

Effects of different selenium sources on duodenum and jejunum tight junction network and growth performance of broilers in a model of fluorine-induced chronic oxidative stress

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ABSTRACT The protective effects and underlying molecular mechanisms of sodium selenite (**SS**) and selenomethionine (**SM**) against chronic oxidative stress-induced duodenum and jejunum tight junction (**TJ**) network disturbance and growth inhibition of broilers were investigated in the current experiment. At the age of 1 d, 720 Lingnan Yellow broiler chicks were allocated to 4 experimental diets (with 6 replicates per diet and 30 birds per replicate) and offered either a control diet (fluorine [**F**] 23 mg/kg, control [**CoN**] group) or test diets (800 mg/kg F, high F [**HF**] group; 800 mg/kg F +0.15 mg selenium [**Se**]/kg as SS [**SS group**] or SM [**SM group**]) for 56 d. The results showed that HF group could induce chronic oxidative stress and subsequently increased ($P < 0.05$) proinflammatory cytokines levels of duodenum and jejunum in comparison with the CoN group. Increased proinflammatory cytokines levels of HF group promoted myosin light chain kinase (**MLCK**) transcription, thus leading to a decrease ($P <$

0.05) in TJ proteins expression of duodenum and jejunum when compared with the CoN group. A reduction of TJ proteins expression destroyed the TJ structures in the HF group, which in turn increased intestinal mucosal permeability of duodenum and jejunum and ultimately induced growth inhibition of broilers. Dietary Se supplementation could ameliorate HF-induced duodenum and jejunum TJ network impairment and growth retardation of broilers, potentially by increasing ($P < 0.05$) the glutathione peroxidase and thioredoxin reductase activities, reducing ($P < 0.05$) the reactive oxygen species and malondialdehyde levels, regulating the secretion of proinflammatory cytokines, and mediating the transcription level of MLCK in the duodenum and jejunum. Additionally, our data also suggested that the protective effects of SM were superior to those of SS. This study will provide a theoretical basis for developing SM into an efficient protective agent for intestinal mucosal barrier in poultry.

Key words: broiler, chronic oxidative stress, selenomethionine, sodium selenite, tight junction network

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INTRODUCTION

Tight junction (**TJ**) is the main connection between intestinal epithelial cells, which plays a key role in regulating intestinal mucosal permeability and preventing bacteria, endotoxin and toxic macromolecules from entering the bloodstream (Lee et al., 2018). Destruction of TJ can increase intestinal mucosal permeability, which causes noxious molecules to enter the bloodstream from the intestinal lumen via the paracellular pathway

and eventually lead to increased intestinal or systemic diseases (Awad et al., 2017). The TJ is a multiprotein complex and composed of the transmembrane proteins (such as Claudin family, Occludin, and junctional adhesion molecules) and the cytoplasmic proteins (such as Zona Occludens-1, 2, 3 [**ZO-1**, **ZO-2**, and **ZO-3**]) (Liang and Weber, 2014; Awad et al., 2017). A reduction of TJ proteins expression can destroy the TJ structures and subsequently result in increased intestinal mucosal permeability (Tian et al., 2020).

Oxidative stress is a state in which the balance between oxidation and anti-oxidation is broken, resulting in superfluous reactive oxygen species (**ROS**) formation (Sinha and Dabla, 2015). In the poultry industry, there are many factors, such as feed quality, debeaking, immunization, temperature, density, and transport, which can

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lead to oxidative stress of broilers easily (Fisinin et al., 2016; Surai and Fisinin, 2016). Intestinal tract is a central target site of oxidative stress due to its special vascular anatomical characteristics and convective oxygen exchange mechanism. Some researchers reported that oxidative stress could increase intestinal mucosal permeability by reducing the expression of TJ proteins in broilers (Uerlings et al., 2018; Zhang et al., 2020).

Selenium (Se) can scavenge ROS indirectly by locating the active center of antioxidant selenoproteins such as glutathione peroxidase (Gpx) and thioredoxin reductase (TrR) (Guillin et al., 2019). Selenium is fed to animals either as inorganic Se or as organic Se, and sodium selenite (SS) is a common inorganic Se additive (Rayman, 2020). Selenomethionine (SM) is an organic Se source, which has been shown to have higher antioxidant capacity, absorptivity and bioavailability as well as lower toxicity than SS (Falk et al., 2020). Our previous research has shown that SM was more effective than SS in alleviating diquat-induced acute oxidative stress in the liver of chicken embryos (Li et al., 2020). Besides, a study on bovine mammary epithelial cells has found that SM was superior to SS in antagonizing oxidative stress induced by hydrogen peroxide (Sun et al., 2020a). However, little information is available in the literature regarding the effects of SM and SS on TJ network in the intestines of broilers during oxidative stress-inducing conditions.

Fluorine (F) is one of the most oxidizing substances, which can easily cross the cell membrane by simple diffusion. Then, it attack oxygen to produce excessive ROS and induce oxidative stress (Song et al., 2017). Our previous study has demonstrated that high F (HF) intake could increase ROS production and induce oxidative stress in the liver and kidney of broilers (Wang et al., 2018). Therefore, it seems to be a reasonable assumption that exposure to HF might induce oxidative stress in the intestines of broilers. Thus, the same HF-induced chronic oxidative stress model was used in this study to investigate the effects of SM and SS on oxidative stress parameters, proinflammatory cytokines levels, myosin light chain kinase (MLCK) mRNA levels, TJ proteins expression, TJ structures, intestinal mucosal permeability in the duodenum and jejunum as well as growth performance of broilers. This study will provide a theoretical basis for future studies which focus on the effect of SM on the intestinal barrier function in poultry.

MATERIALS AND METHODS

The experimental procedures were approved by the Committee for the Animal Care and Use of Zhejiang A & F University, which uses Animal Care and Use guidelines to manage all animals' use in the experimental procedures.

Feeding Experiment

A total of 720 one-day old healthy Lingnan yellow broiler chicks were provided by Qunda Livestock

Breeding Co., Ltd., (Jiaxing, Zhejiang, China) and randomly assigned to one of 4 groups with 6 replicates per group and 30 broilers per replicate (half male and half female). The control (CoN) group was fed with the basal diet; the HF group was given the diet identical to the CoN group except for adding 800 mg F/kg as sodium fluoride (Shanghai Aladdin Bio-Chem Technology Co., Ltd., Shanghai, China; Cat: S111591). The SS and SM groups were added with 0.15 mg Se/kg as SS (Sinopharm Chemical Reagent Co., Ltd., Beijing, China; Cat: 80118660) or SM (Beijing InnoChem Science & Technology Co., Ltd., Beijing, China; Cat: 259,960,000) based on the HF group. According to NRC (1994), the nutrient levels in the basal diets were sufficient for broilers except for Se (Table 1). The basal and experimental diet's Se and F concentrations were detected by atomic fluorescence spectroscopy and F ion-selective electrodes, respectively. Six replicates were done for each feed sample. The measured values of Se and F were shown in Table 2.

The broilers were kept in 24 plastic mesh pens (dimensions: 1.5 m × 1.5 m × 2.0 m). In the first week, the

Table 1. The formulation and nutrient composition of the basal diet¹.

Items	Starter (d 1 to 21)	Grower (d 22 to 56)
Ingredients (%)		
Corn	56.00	61.00
Wheat middlings	3.00	4.00
Extruded soybean	5.50	3.00
Soybean meal (43% CP)	26.00	21.00
Corn gluten meal	5.00	6.00
Soybean oil	0.00	1.00
Monocalcium phosphate	1.75	1.30
Limestone	1.40	1.35
Salt	0.25	0.25
L-Methionine	0.10	0.00
L-Lysine HCl	0.00	0.10
Vitamin-mineral premix ²	1.00	1.00
Analyzed nutrient composition ³		
Metabolizable energy ⁴ (Kcal/kg)	2,897.86	2,979.08
Crude protein (%)	20.97	19.06
Crude fat (%)	3.80	4.49
Crude ash (%)	6.89	6.18
Lysine (%)	1.05	0.96
Methionine (%)	0.49	0.37
Methionine + cysteine (%)	0.85	0.70
Calcium (%)	1.02	0.88
Total phosphorus (%)	0.65	0.58
Nonphytate phosphorus (%)	0.44	0.36

¹The optimal treatment level was reached by adding 800 mg fluoride/kg as sodium fluoride (Shanghai Aladdin Bio-Chem Technology Co., Ltd., Shanghai, China; Cat: S111591) and 0.15 mg selenium/kg as sodium selenite (Sinopharm Chemical Reagent Co., Ltd., Beijing, China; Cat: 80118660) or selenomethionine (Beijing InnoChem Science & Technology Co., Ltd., Beijing, China; Cat: 259,960,000) to the diets at the cost of corn.

²Premix is provided per kg of diet: 9,600 IU vitamin A (all trans-retinol acetate); 3.0 mg vitamin K (menadione sodium bisulfate); 36 IU vitamin E (DL- α -tocopheryl acetate); 3.0 mg thiamin (thiamin mononitrate); 2,700 IU cholecalciferol; 10.5 mg riboflavin; 4.2 mg pyridoxine; 0.03 mg cobalamin; 60 mg niacin; 18 mg d-calcium pantothenate; 1.5 mg folic acid; 0.225 mg d-biotin; 1,000 mg choline (choline chloride); 80 mg iron (FeSO₄•7H₂O); 8 mg copper (CuSO₄•5H₂O); 80 mg manganese (MnSO₄•H₂O); 60 mg zinc (ZnSO₄•7H₂O); 0.35 mg iodine (KI).

³Analytical values were derived from 6 determinations.

⁴The data of metabolizable energy was calculated and organized into the table.

Table 2. Fluorine and selenium contents in experimental diets¹.

	F content ² (mg/kg)		Se content ² (mg/kg)	
	Starter (d 1 to 21)	Grower (d 22 to 56)	Starter (d 1 to 21)	Grower (d 22 to 56)
CoN	20.06	18.88	0.047	0.049
HF	819.82	816.16	0.048	0.046
SS	815.32	810.39	0.198	0.201
SM	824.23	813.58	0.203	0.199

¹Abbreviations: CoN, control group; F, fluorine; HF, high fluorine group; Se, selenium; SS, sodium selenite group; SM, selenomethionine group.

²Values are presented as means (n = 6).

brooding temperature was maintained at $34 \pm 1^\circ\text{C}$, after which the temperature was gradually reduced to 22°C by 2 to 3°C per week. Birds were free to experimental diets and fresh water. Mortality and temperature were recorded on a daily basis.

Growth Performance

Total feed intake for each replicate was recorded once a week. At 21 and 56 d of age, body weight was measured after fasting for 12 h, and pens were considered as replicates. Then, based on the above data, average daily gain (ADG), average daily feed intake (ADFI), and feed to gain (F/G) were calculated. When death occurred, ADFI and F/G were corrected for mortality.

Sample Collection

At the age of 56 d, 48 male broilers were randomly selected (2 from each replicate) and blood samples were collected from the main wing vein in heparinized tubes. The serum was separated by centrifuging at 3,000 rpm for 10 min at 4°C and then stored at -80°C for further analysis. The chickens were narcotized with sodium pentobarbital (50 mg/kg body weight) and autopsied after blood was collected. The mid-duodenal and mid-jejunal samples were quickly removed and opened lengthwise. Then the mucosal samples were collected and used for further analysis.

Sample Preparation

Fresh duodenum and jejunum mucosa samples (0.5 g) were added with 4.5 mL ice-cold phosphate buffer saline (PBS) and homogenized in an ice bath. The homogenate was centrifuged at 4°C for 10 min at 4,000 rpm to obtain the supernatant for the determination of ROS, malondialdehyde (MDA), Gpx, TrR, interleukin-6 (IL-6), interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ) levels.

Oxidative Stress Parameters Analysis

The levels of MDA (Cat: A003-1-2), Gpx (Cat: H545-1), and TrR (Cat: A119-1-1) in the duodenum and jejunum were evaluated with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) and the procedures were followed as provided by

the manufacturer. The levels of ROS (Cat: CSB-EQ027740CH) in the duodenum and jejunum were evaluated using an enzyme-linked immunoassay kit specifically for chicken from Cusabio Biotech Co., Ltd., (Wuhan, Hubei, China).

Inflammatory Cytokines Assays

Test kits used for the determination of IL-6 (Cat: SEA079Ga), TNF- α (Cat: SEA133Ga), and IFN- γ (Cat: SEA033Ga) levels were obtained from Cloud-Clone Corp. (Wuhan, Hubei, China), and IL-1 β (Cat: CSB-E11230Ch) was from Cusabio Biotech Co., Ltd., while the procedures were carried out in accordance with the supplier's instructions.

Intestinal Mucosal Permeability Assay

The intestinal mucosal permeability was assessed by measuring the serum diamine oxidase (DAO), D-Lactic acid and endotoxin levels. The levels of DAO (Cat: A088-1-1) and D-Lactic acid (Cat: H263) were measured by commercial kits (Nanjing Jiancheng Bioengineering Institute). Limulus amoebocyte lysate test (Xiamen Bioendo Technology Co., Ltd., Xiamen, Fujian, China) was used to detect the serum endotoxin level.

Ultrastructural Observation

The duodenum and jejunum tissues were cut open longitudinally and rinsed with cold PBS gently. Then approximately 1 mm³ samples were cut from the middle of the intestinal segment and placed into 2.5% glutaraldehyde for fixing 48 h at room temperature. After being washed 3 times with PBS, the samples were fixed in 1% osmic acid for 2 h. Dehydration was performed with graded alcohols, and the samples were embedded in epoxy resin. The ultrathin cut sections were stained with uranyl acetate and lead citrate before examination under a transmission electron microscope of Hitachi H-7650 (TEM H-7650, Hitachi Ltd., Tokyo, Japan).

Real-Time PCR

Following the manufacturer's protocol, total RNA from duodenum and jejunum mucosa samples was isolated using RNA isolated Plus (Takara Biotechnology Co., Ltd., Tokyo, Japan; Cat: 9109). RNA concentration and purity

Table 3. Primer sequences used in quantitative real-time PCR¹.

Target gene	GeneBank accession. no.	Primer sequence (5'→ 3')	Product size (bp)
Claudin-1	NM_001013611.2	F: CTGATTGCTTCCAACAG R: CAGGTCAAACAGAGGTACAAG	140
Occludin	NM_205128.1	F: CTGCTCTGCCTCATCTGCTTCTT R: CGGTCCCAGTAGATGTTGGCTTT	83
ZO-1	XM_015278975.1	F: CTCAGGTGTTTCTCTTCTCCTC R: CTGTGGTTTCATGGCTGGATC	131
β -actin	NM_205518.1	F: CTGACTGACCGCGTTACTCC R: TTGCACATACCGGAGCCATT	84
MLCK	NM_001322361.1	F: CTGAACAGCTTGATTTCCGTGACA R: GCTCGGCTGGGATTTCTTTC	90

¹Abbreviations: F, forward; MLCK, myocin light chain kinase; R, reverse; ZO-1, Zona Occludens-1.

were determined by Nanodrop technology. According to the supplier's protocol, 1.0 μ g RNA of each sample was used for synthesizing the first-strand cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA; Cat: 1622). Gene-specific primers of ZO-1, Claudin-1, Occludin, MLCK, and β -actin were designed by Primer Premier Software 6.0 (Premier Biosoft International, Palo Alto, CA) and synthesized by Sangon Biotech (Shanghai, China), as listed in Table 3. Real-Time PCR was performed on the Applied Biosystems 7500 Real-Time PCR (RT-PCR) System (Applied Biosystems, Foster City, CA). The reaction was carried out in a 25 μ L reaction volume using Power SYBR Green PCR Master Mix (Applied Biosystems; Cat: 4367659).

Each sample was to be checked in triplicate. The thermo-cycling conditions included denaturation at 95°C for 30 s, followed by 40 cycles (95°C for 15 s, 60°C for 60 s). The mRNA expressions were calculated by the $2^{-\Delta\Delta C_t}$ method and β -actin was used as the housekeeping gene. Data were expressed as one relative level to the CoN value.

Western Blot Analysis

Proteins from duodenum and jejunum mucosa samples were extracted using RIPA buffer (Beyotime Biotechnology, Shanghai, China; Cat: P0013B) containing halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific; Cat: 78445). Bicinchoninic acid assay kit from Beyotime Biotechnology (Shanghai, China; Cat: P0010) was used to detect the total protein concentration. After being mixed with 5 $\times$ SDS loading buffer (Beyotime Biotechnology; Cat: P0015L) and denatured at boiling water for 5 min, the dilute protein solutions (containing 60 μ g protein) were separated on the 10% SDS-PAGE gels and then transferred to polyvinylidene fluoride membrane (Millipore, Boston, MA; Cat: IPVH00010). After being blocked in a commercial blocking buffer (Beyotime Biotechnology; Cat: P0252), the membranes were incubated overnight at 4°C with primary antibody against ZO-1 (1:800; Thermo, Waltham, MA; Cat: 61-7300), Occludin (1:1,000; Thermo; Cat: 40-4700), Claudin-1 (1:1,000; Thermo; Cat: 51-9000) and β -actin (1:1,500, Santa Cruz Biotechnology, Santa Cruz, CA; Cat: SC-47778). After being washed 4 times with TBST, the membranes were

incubated with secondary antibody: goat anti-rabbit (1:5,000; Thermo; Cat: 31210) or goat anti-mouse IgG antibodies (1:5,000; Thermo, Waltham, MA; Cat: 31160) at room temperature for 1 h with TBST 5 times at 5 min intervals. The protein bands were detected with an enhanced chemiluminescence kit (Beyotime Biotechnology) and visualized on X-ray films (Kodak, Rochester, NY). Optical intensity was quantified with ImageJ software (ImageJ, 1.47v, NIH, Bethesda, MD). The relative protein expression was standardized with β -actin.

Statistical Analysis

The data were analyzed using the SPSS 22.0 software (SPSS Inc., Chicago, IL) and Graph Pad 8.0 Software (Graph Pad Software Inc., San Diego, CA). Unidirectional variance analysis (ANOVA) followed by Duncan's multiple range tests were used to compare means between different treatments, and differences were taken as significant different when $P < 0.05$. The number of chickens were usually thought of as an experimental unit except for growth performance. The resulting data were expressed as the mean $\pm$ standard deviation in the figure or as the mean and pooled standard error of mean in the Table 4.

RESULTS

Oxidative Stress Parameters

The effects of different Se sources on oxidative stress parameters in the intestines of broilers were shown in Figure 1. High fluorine group elevated ($P < 0.05$) ROS and MDA levels and decreased ($P < 0.05$) TrR and Gpx activities in the duodenum and jejunum in comparison with the CoN group. The addition of Se effectively reduced ($P < 0.05$) the levels of ROS and MDA and improved ($P < 0.05$) the activities of TrR and Gpx as compared to the HF group (except for jejunum TrR activity in the SS group and jejunum Gpx activity in the SM group). When compared the two groups plus Se, we found that SM group had lower ($P < 0.05$) duodenum and jejunum ROS levels as well as duodenum MDA level. In regard to TrR and Gpx activities, no significant differences ($P > 0.05$) in TrR activities were found between the SM and SS groups in the duodenum and jejunum. However, the addition of SS increased ($P < 0.05$) jejunum

Table 4. Effects of different selenium sources on growth performance of broilers in a model of fluorine-induced chronic oxidative stress^{1,2}.

Items	CoN	HF	SS	SM	SEM	P-value
1 to 21 days of age						
Initial weight (g)	41.33 ^a	41.37 ^a	41.50 ^a	41.07 ^a	0.098	<0.001
ADG (g/d)	15.84 ^a	13.42 ^c	14.60 ^b	15.06 ^b	0.276	<0.001
ADFI (g/d)	27.25 ^a	25.44 ^b	26.40 ^{ab}	26.61 ^{ab}	0.292	0.170
F/G (g/g)	1.72 ^c	1.90 ^a	1.81 ^b	1.77 ^{bc}	0.022	0.005
22 to 56 days of age						
ADG (g/d)	45.73 ^a	41.06 ^c	42.71 ^b	44.69 ^a	0.588	0.001
ADFI (g/d)	103.98 ^a	107.86 ^a	106.02 ^a	104.23 ^a	0.831	0.351
F/G (g/g)	2.27 ^c	2.63 ^a	2.48 ^b	2.33 ^c	0.044	<0.001
1 to 56 days of age						
ADG (g/d)	34.52 ^a	30.70 ^c	32.17 ^b	33.58 ^a	0.459	<0.001
ADFI (g/d)	75.13 ^a	76.65 ^a	76.07 ^a	75.05 ^a	0.513	0.699
F/G (g/g)	2.18 ^c	2.50 ^a	2.37 ^b	2.24 ^c	0.040	<0.001

¹Values are presented with the means and pooled standard error of mean, n = 6/group.

²Abbreviations: ADG, average daily gain; ADFI, average daily feed intake; CoN, control group; F/G: feed/gain; HF, high fluorine group; SS, sodium selenite group; SM, selenomethionine group.

^{a,b,c}In the same line, different superscript letters represent significant difference ($P < 0.05$), the same superscript letters represent no significant difference ($P > 0.05$).

Gpx level more than the SM supplementation, and duodenum Gpx level was not affected ($P > 0.05$) by Se source.

Proinflammatory Cytokines Levels

According to Figure 2, we found that the levels of IL-1 β , IL-6, TNF- α , and IFN- γ in the duodenum and

jejunum were higher ($P < 0.05$) in the HF group than those in the CoN and Se added groups (except for duodenum and jejunum IL-6 as well as duodenum IFN- γ levels in the SS group). Compared with the SS group, SM group had lower ($P < 0.05$) duodenum and jejunum IL-1 β , IL-6, TNF- α , and IFN- γ levels (except for duodenum IL-6 and jejunum IL-1 β levels).

MLCK mRNA Expression

According to Figure 3, based on the comparison with the CoN group, it showed that HF group greatly increased ($P < 0.05$) MLCK mRNA levels in the duodenum and jejunum. Compared with the HF group, Se added groups reduced ($P < 0.05$) duodenum and jejunum MLCK mRNA levels, while the values of SM group were lower ($P < 0.05$) than those of SS group.

The Expression of TJ Proteins

In comparison with the CoN group, the duodenum and jejunum Claudin-1, Occludin and ZO-1 mRNA and protein levels in the HF group were decreased ($P < 0.05$; Figure 4). Compared with the HF group, SM group increased ($P < 0.05$) the expression of Claudin-1, Occludin, and ZO-1 at mRNA and protein levels in the duodenum and jejunum, SS group only improved ($P < 0.05$) Claudin-1 and ZO-1 mRNA levels in the duodenum. The expressions of Claudin-1, Occludin, and ZO-1 at mRNA and protein levels in the jejunum and duodenum in the SM group were higher ($P < 0.05$) than those in

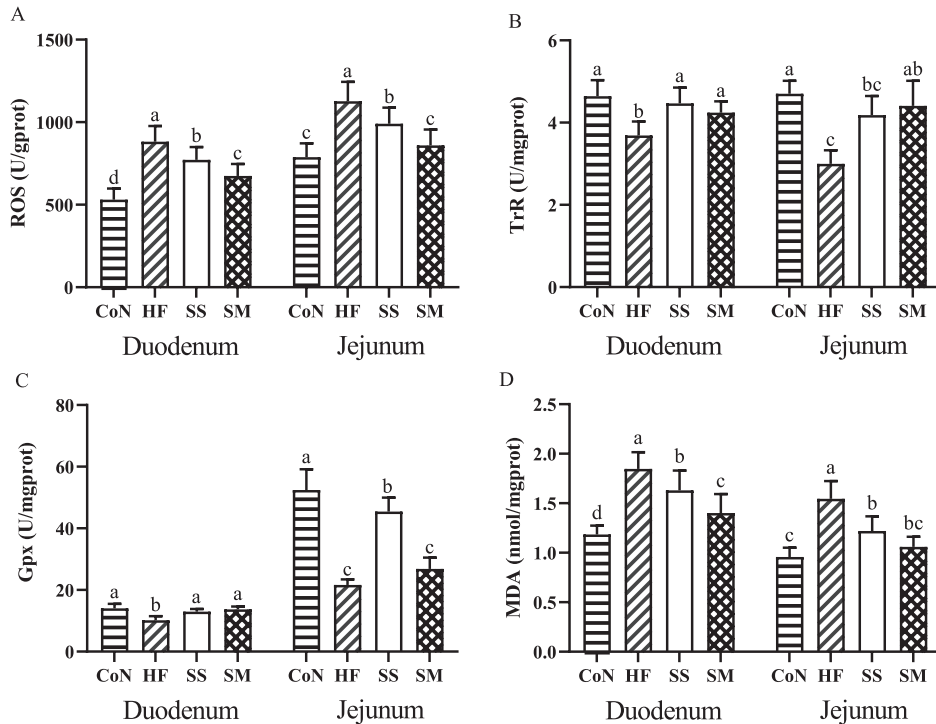


Figure 1. Effects of different selenium sources on oxidative stress parameters in the intestines of broilers in a model of fluorine-induced chronic oxidative stress. (A) ROS, (B) TrR, (C) Gpx, and (D) MDA. Values are presented with the means $\pm$ standard deviation, n = 12/group. Different lowercase letters marked on the bar graph of the same tissue indicate significant differences ($P < 0.05$), and unmarked or the same letters indicate nonsignificant differences ($P > 0.05$). Abbreviations: CoN, control group; Gpx, glutathione peroxidase; HF, high fluorine group; MDA, malondialdehyde; ROS, reactive oxygen species; SS, sodium selenite group; SM, selenomethionine group; TrR, thioredoxin reductase.

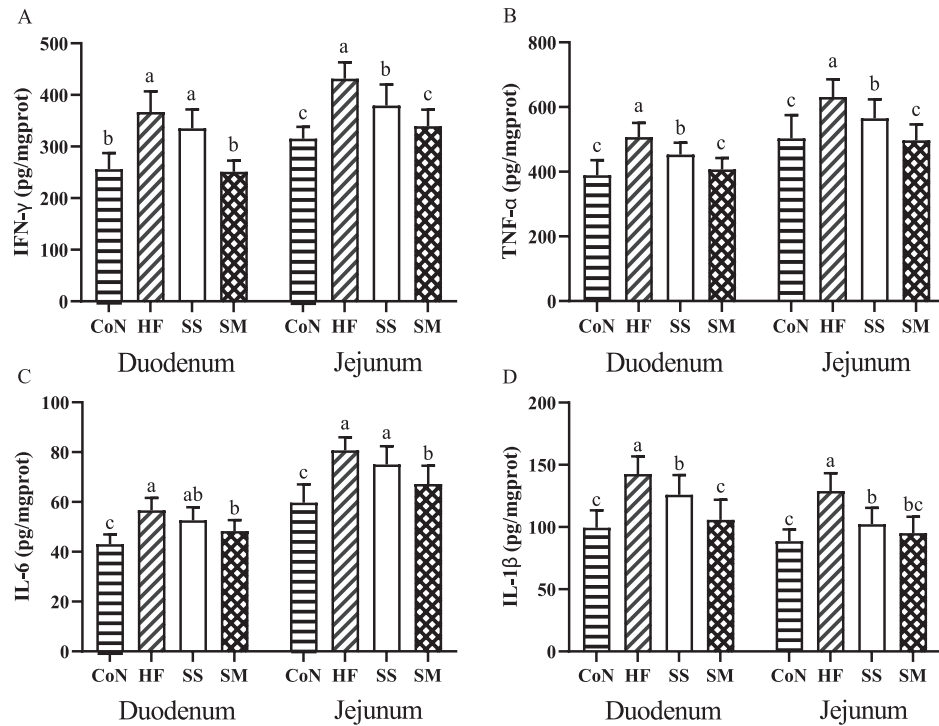


Figure 2. Effects of different selenium sources on proinflammatory cytokines levels in the intestines of broilers in a model of fluorine-induced chronic oxidative stress. (A) IFN- γ , (B) TNF- α , (C) IL-6, and (D) IL-1 β . Values are presented with the means $\pm$ standard deviation, $n = 12$ /group. Different lowercase letters marked on the bar graph of the same tissue indicate significant differences ($P < 0.05$), and unmarked or the same letters indicate non-significant differences ($P > 0.05$). Abbreviations: CoN, control group; HF, high fluorine group; IL-6, interleukin-6; IL-1 β , interleukin-1 β ; IFN- γ , interferon- γ ; SS, sodium selenite group; SM, selenomethionine group; TNF- α , tumor necrosis factor-alpha.

the SS group (except for duodenum Claudin-1 protein abundance).

Ultrastructural Examination

The Figure 5 showed that the TJ structures in the duodenum and jejunum were disrupted and the

intercellular spaces between intestinal epithelial cells observed comparatively enlarge in the HF group compared with the CoN group. Selenium (SS and SM) added groups could alleviate the damage of TJ structures in the duodenum and jejunum. In the SM group, the TJ structures between intestinal epithelial cells were normally distributed, which were close to the level of the CoN group. However, SS was less effective than SM in alleviating the disturbance of TJ structures between intestinal epithelial cells.

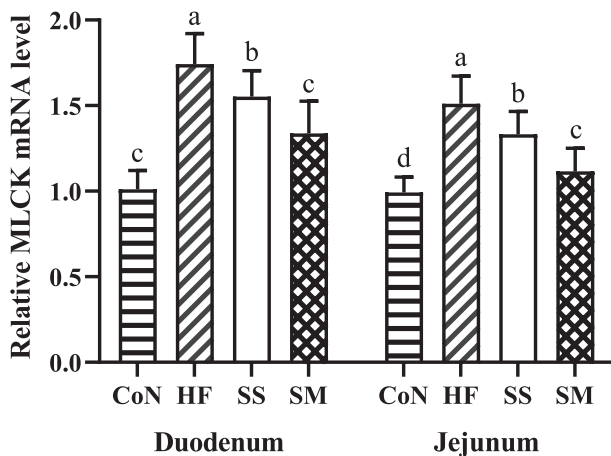


Figure 3. Effects of different selenium sources on MLCK mRNA levels in the intestines of broilers in a model of fluorine-induced chronic oxidative stress. MLCK, myosin light chain kinase. Values are presented with the means $\pm$ standard deviation, $n = 12$ /group. Different lowercase letters marked on the bar graph of the same tissue indicate significant differences ($P < 0.05$), and unmarked or the same letters indicate non-significant differences ($P > 0.05$). Abbreviations: CoN, control group; HF, high fluorine group; SS, sodium selenite group; SM, selenomethionine group.

Intestinal Permeability

The results in Figure 6 showed that HF group resulted in remarkable upregulation ($P < 0.05$) of DAO, D-Lactic acid and endotoxin levels in the serum compared with the other groups (except for endotoxin level in the SS group). The concentrations of DAO and endotoxin in the SM group were lower ($P < 0.05$) than those in the SS group. There was no significant difference ($P > 0.05$) between the SM and SS groups with respect to lactic acid level.

Growth Performance

The results in Table 4 showed that compared with the CoN and Se supplemented groups, HF group increased ($P < 0.05$) F/G and decreased ADG of broilers in any growth period. From 22 to 56 d and 1 to 56 d of age, ADG and feed efficiency in the SM group were higher

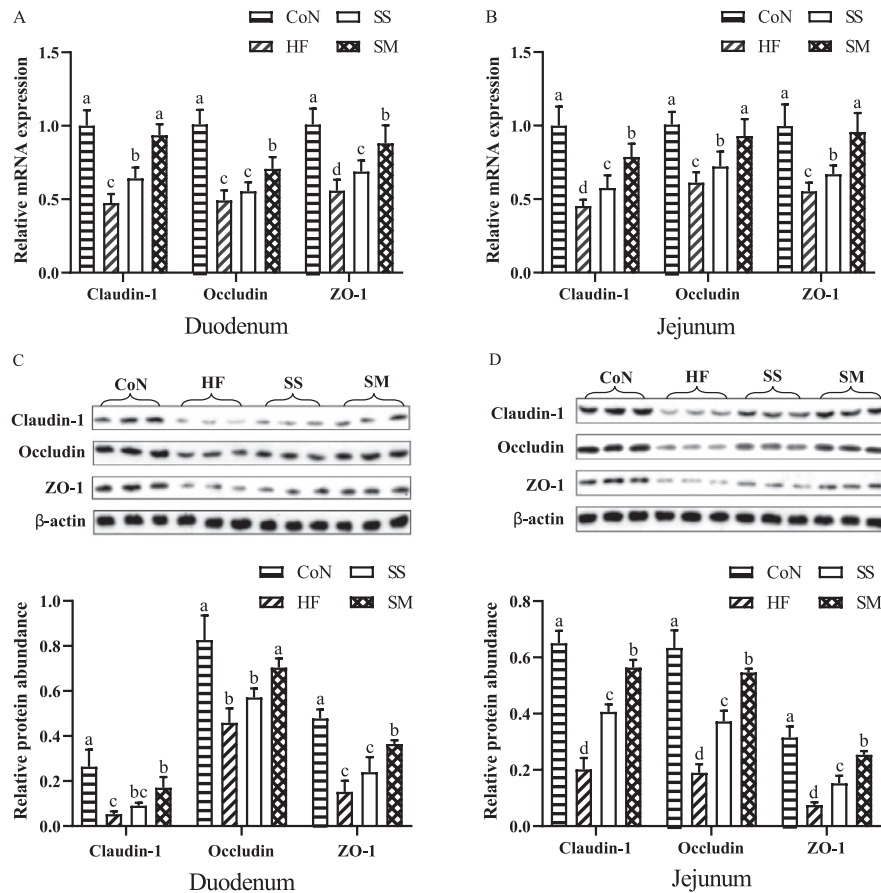


Figure 4. The expression of tight junction proteins in the intestines of broilers at mRNA and protein levels. The mRNA levels of Claudin-1, ZO-1 and Occludin in the duodenum (A) and jejunum (B); the protein levels of Claudin-1, ZO-1 and Occludin in the duodenum (C) and jejunum (D). The relative protein expressions of Claudin-1, ZO-1 and Occludin were normalized to β -actin. Values are presented with the means $\pm$ standard deviation, $n = 12$ /group for mRNA expression and $n = 6$ /group for protein expression. Different lowercase letters marked on the bar graph of the same tissue indicate significant differences ($P < 0.05$), and unmarked or the same letters indicate non-significant differences ($P > 0.05$). Abbreviations: CoN, control group; HF, high fluorine group; SS, sodium selenite group; SM, selenomethionine group; ZO-1, Zona Occludens-1.

($P < 0.05$) than those in the SS group. There were no significant differences ($P > 0.05$) among all groups in ADFI except for in the start period when the HF group had lower ($P < 0.05$) ADFI than the CoN group.

DISCUSSION

Fluorine is one of the most oxidizing substances, which can easily pass through the cell membrane by simple diffusion. Then, it attack oxygen to produce excessive ROS (Song et al., 2017). It has been reported that HF was associated with a high production of ROS in rats (Chouhan and Flora, 2008; Wang et al., 2019). According to our results, ROS levels in the duodenum and jejunum were significantly increased in the HF group compared with those of the CoN group, which further demonstrate that high dietary F can induce excessive production of ROS. Glutathione peroxidase and TrR are vital members of the antioxidant selenoprotein family, both of which play a key role in the elimination of ROS (Guillin et al., 2019). The present experimental results showed that Gpx and TrR activities in the duodenum and jejunum of the HF group were less than those of the CoN group, which were in good accordance

with findings by previous studies (Deng et al., 2014; Akinrinde et al., 2020). The decreased activities of Gpx and TrR in the HF group due to protein synthesis inhibition resulted in the reduced scavenging ability of ROS, thus making excessive ROS accumulation in the duodenum and jejunum.

It has been well established that excessive levels of ROS can cause lipid peroxidation and formation of MDA, which acts as a reliable biomarker of oxidative stress (Gawe et al., 2004). The results of our experiment showed that duodenum and jejunum MDA contents in the HF group were significantly higher than those in the CoN group, which were consistent with previously published results (Chen et al., 2011; Deng et al., 2014), and suggest that oxidative stress in the duodenum and jejunum may be caused by high dietary F in the present study.

It has been confirmed that oxidative stress can cause oxidative injury to mitochondria and endoplasmic reticulum, thus resulting in severe cellular damage, which causes the release of immunomodulatory molecules that recruit and activate the immune system, hence leading to a corresponding increase in the levels of various proinflammatory cytokines (Morris et al., 2018; Fishbein et al., 2020). As expected, the levels of proinflammatory cytokines IL-1 β , IL-6, TNF- α , and IFN- γ in

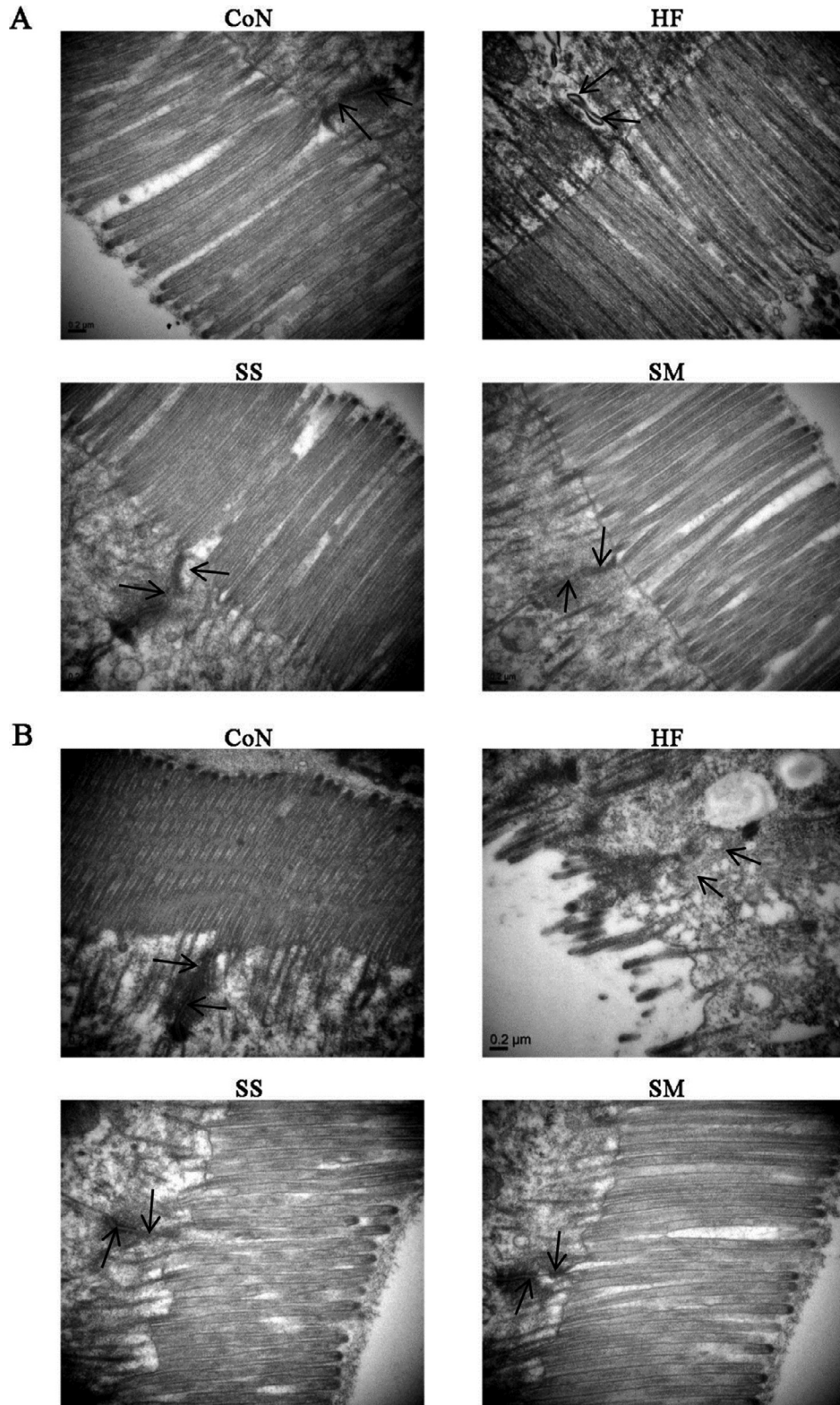


Figure 5. Effects of different selenium sources on tight junction structures (TEM, $\times 40,000$) of duodenum (A) and jejunum (B) in broilers in a model of fluorine-induced chronic oxidative stress. The black arrows in the figure refer to a tight connection. Control group, the tight junction structures (black arrows); HF group, the destroyed tight junction structures and increased intercellular spaces (black arrows) between adjacent intestinal epithelial cells are observed in the HF group in comparison with the CoN group; SS group, the gaps (black arrows) between adjacent intestinal epithelial cells are more smaller than those in the HF group; SM group, the tight junction structures (black arrows) are more integrity than those in the SS group and are similar with those in the CoN group. Abbreviations: CoN, control group; HF, high fluorine group; SS, sodium selenite group; SM, selenomethionine group.

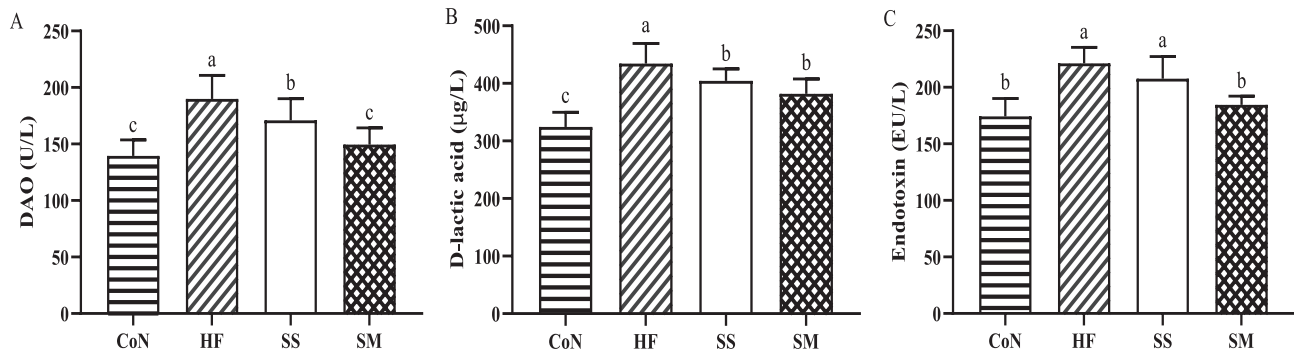


Figure 6. Effects of different selenium sources on the indexes related to intestinal mucosal permeability in broilers in a model of fluorine-induced chronic oxidative stress. The serum levels of (A) DAO, (B) D-lactic acid and (C) endotoxin. Abbreviations: CoN, control group; DAO, diamine oxidase; HF, high fluorine group; SS, sodium selenite group; SM, selenomethionine group. Values are presented with the means $\pm$ standard deviation, $n = 12$ /group. Different lowercase letters marked on the bar graph of the same tissue indicate significant differences ($P < 0.05$), and unmarked or the same letters indicate nonsignificant differences ($P > 0.05$).

the duodenum and jejunum were higher in the HF group than those in the CoN group in the present study, which were consistent with findings by other laboratories showing that the increased proinflammatory cytokines levels caused by hypoxia and food contaminants induced oxidative stress (Butturini et al., 2019; Rives et al., 2020).

Specifically, the overexpression of MLCK was positively correlated with TNF- α and IFN- γ (Chen et al., 2015; Chen et al., 2020). Myosin light chain (MLC) is phosphorylated by MLCK and subsequently lead to decreased TJ proteins expression (Ye and Sun, 2017). A reduction of TJ proteins expression can cause the opening of TJ and subsequently result in paracellular permeability (Tian et al., 2020). In this research, higher MLCK mRNA abundance and lower mRNA and protein levels of Claudin-1, ZO-1 and Occludin in the duodenum and jejunum were noticed in the HF group in comparison with the CoN group. Furthermore, in the present study, we also found that the TJ structures of duodenum and jejunum in the HF group were destroyed, with increased gaps between adjacent epithelial cells when compared with those in the CoN group. Our results imply that HF may disrupt TJ through modulating MLCK transcription level and downregulating the expression of TJ proteins, but the detailed mechanism needs to be further clarified because the MLCK and phosphorylation-MLC protein levels were not measured in the present study.

The destruction of TJ can increase paracellular permeability, and elevated DAO, D-lactate and endotoxin levels in the serum are crucial indicators of increased intestinal mucosal permeability (Guo et al., 2019). Data from the present study indicated that the HF group had higher serum DAO, D-lactate and endotoxin levels as compared to the CoN group. These results were similar to observations by Xin et al. (2020), and suggest that HF can improve intestinal mucosal permeability of duodenum and jejunum.

Increased intestinal mucosal permeability may improve the potential for intestinal inflammation and damage the immune system, which subsequently influences the overall health of the organism (Bischoff et al.,

2014; Tran et al., 2015). As expected, our results indicated that HF group suppressed the growth performance of broilers, this was evidenced by the decreased ADG and increased F/G in the HF group in comparison with the CoN group, which were positively correlated with the findings of Tao et al. (2005) in pigs and Sun et al. (2020b) in rats. On the other hand, oxidative stress can induce severe apoptosis of duodenum and jejunum (Uchida et al., 2017; Ju et al., 2021), which may result in a reduction in intestinal absorption function, and this may represent another explanation for the decreased growth performance of HF group. In addition, under oxidative stress conditions, dietary nutrients are used to maintain the immune response instead of maintaining growth, resulting in growth inhibition of stressed broilers.

In this study, compared with the HF group, Se added groups increased Gpx and TrR activities and decreased ROS, MDA and proinflammatory cytokines levels in the intestines. Besides, Se supplemented groups were more effective in increasing the expression of TJ proteins and decreasing the levels of MLCK mRNA in the intestines than HF group. Furthermore, the structures of TJ in the duodenum and jejunum were better maintained in the Se added groups in comparison with the HF group. In addition, the serum DAO, D-lactate and endotoxin levels in the Se added groups were lower than those in the HF group. Meanwhile, Se supplemented groups had better efficacy in improving growth performance of broilers than HF group. Our results suggest that SS and SM supplementation can effectively protect against the HF-induced duodenum and jejunum TJ network impairment and growth inhibition of broilers, which can be explained by the fact that Se added groups could increase Gpx and TrR activities much more efficiently than HF group. The enhanced Gpx and TrR activities result in the increased ability of the intestines to scavenge toxic ROS, then lead to a decrease in cellular oxidative damage and TNF- α and IFN- γ levels, thus the TJ integrity was maintained by inhibiting the transcription of MLCK and ultimately reduce paracellular permeability and counteract growth restriction caused by HF. Our results were in good accordance with findings by

Liu et al. (2020) and Qiao et al. (2020). In the present study, the protein levels of Claudin-1, ZO-1, and Occludin in the jejunum were higher in the SS group than those in the HF group; but there were no differences with respect to Claudin-1, ZO-1, and Occludin protein levels in the duodenum between the HF and SS groups; it can be concluded that the jejunum reacts more sensitively to SS supply in terms of improving protein levels of TJ proteins.

When we compared the two Se-added groups with each other, we found that SM supplementation was more effective in increasing TJ proteins expression (except for duodenum Claudin-1 protein level) and decreasing ROS, MDA, proinflammatory cytokines, DAO, endotoxin and MLCK mRNA levels (except for jejunum MDA and IL-1 β as well as duodenum IL-6 levels) than SS supplementation. Besides, SM had better efficacy in maintaining TJ structures of duodenum and jejunum than SS. In terms of growth performance, SM group showed a higher potential to improve ADG and feed efficiency of broilers (From 22 to 56 d and 1 to 56 d of age) in comparison with SS group. These results suggest that SM is more effective in mitigating the adverse effects of HF on duodenum and jejunum TJ network and growth performance of broilers than SS. However, in our present study, duodenum and jejunum TrR activities as well as duodenum Gpx activity did not differ between the SM and SS treatments. Additionally, our study also revealed that jejunum Gpx activity was significantly decreased in SM group as compared to SS group. Our outcomes were consistent with the results of Cantor et al. (1975) and Jiang et al. (2009) as well as a series of previous studies from our laboratory (Wang et al., 2011; Zhan et al., 2014; Wang et al., 2018; Li et al., 2020). These results imply that TrR and Gpx may not be the factors that cause the different protective effects of SS and SM against HF-induced disturbance of the TJ network. And it seems likely that other related signal pathways may be responsible for the greater bioavailability of SM compared to SS. A study of Hepa 1c1c7 cells found that the addition of SM could improve erythroid 2-related factor 2 (Nrf2) target enzymes expression, but SS addition could not induce the expression of Nrf2 downstream enzymes (Xiao and Parkin, 2006). Besides, in a chicken trial, SM showed more potential to suppress the TLR4-NF- κ B-NLRP3 signaling pathway than SS in inhibiting the Lipopolysaccharide-induced inflammation of liver tissue (Qu et al., 2020). Thus, we speculate that the mentioned above related signaling pathways may play a pivotal role in regulating the different protective effects of SS and SM against HF-induced disturbance of the TJ network. However, these additional pathways were not examined directly in the present experiment, and further studies should focus on exploring these pathways' parameters.

Conclusively, the results of this study indicated that HF could induce chronic oxidative stress and subsequently increase proinflammatory cytokines levels of duodenum and jejunum. Increased proinflammatory cytokines levels stimulated MLCK gene expression and subsequently decreased the expression of TJ proteins in

the duodenum and jejunum. A reduction of TJ proteins expression impaired the TJ structures, which in turn resulted in increased intestinal mucosal permeability of duodenum and jejunum and ultimately reduced the growth performance of broilers. Dietary supplementation with Se could exhibit antagonistic effects on HF-induced duodenum and jejunum TJ network disturbance and growth inhibition of broilers by regulating the duodenum and jejunum MLCK transcription level, presumably through increasing the Gpx and TrR activities and reducing the ROS and MDA levels as well as suppressing the secretion of various proinflammatory cytokines in the intestines. Moreover, SM seemed to be more effective in mitigating the deleterious effects of HF on duodenum and jejunum TJ network, compared with SS. Our findings will bring a promising tactics for the utilization of SM as an efficient antioxidant additive for reducing the intestinal damage caused by oxidative stress in poultry.

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DISCLOSURES

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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