacteria's outer membrane, lipopolysaccharide (LPS) induces immune stress by causing an imbalance in the inflammatory response and antioxidant system (Izadparast et al., 2022). Recognized by host toll-like receptors (TLRs), LPS activates the downstream nuclear factor kappa-B (NF-κB) pathway which triggers the intrinsic immune response and promotes the secretion of pro-inflammatory cytokines, causing an inflammatory response and disrupting intestinal barrier function (Ciesielska et al., 2021, Peace and O'Neill, 2022). With its accessibility and ease of handling, the in ovo feeding is an attractive way for research in the fields of nutrition and toxicology (Kadam et al., 2013, Retes et al., 2018). Past studies have confirmed that broiler embryos begin to ingest amniotic fluid through the oral cavity at embryonic day 17 (E17) (Pan et al., 2023), and in ovo feeding through the amniotic cavity at late hatching has been applied to an LPS exposure model. Therefore, this study aimed to evaluate the effect of zinc glycinate (Zn-Gly) on zinc homeostasis under LPS exposure and the ameliorative effects on the inflammatory response, oxidative stress and intestinal barrier function.

2. Materials and methods

All experimental procedures were approved by the Ethics Committee of Shandong Agricultural University (No. SDAUA-2022–50) and conducted in accordance with the Guide for Laboratory Animals of the Ministry of Science and Technology (Beijing, People's Republic of China). The experiment was conduct in Shandong Agricultural University (Taian, China).

2.1. Experimental design and treatments

The optimal embryonic age for sampling needed to be determined first by a pilot study prior to in ovo injection. The challenge model for in ovo feeding of LPS and the dose of LPS has been reported in past study, identifying 32 μg and 12 h as the dose and sampling time for LPS, respectively (Kong et al., 2023). Therefore, we still required standardization of sampling times only by specifying the length of time required for zinc to be able to be utilized in the embryos. The age of the injected embryos and the dose of zinc were sourced from past studies about zinc in ovo administration, and 40 Arbor Acres (AA) broiler fertilized eggs of similar weights were weighed, labeled, and incubated, before 0.5 mL of Zn-Gly solution (at a zinc concentration of 160 μg/mL) was injected into the amniotic cavity at E17.5 (Meriwether et al., 2010). Yolk samples were collected before injection and after 6, 12, and 24 hours of continued incubation for a zinc content assay.

A total of 160 AA broiler fertilized eggs with similar weights (71.5 ±2 g) were weighed and labeled for the formal experiment, and all eggs were randomly divided into four treatment groups (abbreviated as CON, LPS, Zn-Gly and Zn-Gly+LPS) with eight replicates of five eggs each. At E17.5, 0.5 mL of saline, LPS solution (64 μg/mL), Zn-Gly solution (160 μg/mL), or a mixture of LPS and Zn-Gly solution were injected.

2.2. Eggs and incubation management

All eggs were purchased from Taian Liuhe Broiler Company (Taian, China), stored at 20°C for 24 h and sterilized by fumigation with formaldehyde gas before being transferred to an automatic incubator (Jiayu Electronic Technology Company, Dezhou, China) for incubation with a relative humidity of 60% and a temperature of 37.8°C. At E17.5, we candled all the fertilized eggs in warm environment, discarded the dead embryos, injected in ovo, and incubated the remaining fertilized eggs until samples were collected.

2.3. Solution preparation and in ovo injection procedure

The reagent grade Zn-Gly ($C_4H_8N_2O_4Zn$) was prepared at Jining HeShi Biological Co., Ltd. According to the manufacturer's procedure, the molar ratio of 2:1 glycine and alkaline zinc carbonate were dissolved in water in a reaction kettle and then heated to 80°C for 40 min to produce zinc glycinate mother liquor. The Zn-Gly product was obtained by centrifugation to extract the crystals and then dried. The zinc content was measured and found to be 21.2% while the chelation strength Q_f value was 11.2. The solution was diluted with saline to obtain a theoretical value of 160 μg/mL. All prepared solutions were filtered through a 0.22 μm acetate filter and verified for Zn-Gly solution to obtain a zinc content of 157.8 μg/mL, thus determining that the actual zinc content of the 0.5 mL of zinc glycinate solution used for injection was 78.9 μg. LPS reagent (L2880, Sigma-Aldrich I, St. Louis, USA) was purchased from supplier.

The protocol was conducted as previously described (Kong et al., 2023). The eggs were candled and the location of the air chamber was marked using a pencil, the shell was sterilized with alcohol swabs after which a 1 mm diameter hole was drilled in the middle, while 0.5 mL solution of different treatments were injected into the amniotic cavity by a needle of twenty-one gauge. The depth of injection was 2 cm and the operation was assisted by illumination. Immediately after injection, the hole was covered with paraffin and the return the eggs to the incubator. The entire procedure was performed in a laminar flow system, and all solutions were freshly prepared and then heated to 37°C to prevent embryonic cold shock. The entire procedure of each egg took no more than 15 minutes outside the incubator.

2.4. Sample collection

The timing of sample collection was determined according to the results of the pretest and the Taipan Blue stain localization method. Briefly, yolk samples were collected from pre-tested chicken embryos and their yolk zinc content was measured to infer the utilization of the zinc solution by the embryos. The results showed that the yolk zinc content was 713.38 μg, 700.32 μg, 707.43 μg and 680.98 μg before and 6, 12 and 24 h after injection, respectively. It was possible to determine that the exogenous zinc was efficiently absorbed by the embryos after 12 h of injection. Combined with the photographs after in ovo injection with 0.5 mL of Taipan Blue staining solution (dilution 1:500) as

described and at 12 h (Fig. 1.), it was shown that the solution was completely utilized by the embryos after 12 h of in ovo injection. Therefore, 12 h was chosen as the sampling timepoint after in ovo injection.

Eight samples per treatment were obtained for analysis of various indicators. Samples were collected at E18 and four embryos per replicate were randomly selected for execution with the neck transfer method. The blood was drawn from the embryonic heart using a sterile syringe and approximately 0.2 mL of blood was transferred into a tube without anticoagulant. After 30 min of settling, the upper layer of fluid was separated by centrifugation using a speed of 3000 × *g* for 10 min to obtain the serum. The liver and small intestine were immediately removed, stored in a freezer at −80°C after being rapidly frozen in liquid nitrogen. After removing the skin and bones from the right thigh, the meat was removed and stored at −80°C after being stored at room temperature. Samples from every three consecutive embryos with same weight were combined into one sample for zinc content analysis. Jejunum and liver samples were obtained from one additional chicken embryo in each replicate, rapidly snap-frozen in liquid nitrogen and stored at −80°C for subsequent index analysis.

2.5. Determination of zinc concentration

After soaking in ether for 96 hours, the tibia were degreased, dried at 105°C, then ash in a muffle furnace (550°C, 24 h) at a constant weight. The ashed samples were dissolved using 5 mL of hydrochloric acid solution with a concentration of 6 mol/L and then fixed in a 25 mL volumetric flask for measurement. The samples of small intestine, liver and leg muscle were freeze-dried, and 0.2 g dried powder was added to 2 mL of nitric acid (70%) and 1 mL of hydrogen peroxide solution (30%), placed in a 50 mL plastic centrifuge tube and digested in a water bath at 95°C for 6 h. After cooling, the samples were fixed in a 25 mL volumetric flask and filtered for measurement. The zinc content of tissues was analyzed by an inductively coupled plasma spectrometer (ICAP 7000, Thermo, Waltham, MA, USA).

2.6. Detection of immune parameters and zinc-related enzyme activity

The levels of immune factors interleukin 1β (IL-1β), interleukin 6 (IL-6), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) in the jejunum and liver were measured with enzyme-linked immunosorbent assay kits from MLBIO (Shanghai, China). Zinc metabolism-related markers alkaline phosphatase (ALP) activity, MT levels and copper-zinc superoxide dismutase (Cu,Zn-SOD) activity were also assayed using kits from the same company. According to the manufacturer's instructions, all assay procedures were performed strictly and the intra-batch coefficient of variation (CV) of the data was 5% and the inter-batch CV was 8%.

2.7. Oxidative status assay

Catalase (CAT) activity, total antioxidant capacity (T-AOC) levels, inducible nitric oxide synthase (iNOS) activity and malondialdehyde (MDA) levels were measured in the jejunum and liver using kits provided by Nanjing Jiancheng Institute of Biotechnology (Nanjing, China) for the assessment of antioxidant capacity. According to the manufacturer's instructions, all assay procedures were performed strictly and the intra-batch CV of the data was 5% and the inter-batch CV was 8%.

2.8. RNA isolation and real-time quantitative PCR

We isolated total RNA from the jejunum and liver using Trizol reagent (Invitrogen, San Diego, USA). NanoDrop spectrophotometers (ND-2000, Thermo Scientific, Wilmington, USA) were used to measure the concentration and purity of each RNA sample. An agarose gel electrophoresis of 1% was used to test for RNA integrity. A PrimeScript® RT kit (RR047A, TaKaRa) was used to reverse transcribe 1 g of total RNA. To determine gene expression, RT-PCR analysis was performed using the TB Green Premix Ex Taq (RR820A, Takara, Japan) in an ABI 7500 real-time PCR system (Thermo Scientific, Wilmington, USA). Each reaction was repeated in three wells, and the first sequence is shown in Table 1. Each reaction consisted of: predenaturation at 95°C for 10 s, denaturation at 95°C for 5 s, and annealing and extension at 60°C for 40 s. Based on a standard curve from the software, the primer amplification efficiencies were calculated. Melting curve analysis verified that the amplification products were specific. In order to analyze the relative gene expression levels for all the target genes, we normalized with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression.

2.9. Data analysis

All data were expressed as mean and total standard error of the mean, and data were counted and analyzed in replicates. Data were analyzed using the Shapiro-Wilk test (95% confidence interval) using the software SPSS 22.0 (SPSS. Inc., Chicago, USA) followed by two-way ANOVA with general linear model, and Tukey's post-hoc test was

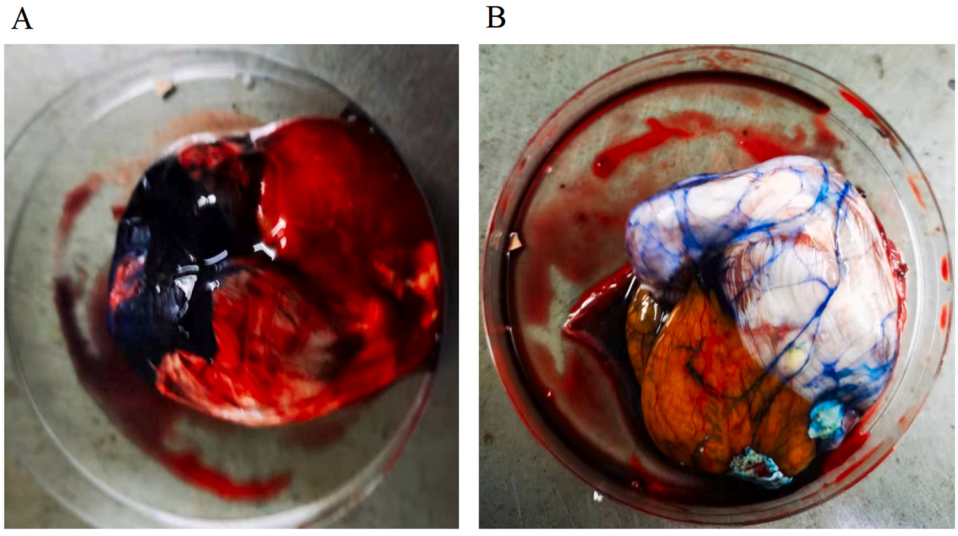

Fig. 1. Embryo anatomy after 0 h (A) and 12 h (B) of in *ovo* feeding Taipan Blue stain solution.

Table 1
Primer sequences used for real-time quantitative PCR.

Gene	Accession Number	Primer sequence, 5′→ 3′	Size (bp)	Reference
IL-1β	NM_204524.1	GGTCAACATCGCCACCTACA CATACGAGATGCAAACCAGCAA	86	Kong et al. (2022)
1 L-6	NM_204628.1	AAATCCCTCCTCGCCAATCT CCCTCACGGTCTTCTCCATAAA	106	
TLR4	NM_001030693.1	AGGCACCTGAGCTTTTCCTC TACCAACGTGAGGTTGAGCC	96	
NF-κB p65	NM_001396038.1	CAGCCCATCTATGACAACCG TCAGCCCAGAAACGAACCTC	152	
ZO-1	XM_015278981.2	CTTCAGGTGTTTCTCTTCCTCCTCTC CTGTGGTTTCATGGCTGGATC	131	
Occludin	NM_205128.1	TCATCGCCTCCATCGTCTAC TCTTACTGCGCGTCTTCTGG	142	
Claudin-1	NM_001013611.2	CTGATTGCTTCCAACCAG CAGGTCAAACAGAGGTACAAG	140	
MT	NM_205275.1	GCAACAACTGTGCCAAGGGC TTTCGTGGTCCCTGTCACCC	138	Li et al. (2015)
Cu,Zn-SOD	NM_205064.1	TTGTTCTGATGGAGATCATGGCTTC TGCTTGCCTTCAGGATTAAAGTGAG	98	
MTF-1	XM_015297695.3	CCTGGTTCAACTCCTATGC TCAAACGGCTTCTCCTTA	278	
A20	XM_003640919.2	GACATCGTGCTAACAGCTTGGA AGAAAAGAGGTATCAGGCACAAC	141	
ZnT1	NM_001389457.1	CTTCGCTTAGCATTTCTT TCTCCGATTTAGTCCTTCT	75	Wu et al. (2020)
DMT1	NM_001128102.2	AGCCGTTCACCACTTATTTCG GGTCCAAATAGGCGATGCTC	129	
Zip8	XM_040671236.1	TGTAAATGTCTCGGTGGG CAAGATGGCTATGGAGGT	159	
Zip14	XM_040689606.1	GTTCTGCCCCGCTGTCCT GGTCTGCCCTCCTCCGTCT	96	
GADPH	NM_204305.1	GCCCAGAACATCATCCCA CGGCAGGTCAGGTCAACA	134	Kong et al. (2023)

TLR4 = toll-like receptor 4; NF-κB p65 = nuclear factor kappa-B p65; ZO-1 = zonula occludens 1; MTF-1 = metal transcription factor-1; ZnT = zinc transporter; Zip = zinc-regulated transporter, iron-regulated transporter-like protein; MT = metallothionein; Cu,Zn-SOD = copper-zinc superoxide dismutase; GADPH = glyceraldehyde-3-phosphate dehydrogenase.

applied to determine differences between groups. Data were considered statistically significantly different when $P < 0.05$.

3. Results

3.1. Inflammatory cytokine levels

The effects of experimental treatments on cytokine levels are shown in Table 2. A significant interaction between Zn-Gly and the LPS challenge was observed in the liver and jejunal cytokines of embryos , where exogenous zinc injection significantly reduced jejunal IL-1β content in challenged embryos ($P = 0.003$). The LPS exposure increased the levels of IL-1β, IL-6 and IFN-γ in the liver ($P < 0.05$), the same trend was found in the jejunum. In addition, the main effect of Zn-Gly was significant, reducing the contents of jejunal IL-1β ($P < 0.001$).

3.2. Antioxidant status

Table 3 demonstrates the effect of Zn-Gly on the antioxidant capacity of the embryonic liver and jejunum as a result of the LPS challenge. The

Table 2
Effects of in ovo feeding of Zinc Glycine on immune parameters (pg/mL) of lipopolysaccharide-challenged embryos.

Item	Liver				Jejunum			
	IL-1β	IL-6	IFN-γ	TNF-α	IL-1β	IL-6	IFN-γ	TNF-α
CON	479.23^{b}	29.07^{b}	49.33^{b}	68.65	512.43^{ab}	25.69^{b}	54.64^{b}	63.02
LPS	542.16^{a}	41.73^{a}	69.21^{a}	63.17	561.53^{a}	35.09^{a}	66.25^{a}	71.26
Zn-Gly	550.94^{a}	36.96^{ab}	52.03^{b}	65.24	466.16^{b}	32.03^{ab}	61.26^{ab}	68.41
Zn-Gly+LPS	522.79^{ab}	39.61^{a}	64.05^{a}	65.68	475.53^{b}	30.45^{ab}	58.94^{ab}	69.96
Pooled SEM	24.98	4.12	2.63	4.52	14.23	1.95	2.45	5.03
Zinc								
No zinc	510.70	35.41	59.27	65.91	536.98	30.39	60.45	67.14
Gly-Zn	536.87	38.29	58.04	65.46	470.85	31.24	60.10	69.19
LPS Challenge								
No Challenge	515.09	33.02	50.68	66.95	489.30	28.86	57.95	65.72
Challenge	532.48	40.67	66.63	64.43	518.53	32.77	62.59	70.61
P value								
Zn-Gly	0.076	0.062	0.286	0.802	< 0.001	0.308	0.811	0.124
LPS	< 0.001	< 0.001	< 0.001	0.166	< 0.001	< 0.001	0.001	0.029
Interaction	0.01	< 0.001	0.002	0.106	0.003	< 0.001	< 0.001	0.339

Values are expressed as the mean pooled SEM (n = 8).
Means in a row with no common superscript are significantly different ($P < 0.05$), SEM = standard error of the mean.
IL-1β = interleukin 1β; IL-6 = interleukin 6; *IFN-γ* = interferon gamma; TNF-α = tumor necrosis factor alpha.

Table 3
Effects of in ovo feeding of Zinc Glycine on oxidative status of lipopolysaccharide-challenged embryos.

Item	Liver				Jejunum			
	CAT	T-AOC	iNOS	MDA	CAT	T-AOC	iNOS	MDA
	pg/mg prot	nmol/mg prot	nmol/mg prot	nmol/mg prot	pg/mg prot	nmol/mg prot	nmol/mg prot	nmol/mg prot
CON	16.14[a]	0.19	0.88	1.58	19.38	0.18	0.23	1.07
LPS	10.69[c]	0.06	0.63	1.9	18.03	0.19	0.34	2.08
Zn-Gly	12.98[b]	0.16	1.26	1.76	17.34	0.19	0.21	2.06
Zn-Gly+LPS	11.31[c]	0.09	0.71	2.55	15.54	0.2	0.26	2.25
Pooled SEM	1.92	0.01	0.18	0.42	2.36	0.03	0.04	0.32
Zinc								
No zinc	13.42	0.13	0.76	1.74	18.71	0.19	0.29	1.58
Gly-Zn	12.15	0.13	0.99	2.16	16.44	0.20	0.24	2.16
LPS Challenge								
No Challenge	14.56	0.18	1.07	1.67	18.36	0.19	0.22	1.57
Challenge	11.00	0.08	0.67	2.23	16.79	0.20	0.30	2.17
P value								
Zn-Gly	0.025	0.896	0.101	0.064	0.262	0.829	0.062	0.057
LPS	0.001	0.001	0.27	0.009	0.428	0.82	0.012	0.033
Interaction	0.003	0.232	0.08	0.244	0.908	0.41	0.313	0.134

Values are expressed as the mean and pooled SEM (n = 8).
Means in a row with no common superscript are significantly different ($P < 0.05$), SEM = standard error of the mean.
CAT = catalase; T-AOC = total superoxide dismutase; iNOS = inducible nitric oxide synthase; MDA = malondialdehyde.

interaction showed that Zn-Gly did not alleviate the effect of LPS challenge on embryonic antioxidant enzyme activity. The main effect showed that the LPS challenge reduced CAT activity and T-AOC levels, and elevated MDA levels in the liver, while contributing to a significant increase in the jejunal iNOS activity and MDA level ($P < 0.05$). Whereas, Zn-Gly did not show a significant positive effect on the antioxidant capacity of embryos.

3.3. Zinc concentration in serum, tibia and tissues

As demonstrated in Fig. 2, Zn-Gly caused a redistribution of zinc in embryonic tibia, serum and tissues under LPS challenge. A significant interaction between Zn-Gly and the LPS challenge on zinc content was observed in the tibia ($P < 0.001$) and small intestine ($P = 0.038$). Moreover, the LPS challenge upregulated the tibial zinc content ($P = 0.04$), while Zn-Gly alleviated the LPS challenge-induced increase in the zinc concentration of the tibia. Both exogenous zinc ($P = 0.014$) and the LPS challenge ($P = 0.037$) resulted in the upregulation of zinc concentrations in the small intestine, while the LPS challenge enhanced the increase in small intestine zinc due to exogenous zinc. In addition, the LPS challenge reduced zinc levels in the serum and liver but increased zinc levels in the thigh meat ($P < 0.05$). Furthermore, Zn-Gly supplementation increased zinc content in the serum ($P = 0.004$).

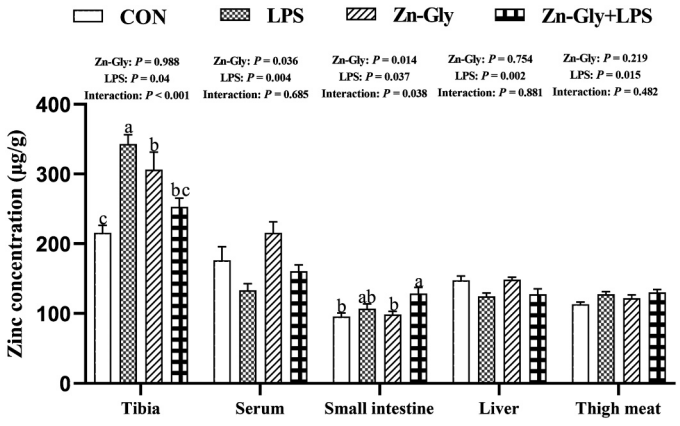

Fig. 2. Effects of in ovo feeding of zinc glycine on zinc concentrations in the tibia, serum and tissues of lipopolysaccharide-challenged embryos. Means with no common superscripts differ significantly ($P < 0.05$).

3.4. Zinc-related enzyme activity and MT

To further clarify the mechanism of zinc redistribution under LPS challenge, ALP activity (Fig. 3A), MT concentration (Fig. 3B), and Cu, Zn-SOD activity (Fig. 3C) were examined in the liver and jejunum. There was a significant interaction between Zn-Gly and LPS challenge on the jejunal ALP activity (P = 0.04), with the LPS challenge attenuating the upregulation of ALP activity by Zn-Gly. Zn-Gly treatment resoundingly in elevated jejunal ($P = 0.018$) and liver ($P = 0.047$) ALP activity, while LPS exhibited a significant negative effect in liver ($P = 0.043$). In contrast, there was no significant interaction between the two factors on the levels of MT in the jejunum and liver ($P > 0.05$), Zn-Gly treatment down-regulated the levels of MT in the jejunum ($P = 0.01$) and liver ($P = 0.003$) tissues, and the LPS challenge showed a similar reduction in the levels of MT in the liver ($P < 0.001$). The effect of Zn-Gly and LPS challenge on Cu,Zn-SOD activity in the embryonic liver caused a significant interaction ($P < 0.001$), with LPS inhibiting the Zn-Gly-induced upregulation of Cu,Zn-SOD activity. In addition, the main effect showed that LPS challenge caused an upregulation of embryonic jejunal Cu,Zn-SOD activity ($P = 0.05$).

3.5. Zinc transport-related gene expression

The regulatory effects of Zn-Gly injection on the major zinc importing and zinc exporting genes in the embryonic liver and jejunum under LPS challenge are shown in Fig. 4A and Fig. 5A respectively. In the liver, a significant interaction between Zn-Gly and the LPS challenge on the gene expression of *ZnT-1*, divalent metal transporter 1 (*DMT1*), and *Zip3* ($P < 0.05$) indicated that Zn-Gly attenuated the enhanced effect of LPS on the liver zinc transport genes *ZnT-1*. While the main effect analysis showed that LPS challenge significantly upregulated the gene expression of embryonic liver *ZnT-1*, *DMT1*, *Zip3* and *Zip8* ($P < 0.05$), exogenous zinc injection similarly upregulated the expression of *DMT1* ($P = 0.017$). In addition, the main effect showed that LPS challenge resulted in elevated expression of the zinc export gene *ZnT-1*, with the zinc import genes *DMT1*, *Zip3*, *Zip8* and *Zip14* reflecting the same trend ($P < 0.05$). In ovo feeding with Zn-Gly similarly upregulated the expression of the zinc export gene *ZnT-1* ($P = 0.05$) and the zinc import gene *DMT1* ($P = 0.017$).

Having demonstrated that Zn-Gly and LPS challenge regulated the embryonic zinc transport-related genes, the embryonic liver and jejunal zinc transport regulatory gene *A20* was also examined, alongside metal transcription factor-1 (*MTF-1*), and zinc concentration markers *MT* and

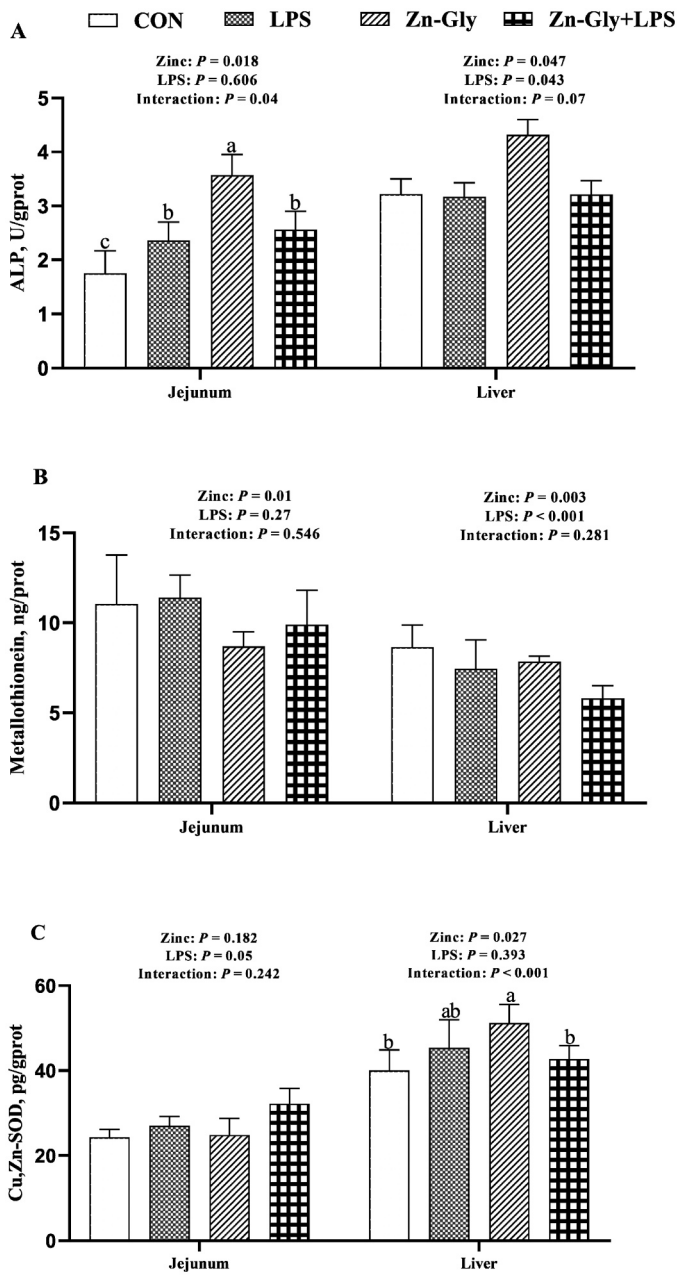

Fig. 3. Effects of in ovo feeding of zinc glycine on ALP activity, metallothionein concentration and Cu,Zn-SOD activity in the jejunum and liver of lipopolysaccharide-challenged embryos. Means with no common superscripts differ significantly ($P < 0.05$). ALP: alkaline phosphatase; Cu,Zn-SOD: copper-zinc superoxide dismutase.

Cu,Zn-SOD gene expression, as shown in Fig. 4B and Fig. 5B. The significant interaction between in ovo feeding with Zn-Gly and the LPS challenge was observed in the liver for the gene expression of *A20*, *MTF-1* and *Cu,Zn-SOD* ($P < 0.05$), while no interaction was observed for this series of gene expression in the jejunum. An upregulation of the liver *A20* gene expression after LPS injection was observed, while Zn-Gly injection suppressed the alteration. Main effects analysis showed that LPS decreased the gene expression of liver *MT* ($P = 0.004$) and upregulated expression of jejunal *MTF-1* ($P = 0.005$) and *Cu,Zn-SOD* ($P = 0.031$). Additionally, Zn-Gly upregulated the gene expression of jejunal *A20* and decreased expression of *MT* ($P < 0.001$).

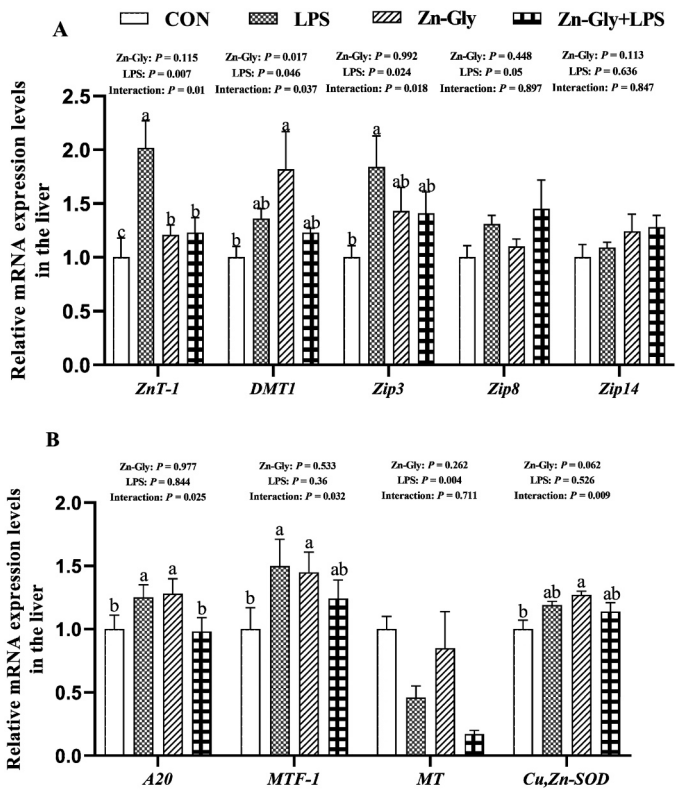

Fig. 4. Effects of in ovo feeding of zinc glycine on the gene expression levels of zinc transporter (A) and zinc regulation-related genes (B) in the liver of lipopolysaccharide-challenged embryos. Means with no common superscripts differ significantly ($P < 0.05$). *ZnT*: zinc transporter; *DMT1*: divalent metal transporter 1; *ZIP*: zinc-regulated transporter, iron-regulated transporter-like protein; *MTF-1*: metal transcription factor-1; *MT*: metallothionein; *Cu,Zn-SOD*: Copper-zinc superoxide dismutase.

3.6. TLR4/NF-κB pathway

Fig. 6A shows that the interaction between Zn-Gly and LPS challenge significantly affected the liver *TLR4* ($P = 0.022$) and nuclear factor kappa-B p65 (*NF-κB p65*) ($P < 0.001$), and *IL-1β* ($P = 0.022$). Embryos in the LPS-treated group had higher levels of liver *TLR4*, *NF-κB* and *IL-1β* gene expression than those of control embryos ($P < 0.05$). The main effect showed that LPS significantly upregulated the gene expression of embryonic liver *TLR4*, *NF-κB p65*, *IL-1β* and *IL-6* ($P < 0.05$).

In the embryonic jejunum, a significant interaction between Zn-Gly and LPS exposure was observed in *TLR4* ($P < 0.001$), *NF-κB p65* ($P = 0.045$), *IL-1β* ($P = 0.05$) and *IL-6* ($P = 0.035$) (Fig. 6B). Zn-Gly significantly increased the expression of *TLR4* and *IL-6* compared to the control group. The gene expression of *TLR4*, *NF-κB p65*, *IL-1β* and *IL-6* was down-regulated in the Zn-Gly+LPS group compared to the LPS group ($P < 0.05$). Furthermore, the significant main effect of LPS in the jejunum was consistent with that seen in the liver, exhibiting upregulation of all pathway genes, while Zn-Gly downregulated gene expression of *TLR4* and *IL-1β*.

3.7. Expression of jejunal barrier genes

Fig. 7 presented the effect of the experimental treatments on the expression of genes responsible for the embryonic jejunal barrier is presented. The interaction between Zn-Gly and LPS challenge significantly affected the gene expression of Claudin-1 ($P = 0.035$). Supplementation with Zn-Gly rescued the reduction in Claudin-1 expression caused by LPS. Main effects analysis showed that LPS challenge downregulated Claudin-1, Occludin and zonula occludens 1 (*ZO-1*) gene

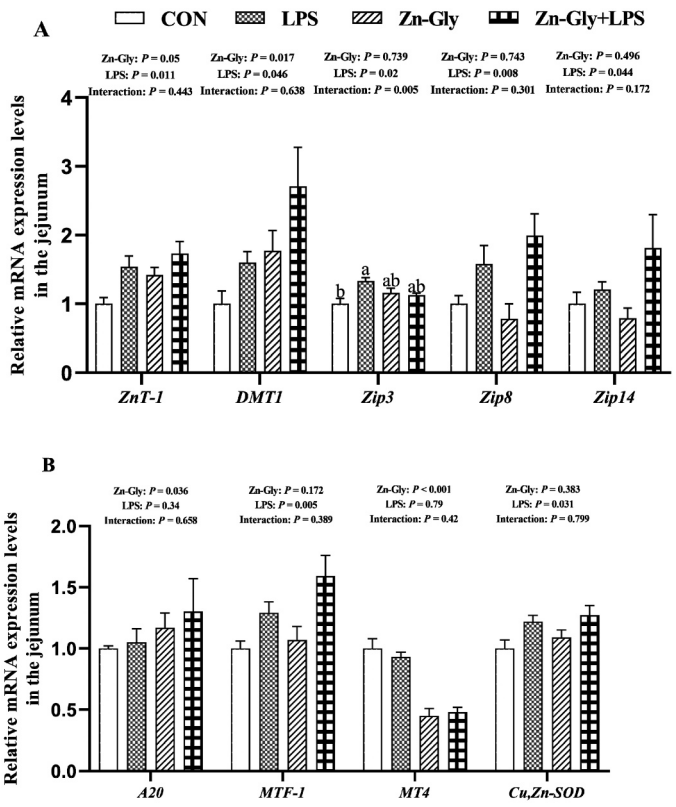

Fig. 5. Effects of in ovo feeding of zinc glycine on the gene expression levels of zinc transporter (A) and zinc regulation-related genes (B) in the jejunum of lipopolysaccharide-challenged embryos. Means with no common superscripts differ significantly ($P < 0.05$). *ZnT*: zinc transporter; *DMT1*: divalent metal transporter 1; *ZIP*: zinc-regulated transporter, iron-regulated transporter-like protein; *MTF-1*: metal transcription factor-1; *MT*: metallothionein; *Cu,Zn-SOD*: Copper-zinc superoxide dismutase.

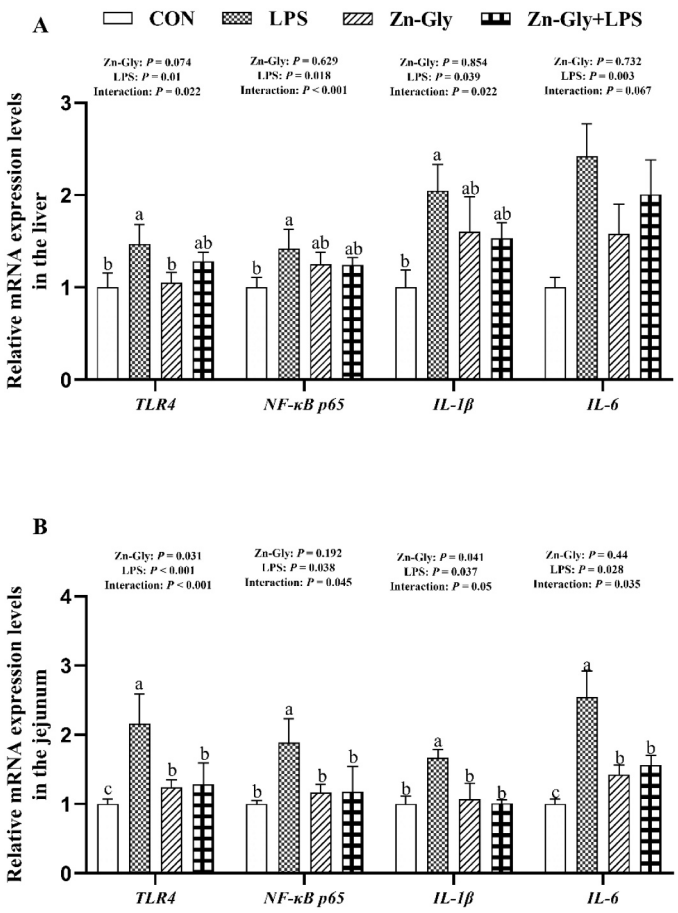

Fig. 6. Effects of in ovo feeding of zinc glycine on the gene expression levels of TLR4 /NF-κB p65 pathway in the liver and jejunum. Means with no common superscripts differ significantly ($P < 0.05$). *TLR4* = toll-like receptor 4; *NF-κB p65* = nuclear factor kappa-B p65.

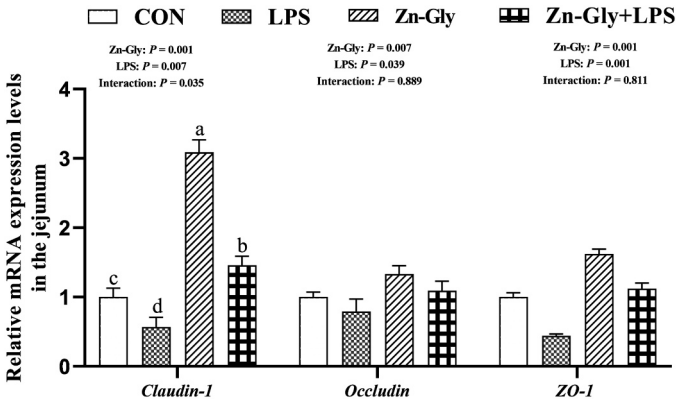

Fig. 7. Effects of in ovo feeding of zinc glycine on the gene expression levels of intestinal barrier genes in the jejunum of lipopolysaccharide-challenged embryos. Means with no common superscripts differ significantly ($P < 0.05$). *ZO-1* = zonula occludens −1.

expression in the embryonic jejunum ($P < 0.05$), while Zn-Gly showed a significant upregulation effect on all these genes ($P < 0.05$). These data suggested that in ovo feeding with Zn-Gly ameliorated the decreased intestinal barrier function due to LPS treatment of avian embryos.

4. Discussion

Past study showed that zinc is a classical type 2 essential trace nutrient consumed frequently by animals and maintained dynamically in their tissues to sustain regular metabolic functions (Kim et al., 2023). Zinc is added to feed to cover nutritional requirements, improve gut health and enhance innate immune defenses in animals, reducing the pro-inflammatory response to infection (Oh et al., 2021, Ogbuewu and Mbajiorgu, 2023). However, the alteration of zinc metabolism in animals undergoing immune challenge and the association of changes with immune regulation have not been explored hitherto. The purpose of this study was to investigate systemic alterations in zinc homeostasis under LPS exposure and the effects of zinc-glycine on innate immunity in broiler embryos.

Research has shown that the integrity of the intestinal barrier plays an important role in protecting animals from pathogens (Camilleri, 2019). By decreasing the expression of tight junction proteins, LPS affects intestinal permeability and impairs intestinal barrier function (Pothuraju et al., 2018). Moreover, LPS exposure leads to impaired intestinal barrier function whereupon it invades the circulatory system before circulating through the hepatic portal system to reach the liver. As a result, this exposure triggers a hepatic inflammatory response and activates the production of inflammatory cytokines IL-1β, IL-6, IFN-γ, and TNF-α (Arab et al., 2018). In this study, LPS exposure caused downregulation of the expression of the gut barrier genes Claudin-1, Occludin, and *ZO-1*, and upregulation of the levels of immune factors such as IL-1β, IL-6, and IFN-γ in the embryonic liver and jejunum, which is similar to previous findings (Bavananthasivam et al., 2019). Zinc acts as a second messenger for immune cells not only to regulate cell-mediated immune function but also as an antioxidant and anti-inflammatory agent (Prasad, 2013). It has been shown to

significantly reduce the levels of the inflammatory factors IL-1β, IL-6 and TNF-α to block the production of cytokine storms (Wu et al., 2022). Deficiency in zinc is known to induce the secretion of IL-1β through activation of macrophage NLRP3 (NOD-, LRR- and pyrin domain-containing 3) inflammasomes followed by inflammatory response (Summersgill et al., 2014). We demonstrated that Zn-Gly reversed the up-regulation of pro-inflammatory factor IL-1β in the jejunum and enhanced the expression levels of barrier genes under LPS challenge. This would suggest that Zn-Gly is capable of attenuating the aforementioned inflammatory response by protecting against the potential loss of intestinal tight junction proteins as a result of LPS exposure.

The toll-like receptor family is the initiator of innate immunity, and TLR4 is the most characteristic member of the family that recognizes lipopolysaccharides and subsequently triggers a series of cellular signals ultimately activating the NF-κB signaling pathway (Doyle and O'Neill, 2006). It regulates the inflammatory response by binding to nucleotide sequences upstream of pro-inflammatory cytokines and chemokine genes (Zhang et al., 2017). We confirmed the activation of the embryonic TLR4/NF-κB pathway by LPS, which is consistent with previous work (Xiong et al., 2022). Zn-Gly reversed the upregulation of *TLR4* and *NF-κB p65* expression induced by LPS exposure and modulated the expression of the pro-inflammatory factors *IL-1β* and *IL-6*. This suggests that Zn-Gly alleviates the LPS-induced inflammatory response through the TLR4/NF-κB pathway. However, the NF-κB signaling pathway also induced the zinc import protein *Zip8* to promote an increase in intracellular zinc levels, which in turn inhibited the release of *IκB* kinase to counteract oxidative stress (Liu et al., 2013). IL-6 was also shown to upregulate the expression of *Zip14* in the liver in murine models to mobilize free zinc to counteract inflammatory responses (Liuzzi et al., 2005). Weakened *NF-κB p65* expression due to zinc supplementation has also been shown to be associated with improved intestinal barrier function (Chen et al., 2021). Thus, Zn-Gly regulates the LPS-induced inflammatory responses and protects against impairment of intestinal barrier function due to LPS exposure via the TLR4/NF-κB pathway, as evidenced by its involvement in zinc homeostasis remodeling and inhibition of proinflammatory cytokines.

Stimulation of cells by LPS induces excess production of reactive oxygen species (ROS) by the NADPH oxidase system, which activates the antioxidant system and regulates the expression of antioxidant enzymes (Zhang et al., 2019, Yang et al., 2020). ROS production is a double-edged sword that both kills invading pathogens and causes oxidative damage to tissues during the inflammatory response (Lauridsen, 2019). The occurrence of oxidative stress represents a disruption in the balance between antioxidant and pro-oxidant factors in animals (Li et al., 2017). MDA and iNOS are biomarkers of lipid damage caused by the oxidative stress (Al-Hakeim et al., 2023). For the purpose of this study, LPS exposure up-regulated the levels of MDA in the liver and jejunum as well as the jejunal iNOS. The activation of the antioxidant enzyme system is essential to maintain oxidative stress homeostasis and LPS caused a decrease in the liver antioxidant enzymes of CAT and T-AOC (Forman and Zhang, 2021). In agreement with previous findings in birds, this study indicates that LPS challenge induces oxidative stress (Pang et al., 2023). By stabilizing protein sulphydryl structures to avoid oxidation and engaging in metal-catalyzed reactions, zinc participates in antioxidant processes to ensure inhibition of inflammation and oxidative stress before cytokine storms are induced (Nakamura et al., 2019, Liao et al., 2021). In the present study, exogenous zinc didn't show a positive effect on the antioxidant capacity of the embryos, which may be related to the stage at which the oxidative reaction occurs.

An acute inflammatory or stress status of the organism is accompanied by a loss of homeostasis of essential ions such as potassium, calcium, selenium and zinc, which affects the efficiency of the metalloenzyme antioxidant defense system against oxidative stress (Borkowski et al., 2011). The inflammatory response due to an LPS challenge is known to cause hypozincemia, due to the coordinated ectopic movement of zinc ions in the blood towards damaged tissues to increase free zinc ions inside cells to combat inflammation (Kirsten et al., 2015). Nutritional interventions have been found to be an effective addition to pharmacological treatment of acute inflammation (Chua et al., 2012). In previous studies, low blood zinc levels have also been observed in broilers challenged with *Salmonella* or *Coccidioides* (He et al., 2019, Wu et al., 2020). The jejunum is the main site of zinc absorption, with zinc being absorbed by the intestine through transport protein-mediated ion channels and entering the portal venous system to the liver (Kambe et al., 2015, Hennigar et al., 2022). Benefiting from the abundant zinc reserves and the rapidity of its ion exchange capacity, the liver is crucial in the regulation of zinc homeostasis (Stamoulis et al., 2007). After 12 hours of LPS challenge, zinc was redistributed across embryonic tissues, with reduced zinc levels in the serum and liver in contrast to increased zinc levels in intestinal and muscle tissues as well as increased zinc deposition in the tibia. The expression of the liver zinc export gene *ZnT-1* and the import genes *DMT1*, *Zip3* and *Zip8* was up-regulated and a similar trend was identified in the jejunum. As the exogenous zinc was injected, the expression of the liver and jejunum zinc import gene *DMT1* and the jejunum zinc export gene *ZnT-1* were also up-regulated and the gene expression of jejunum *MT* was down-regulated. The administration of Zn-Gly and LPS had a mutually suppressive effect on embryonic zinc transporter *ZnT-1* gene expression. It has been suggested that embryos in an immune-challenged state increase the rate of zinc transport through the regulation of zinc transport genes and metallothionein leading to a remodeling of zinc homeostasis, with zinc supplementation alleviating this trend.

Zinc homeostasis is directly regulated by zinc transporter proteins and MT, with cysteine-rich MT acting as intracellular zinc buffers to maintain the normal concentration (Jarosz et al., 2017). Theoretically, zinc intake leads to an increase in MT level of tissue cells, and the increased MT inhibits zinc uptake through signaling. It appears that the MT level is not the only marker of zinc homeostasis, as MT undergoes complex signaling, receiving both metal-regulated transcription factor (MTF-1), which regulates zinc concentration signaling, and nuclear factor E2-related factor 2 (Nrf2), which regulates the redox pathway (Hübner and Haase, 2021). A member of the nucleic acid exonuclease family, ALP, is an enzyme containing two zinc ions and has the function of dephosphorylating endotoxins to attenuate biotoxicity (Zaher et al., 2020). Cu,Zn-SOD enzyme is an antioxidant enzyme directly involved in the resistance of zinc to oxidative stress (Qi et al., 2019). The present study demonstrated that an LPS challenge down-regulated the liver MT and ALP levels and caused an up-regulation of jejunum Cu,Zn-SOD level in terms of the protein content and gene expression levels, suggesting that LPS exposure induced the release of free zinc from liver MT, which together with ALP resisted the inflammatory response induced by LPS. The jejunum, as an important immune organ, is also involved in the antioxidant process through the enzyme Cu,Zn-SOD. Zn-Gly increased ALP levels in the embryonic liver and jejunum and down-regulated MT levels, suggesting that more zinc was mobilized for the body's defense mechanisms, which was also reflected in the inhibition of LPS-induced increases in Cu,Zn-SOD levels by Zn-Gly.

Through the overall regulation of ubiquitin-dependent signaling pathways, zinc finger protein A20 (TNFAIP3) might serve as a susceptibility gene for inflammatory diseases (Li et al., 2016). A20 regulates inflammatory factors by regulating zinc concentration, with zinc supplementation having been shown to reduce inflammatory factor gene expressions by regulating *A20* level (Shembade and Harhaj, 2012, Maares and Haase, 2016). As an upstream gene of *MT*, *MTF-1* also modulates *MT* expression and thus participates in the anti-inflammatory response of the body by sensing zinc concentration (Jia et al., 2021). The present study reveals the effects of LPS exposure and zinc supplementation on these two zinc-sensitive genes. The imbalance in zinc homeostasis induced by LPS challenge leads to elevated embryonic liver *A20* and *MTF-1* expression, while Zn-Gly supplementation inhibited the adjustment of *A20* gene expression. A study in rats demonstrated that

zinc was capable of preventing inflammatory responses Inhibiting the NF-B pathway through upregulation of A20, while MTF-1 modulates MT to regulate zinc concentration through its modulation (Yan et al., 2016, Chen et al., 2020). Thus, the reconfiguration of zinc homeostasis and the treatment of Zn-Gly against LPS challenge-induced inflammatory responses are inextricably linked to two zinc signaling sensing factors, A20 and MTF-1.

5. Conclusion

In conclusion, this study demonstrated that the systemic zinc homeostasis in broiler embryos was modulated by immune challenge and the positive effects of exogenous zinc. In response to an LPS challenge, zinc transporter proteins and MT were involved in the remodeling of host zinc homeostasis. Zn-Gly mediated the inflammatory response and impairment of intestinal barrier function induced by LPS exposure by participating in the regulating of zinc homeostasis and inhibiting the production of pro-inflammatory cytokines via the TLR/NF-κB signaling pathway.

Author contributions

CX, ZS conceived and designed the experiments and wrote the paper. XP and LK performed the experiments and analyzed the data. LC, NE and MS provided advice and revised the article. All authors have read and approved the final manuscript.

CRediT authorship contribution statement

Schroyen Martine: Supervision, Writing – review & editing. **Song Zhigang:** Funding acquisition, Project administration, Supervision. **Pan Xue:** Data curation, Methodology. **Everaert Nadia:** Supervision. **Xiao Chuanpi:** Investigation, Writing – original draft, Writing – review & editing. **Comer Luke:** Data curation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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