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Maternal dietary supplementation with different sources of selenium on antioxidant status and mortality of chicken embryo in a model of diquat-induced acute oxidative stress

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ABSTRACT

The experiment was conducted to investigate the effects of maternal dietary supplementation with different sources of selenium (Se) on antioxidant status and mortality of chicken embryo in a model of diquat-induced acute oxidative stress. A total of 180 Meihuang-4 broiler breeders (40wk old) were randomly assigned into three dietary treatments: basal diet (Control), basal diet supplemented with 0.15 mg Se/kg of sodium selenite (SS) or selenomethionine (SM)). Birds were raised for 12 wk. From 10th to 11th week of the experiments, 960 eggs were collected (40 effs per replicate) and assigned into 2 diquat-injected treatments (0 or 25 μg diquat injected on 17th day). There were 3 dietary Se treatments × 2 diquat-injected treatments (CON, SS, SM, CON-DIQ, SS-DIQ, SM-DIQ) in this experiment, each of which was replicated eight times. Then, 240 eggs were slaughtered for analysis on 18th day of hatching, and 720 eggs were used for mortality statistics on 21st day. Results showed that diquat significantly affected chicken embryos mortality and nitric oxide (NO) content, total antioxidation capability (T-AOC), protein carbonyl content, myeloperoxidase (MPO) activity, xanthine oxidase (XOD) activity, total nitric oxide synthase (NOS) activity, monoamine oxidase (MAO) activity and B-cell Leukemia Lynmphoma 2 (bcl-2) content in liver ($P < 0.05$). Compared with the control, broiler breeders fed with Se had lower reactive oxygen species (ROS) content, NO content, malondialdehyde (MDA) content, protein carbonyl,caspase-3 content and higher T-AOC, glutathione peroxidase (GPx) activity, mitochondrial membrane potential (MMP), MPO activity, XOD activity, MAO activity, bcl-2 content in liver ($P < 0.05$). There were interactions between dietary Se and diquat in chicken embryo mortality and NO content, CAT activity, MPO activity, MAO activity, caspase-3 content and bcl-2 content ($P < 0.05$). After diquat-injected, SM-DIQ group had lower chicken embryo mortality, NO content, caspase-3 content, bcl-2 content and higher MPO activity, MAO activity than CON-DIQ group ($P < 0.05$). Inconclusion, this study indicated that diquat significantly increased free

Abbreviations: Se, Selenium; SS, Sodium selenite; SM, Selenomethionine; DIQ, Diquat; CON, Control; T-AOC, Total antioxidation capability; CAT, Catalase; T-SOD, Total superoxide dismutase; 8-OHDG, 8-hydroxydeoxyguanosine; MPO, Myeloperoxidase; NO, Nitric oxide; XOD, Xanthine oxidase; NOS, Nitric oxide synthase; MAO, Monoamine oxidase; Bcl-2, B-cell Leukemia Lynmphoma 2; ROS, Reactive oxygen species; MDA, Malondialdehyde; GPx, Glutathione peroxidase; MMP, Mitochondrial membrane potential; H&E, Hematoxylin and eosin

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radicals and induced oxidative stress in chicken embryos; maternal dietary supplementation with Se could alleviate the negative effects of diquat in chicken embryos, and SM had better protective effects against oxidative stress induced by diquat than SS.

1. Introduction

It is well known that the incubation of chicken embryos is an important part of poultry production. Therefore, in the process of incubation, the mortality of chicken embryo has a direct impact on the economic benefits of chicken farms. There are two death peaks throughout the chicken embryo hatching process. The first death peak appears on the 2nd to 4th day of the chicken embryo hatching process and the second one occurs on the 19th to 21st day (JASSIM et al., 1996; Penuela, 2018). The mortality in the second period accounts for about half of the total deaths during the entire incubation process. There are many reasons, such as temperature, humidity, oxygen, etc (Moran, 2007; Al-Zghoul et al., 2019). The main factor is that the chicken embryo is converted from the chorioallantoic respiration to the lung respiration and with 60 % oxidative metabolism accelerating, it leads to the second peak of death (Visschedijk, 1968; Giussani et al., 2007; Jr. E. T. Moran, 2007; Surai et al., 2016). In this process, a lot of free radicals are produced, including active oxygen and reactive nitrogen radicals. Oxidative stress occurs due to the imbalance between the ability of antioxidant defense system to eliminate free radicals and the production of reactive oxygen species (**ROS**) (Halliwell, 2007). Excessive levels of ROS can lead to cellular injury, apoptosis and oxidation of proteins, lipids and DNA, which ultimately causes the death of chicken (Brenneisen et al., 2005; Yang et al., 2010). Thus, we need to find an effective way to reduce the mortality of chicken embryo incubation through oxidative stress.

Selenium (**Se**) plays an important role in antioxidative (Cantor and Scott, 1974a; Yuan et al., 2011), immunity (Sun et al., 2018) and anti-apoptosis (Jin et al., 2018; Chi et al., 2019) for poultry. It could eliminate free radicals by acting the active site of glutathione peroxidases (**GPx**) (Tapiero et al., 2003). Sources of Se added into maternal dietary includes inorganic sodium selenite (**SS**) and organic Se such as selenized yeast and selenomethionine (**SM**). Previous studies showed that organic Se has better bioavailability (Fairweather-Tait, 1997; Rayman, 2004; Baylan et al., 2011) and lower biotoxicity than inorganic Se (Mihajlović, 1992; Tiwary et al.,

Table 1
Composition and nutrient levels of broiler breeder basal diets (g/kg, unless otherwise stated).

Item	
Ingredient	
Maize	646.0
Soybean meal	250.0
$CaHPO_4$	18.0
Limestone	70.0
Salt	3.0
DL-Methionine	3.0
Premix[1]	10.0
Nutrient levels	
ME[2] ($MJ{\cdot}kg^{-1}$)	11.2
Crude protein	161.1
Calcium	30.2
Total phosphorus	6.5
Available phosphorus	4.4
Lysine	8.2
Methionine	5.5
Met + Cys	8.1
Threonine	6.1
Tryptophan	1.8
Histidine	4.2
Arginine	10.7
Leucine	14.2
Isoleucine	6.4
Phenylalanine	7.9

[1] Supplied the following per kilogram of diet: iron, 72 mg; copper, 7 mg; zinc, 72 mg; manganese, 90 mg; iodine, 0.9 mg; vitamin A, 10,800 IU; vitamin D3, 2,160 IU; vitamin E, 27 IU; menadione, 1.4 mg; thiamin,1.8 mg; riboflavin, 8 mg; pyridoxine, 4.1 mg; vitamin B12, 0.01 mg; niacin, 32 mg; calcium pantothenate,11 mg; folic acid, 1.08 mg; biotin, 0.18 mg.
[2] ME was calculated from data provided by Feed Database in China.

2016) and maternal diet supplementation with Se was significantly positively correlated with Se content in eggs, chicken embryos and chicks (Surai, 2000; Pan et al., 2007; Wu et al., 2011; Yuan et al., 2011). Our research proved that maternal dietary supplementation with 0.15 mg Se/kg L-SM elevated the percentage of egg production, promoted the hatchability and birthrate (Wu et al., 2011; Dong et al., 2014),and feeding diet supplemented with 0.15 mg Se/kg SM for the broiler breeder significantly decreased the embryo mortality, H_2O_2 content and MDA content and increased GPx activity compared with SS during the late period of incubation (Dong et al., 2014). Therefore, we speculated that SM may have better antioxidant effect on chicken embryos than SS.

Diquat (**DIQ**) can utilize molecular oxygen to produce superoxide anion radical. It is often used as a source of oxidative stress because of its low usage rate, ease of control, and ease of handling. Several studies have successfully established oxidative stress models by diquat (Wolfgang et al., 1991; Nisar et al., 2015a; Song et al., 2017a). Thus, the purpose of this experiment was to establish an acute oxidative stress model of diquat-induced chicken embryos to study the effects of maternal supplementation with different Se sources on antioxidant status and mortality during chicken embryo hatching.

2. Materials and methods

The trial was approved and conducted under the supervision of the Zhejiang University Animal Care and Use Committee, which have adopted Animal Care and Use Guidelines governing all animal use in experimental procedures. All efforts were made to minimize suffering.

2.1. Experimental design and treatments

A completely randomized design involving 3 dietary Se treatments × 2 diquat treatments were used in this study. Three supplemental Se treatments were basal diet (0.04 mg/kg of Se) without Se supplementation (**CON**) and basal diet supplemented with 0.15 mg Se/kg SS (Sigma-Aldrich Chemical Co., St. Louis, MO) or 0.15 mg Se/kg SM (Sigma-Aldrich Chemical Co.). Two diquat treatments were injection of 100 μl sterile water or 25 μg diquat (National Institute of Metrology., China, Beijing) dissolved in 100 μlsterile water per egg on the 17th day of chicken embryo. Therefore, there were six treatments (**CON, CON-DIQ, SS, SS-DIQ, SM, SM-DIQ**) in this experiment.

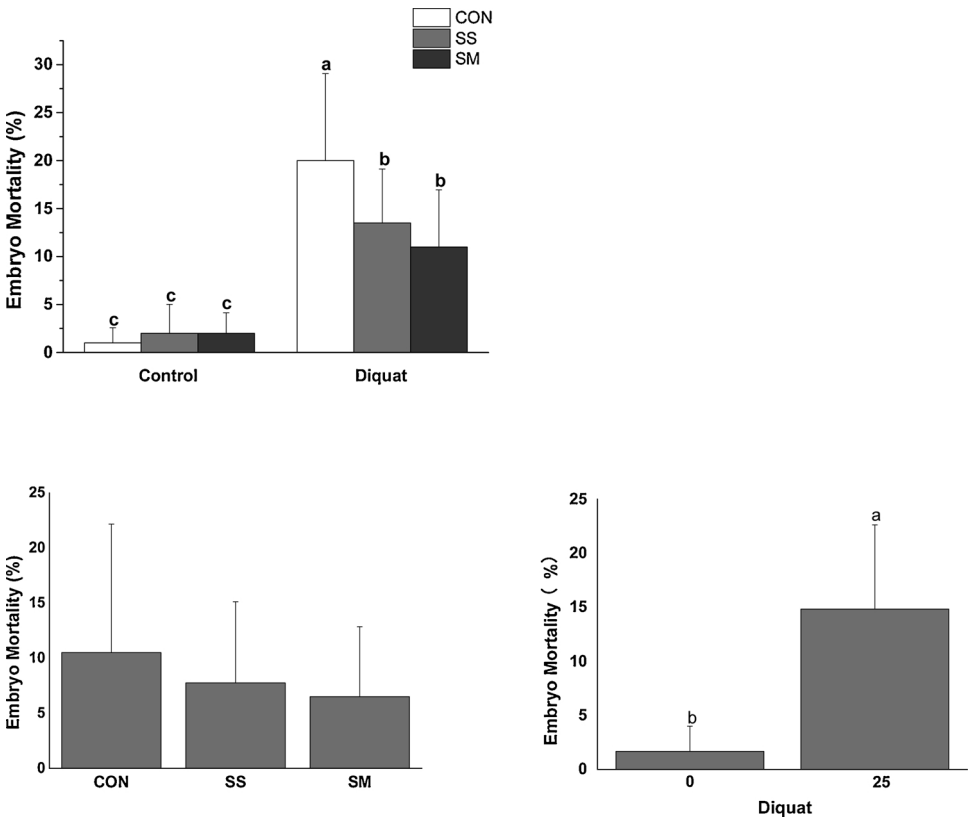

Fig. 1. Effects of maternal dietary supplementation with different selenium sources on embryo (n = 8) mortality to against oxidative stress induced by diquat. Chicken embryo. received diquat (25 μg) injection on 17th day. Mortality was analyzed on 21th day. The embryo mortality was calculated according to the following formula: embryo mortality (%) = (the number of chicken embryo death/15) × 100. Data are represented as mean ± SD. *P* value less than 0.05 is considered significant different. CON = control; SS = sodium selenite; SM = selenomethionine.

2.2. Birds and diets

One hundred and eighty Meihuang-4 broiler breeders (40 wk old) were randomly assigned to three dietary treatments. The broiler breeders were housed in laying battery cages in the same house, 2 hens per cage and provided with clean water and fed 125 g diet per day at 7.30 a.m. every day during the experimental period. Broiler breeders were fed for 12 wk. During the first 4 wk, the broiler breeders were fed the same basal diet (0.04 mg/kg of Se) to deplete body reserves of Se. Then, birds were fed with three different test diets according to treatment group for 8 wk. The basal diet was formulated to meet the nutrient requirements for laying broiler breeders according to Feeding Standard of Chicken (NY/Y33-2004) except for Se (Table 1). The photoperiodic lighting was programmed with 16 h and 20 lx of light per day. The house temperature was maintained between 24 and 30℃.

From 10th to 11th week of the experiment period, 320 eggs per dietary treatment were collected and randomly assigned into 2 diquat-injected treatments (0 or 25 μg diquat-injected), which made up to a total of 960 eggs. All eggs were incubated in a commercial incubator at 37.5℃ and 55 % RH with automatic egg turning. The diquat-injected treatment (160 eggs per dietary treatment, a total of 480 eggs) received 0 or 25 μg diquat injection on 17th day. Then all eggs were divided into 2 parts for experiments:

1) Two hundred and forty eggs were slaughtered for analysis on 18th day. 40 eggs from each treatment were randomly divided into 8 replicates with 5 eggs per replicate and slaughtered humanely 24 h after injection. Liver was collected carefully, blotted free of blood. Liver samples were either stored in 2.5 % glutaraldehyde for electron microscopy analysis or 4 % paraformaldehyde for H&E staining analysis or frozen immediately in liquid nitrogen and stored at −80℃ for further analysis.
2) Seven hundred and twenty eggs were used for mortality statistics on 21st day. 120 eggs of each treatment were randomly divided into 8 replicates with 15 eggs per replicate. The eggs were transferred to a hatcher on the 19th day. Hatchability was showed in Fig. 1.

Livers samples from each replicate were merged into one single sample, weighed and then homogenized (4000 rpm for 15 min at 4℃) in nine volumes of PBS solutionwith an Ultra-Turrax homogenizer (Tekmar Co., Cincinnati, OH). Homogenates were centrifuged at 3500 rpm for 15 min under 4℃ and the supernatant was collected for further analysis.

2.3. Detection of total antioxidation capability (T-AOC), catalase (CAT), total superoxide dismutase (T-SOD) and glutathione peroxidase (GPx) in chicken embryo liver

The T-AOC (Cat:A015-1), CAT (Cat:A007-1-1), T-SOD (Cat:A001-1-1) and GPx (Cat:A005) were determined using kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Company, Jiangsu, China).(Wang et al., 2011; Li et al., 2018)

2.4. Detection of ROS and mitochondrial membrane potential (MMP) in chicken embryo liver

The levels of ROS (Cat: S0033) and MMP (Cat: C2006) in fresh chicken embryo livers were detected using ROS detection kit and MMP kit according to the manufacturer's instructions (Beyotime Company, China). The ROS was analyzed by using flow cytometry. (Guo et al., 2018; Wang et al., 2018; Zhang et al., 2019a)

2.5. Detection of nitric oxide (NO), malondialdehyde (MDA), Protein carbonyl and 8-hydroxydeoxyguanosine (8-OHDG) in chicken embryo liver

The contents of nitric oxide (Cat: A012-1), MDA (Cat: A003-1), protein carbonyl (Cat: A087-2) and 8-OHDG (Cat: H165) were determined using kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Company, Jiangsu, China). (Liu, 2013)

2.6. Detection of myeloperoxidase (MPO), monoamine oxidase (MAO), total nitric oxide synthase (NOS) and xanthine oxidase (XOD) in chicken embryo liver

The enzymatic activities of MPO (Cat: A044), MAO (Cat: A034), NOS (Cat: A014-2-1) and XOD (Cat: A002-1) were determined using kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Company, Jiangsu, China).(Zhang et al., 2019)

2.7. Detection of B-cell Leukemia Lynmphoma 2 (bcl-2) and Caspase-3 in chicken embryo liver

The contents of bcl-2 (Cat: H073) and Caspase 3 (Cat: H076) were determined using ELISA kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Company, Jiangsu, China).

2.8. Analysis of liver cell apoptosis

The specimen was incubated sections in 2 changes of xylene for 15 min, dehydrated in 2 changes of pure ethanol for 5 min, and then dehydrated in gradient ethanol of 95 %, 90 %, 80 %, 70 % ethanol and distilled water for 5 min respectively. The slides were

incubated in DNase-free proteinase K working solution for 15–30 min s at 21-37℃ and washed twice by PBS solution. Each tissue was incubated at 37℃ for 60 min. in the dark with 50 μL of TUNEL reaction solution. The negative control was added 50 μL of Label solution and washed twice by PBS solution. The specimen which in wet box was incubated at 37℃ for 10 min. in dark with 50 μL of DAPI working solution and washed twice by PBS solution. Finally, the slides were coverslip with water mounting medium. Images of the slices were obtained using the NIKON Eclipse TI-SR and NIKON DS-U3.

2.9. Light microscopy

The specimen which was fixed in 4 % paraformaldehyde embedded in paraffin, followed by slicing and staining with hematoxylin and eosin (H&E). Images of the slices were obtained using the Leica DM3000 Microsystem.

2.10. Electron microscopy observation

After fixing in 2.5 % glutaraldehyde in phosphate buffer (0.1 M, pH7.0) for more than 4 h, the liver was washed three times in the phosphate buffer (0.1 M, pH7.0) for 15 min at each step. Then the specimen was post fixed with 1 % OsO4 in phosphate buffer (0.1 M, pH7.0) for 1 − 2 h and washed three times in the phosphate buffer (0.1 M, pH7.0) for 15 min at each step. Afterwards, specimen was dehydrated by a graded series of ethanol (30 %, 50 %, 70 %, 80 %, 90 %, 95 % and 100 %) for about 15–20 min at each step and transferred to absolute acetone for 20 min. Next, the specimen was placed in 1:1 mixture of absolute acetone and the final Spurr resin mixture for 1 h at room temperature, then transferred to 1:3 mixture of absolute acetone and the final resin mixture for 3 h and final Spurr resin mixture for overnight. Finally, specimen was placed in Eppendorf contained Spurr resin and heated at 70℃ for more than 9 h. The specimen was sectioned in LEICA EM UC7 ultratome and sections were stained by uranyl acetate and alkaline lead citrate for 5–10 min respectively and observed in Hitachi Model H-7650 TEM.

2.11. Statistical analysis

All data were statistically evaluated with SPSS software for windows (version 19.0; SPSS Inc., Chicago, IL, USA). A level less than 0.05 ($P < 0.05$) was taken as significant. The results were presented as means ± standard deviation.

3. Results

3.1. Chicken embryo mortality

In this study, there was an interaction ($P = 0.028$) between the diquat and Se. Diquat significantly increased ($P < 0.001$) chicken embryo mortality in the late incubation period. Compared with CON groups, SM supplement groups significant reduced ($P < 0.05$) the chicken embryo mortality. The mortality of SS-DIQ and SM-DIQ was significantly decreased ($P < 0.05$) compared with the CON-DIQ group. But it showed no significant difference ($P > 0.05$) between the SS-DIQ group and the SM-DIQ group (Fig. 1).

Table 2
Effects of maternal dietary supplementation with different selenium sources on ROS and NO against oxidative stress induced by diquat in chicken embryo liver.

Se	Diquat (μg)	ROS (FD/mg prot)	NO (μmol/g prot)
CON	0	20.80	0.117^{d}
	25	25.20	0.265^{a}
SS	0	19.99	0.103^{d}
	25	20.61	0.208^{b}
SM	0	19.61	0.112^{d}
	25	19.76	0.173^{c}
SEM		0.506	0.010
CON		23.00^{a}	0.191^{a}
SS		20.30^{ab}	0.156^{b}
SM		19.69^{b}	0.143^{b}
	0	20.13	0.111^{b}
	25	21.86	0.215^{a}
P value	Se	0.009	< 0.001
	Diquat	0.060	< 0.001
	Se × Diquat	0.114	0.002

Chicken embryo (n = 8) received diquat (25 μg) injection on 17th day. Liver samples were collected on 18th day. Data are represented as mean ± SD. *P* value less than 0.05 is considered significant different.
CON = control; SS = sodium selenite; SM = selenomethionine.
ROS = reactive oxygen species; NO = nitric oxide.

3.2. Liver biochemical parameters

The ROS content and NO content in the liver of chicken embryos were shown in Table 2. There was a significant difference ($P < 0.05$) in ROS content between CON-DIQ group and CON group. Se supplementation reduced ($P < 0.05$) ROS content and SM groups was significantly lower than CON groups. There was an interaction ($P < 0.05$) between Se and diquat on NO content. The content of NO in all diquat-induced groups significantly increased ($P < 0.05$). Hens fed diet with supplemented Se had significant reduced ($P < 0.05$) NO content. But it showed no difference ($P > 0.05$) between the SS and SM. Compared with CON-DIQ group, the NO content was significantly reduced ($P < 0.05$) in the SS-DIQ group and SM-DIQ group. The SM-DIQ group was better ($P < 0.05$) than the SS-DIQ group.

Table 3 showed that diet supplement with Se significantly increased ($P < 0.05$) the T-AOC activity in the liver of chicken embryos. The T-AOC activity of SM groups were significantly increased ($P < 0.05$) compared with CON groups. Diquat also significantly reduced ($P < 0.05$) T-AOC activity in liver of chicken embryos. Data showed that Se and diquat had an interaction ($P < 0.05$) on CAT activity. Se supplementation significantly affected ($P < 0.05$) liver CAT activity. The CAT activity in CON-DIQ group was higher ($P < 0.05$) than that of SM-DIQ group. The SS group had the highest ($P < 0.05$) CAT activity. Se and diquat had no effect ($P > 0.05$) on T-SOD activity in chicken embryos. SM significantly increased ($P < 0.05$) liver GPx activity compared with the CON group. But there were no differences ($P > 0.05$) between diquat-injected groups.

The data of protein carbonyl content, 8-OHDG content, MDA content and MMP were shown in Table 4. No interactions between Se and diquat were observed in these 4 liver indices. Diquat significantly increased ($P < 0.05$) protein carbonyl content. Se significantly reduced ($P < 0.05$) protein carbonyl content and MDA content. Compared with the CON groups, diquat-injected groups had higher ($P < 0.05$) protein carbonyl content. SM significantly decreased ($P < 0.05$) the protein carbonyl content and MDA content. SS only had a significant effect ($P < 0.05$) on protein carbonyl content.

In Table 5, dietary Se significantly increased ($P < 0.05$) MPO activity, XOD activity and MAO activity, but did not influence the NOS activity. Diquat significantly increased ($P < 0.05$) all of these 4 indices. There were interactions ($P < 0.05$) on MPO activity and MAO activity between Se and diquat. Compared with the CON-DIQ group, the SS-DIQ and SM-DIQ had higher ($P < 0.05$) MPO activity. The activities of MAO in the SS-DIQ group and SM-DIQ group were significantly increased ($P < 0.05$) compared with the CON-DIQ group.

3.3. Liver cell apoptosis parameters

Compared with CON, SS and SM significantly reduced ($P < 0.05$) liver cell apoptosis of chicken embryos. The rapid induction of diquat significantly increased ($P < 0.05$) the apoptotic index of liver cells. However, the results of CON-DIQ group and SS-DIQ group were similar($P > 0.05$). SM significantly inhibited ($P < 0.05$) apoptosis in oxidative stress status which induced by diquat (Fig. 2).

Table 6 showed that dietary Se significantly affected ($P < 0.05$) caspase-3 content and bcl-2 content in chicken embryos. Diquat had effects($P < 0.05$) on bcl-2 content, but did not influence ($P > 0.05$) the caspase-3 content. There were interactions ($P < 0.05$) on the content of caspase-3 and bcl-2 between Se and diquat. Compared with the CON group, caspase-3 content in CON-DIQ group was significantly increased ($P < 0.05$). The SM-DIQ group had a lower ($P < 0.05$) caspase-3 content compared with the CON-DIQ group. The contents of bcl-2 in the SS-DIQ group and SM-DIQ group were higher ($P < 0.05$) compared with CON-DIQ group. The SM groups had higher ($P < 0.05$) bcl-2 content and lower caspase-3 content than SS.

Table 3
Effects of maternal dietary supplementation with different selenium sources on T-AOC, CAT, T-SOD and GPx activity against oxidative stress induced by diquat in chicken embryo liver.

Se	Diquat (μg)	T-AOC (U/mg prot)	GPx (U/mg prot)	CAT (U/mg prot)	T-SOD (U/mg prot)
CON	0	1.12	10.30	23.92^{c}	61.70
	25	1.04	11.97	26.94^{b}	65.78
SS	0	1.23	11.93	30.09^{a}	67.95
	25	1.09	12.72	26.10^{bc}	63.81
SM	0	1.39	13.33	25.16^{bc}	63.11
	25	1.19	13.56	23.70^{c}	57.97
SEM		0.026	0.307	0.477	1.021
CON		1.08^{b}	11.13^{b}	25.43^{b}	63.74
SS		1.16^{b}	12.32^{ab}	28.10^{a}	65.88
SM		1.29^{a}	13.45^{a}	24.43^{b}	60.54
	0	1.25^{a}	11.86	26.39	64.25
	25	1.10^{b}	12.75	25.58	62.52
P value	Se	0.001	0.006	0.001	0.087
	Diquat	0.002	0.115	0.293	0.375
	Se × Diquat	0.541	0.568	0.002	0.115

Chicken embryo (n = 8) received diquat (25 μg) injection on 17th day. Liver samples were collected on 18th day. Data are represented as mean ± SD. *P* value less than 0.05 is considered significant different.
CON = control; SS = sodium selenite; SM = selenomethionine.
T-AOC = total antioxidation capability; GPx = glutathione peroxidase; CAT = catalase; T-SOD = total superoxide dismutase.

Table 4
Effects of maternal dietary supplementation with different selenium sources on protein carbonyl, 8-OHDG, MDA content and MMP against oxidative stress induced by diquat in chicken embryo liver.

Se	Diquat (μg)	Protein carbonyl (nmol/mg prot)	8-OHDG (ng/ml)	MDA (nmol/mg prot)	MMP (%)
CON	0	1.66	13.47	0.512	0.587
	25	1.84	14.19	0.544	0.558
SS	0	1.45	12.53	0.496	0.601
	25	1.60	13.13	0.503	0.562
SM	0	0.77	12.35	0.417	0.612
	25	1.10	12.76	0.434	0.635
SEM		0.062	0.271	0.011	0.008
CON		1.75^{a}	13.83	0.528^{a}	0.573^{b}
SS		1.52^{b}	12.83	0.500^{a}	0.582^{b}
SM		0.93^{c}	12.56	0.426^{b}	0.623^{a}
	0	1.30^{b}	12.78	0.475	0.600
	25	1.51^{a}	13.36	0.493	0.585
P value	Se	< 0.001	0.291	< 0.001	0.027
	Diquat	0.002	0.141	0.330	0.344
	Se × Diquat	0.528	0.972	0.867	0.233

Chicken embryo (n = 8) received diquat (25 μg) injection on 17th day. Liver samples were collected on 18th day. Data are represented as mean ± SD. *P* value less than 0.05 is considered significant different.
CON = control; SS = sodium selenite; SM = selenomethionine.
8-OHDG = 8-hydroxydeoxyguanosine; MDA = malondialdehyde; MMP = mitochondrial membrane potential.

Table 5
Effects of maternal dietary supplementation with different selenium sources on MPO, XOD, NOS and MAO activity against oxidative stress induced by diquat in chicken embryo liver.

Se	Diquat (μg)	MPO (U/g)	XOD (U/L)	NOS (U/mg prot)	MAO (U/ml)
CON	0	0.953^{a}	6.66	0.864	0.877^{cd}
	25	0.343^{c}	6.19	0.745	0.607^{e}
SS	0	0.902^{a}	8.35	0.984	1.179^{a}
	25	0.676^{b}	6.13	0.768	0.800^{d}
SM	0	0.924^{a}	8.46	0.923	1.060^{ab}
	25	0.669^{b}	6.91	0.833	0.960^{bc}
SEM		0.036	0.203	0.023	0.034
CON		0.648^{b}	6.42^{b}	0.804	0.742^{b}
SS		0.789^{a}	7.24^{a}	0.876	0.989^{a}
SM		0.796^{a}	7.69^{ab}	0.878	1.010^{a}
	0	0.926^{a}	7.82^{a}	0.924^{a}	1.039^{a}
	25	0.563^{b}	6.41^{b}	0.782^{b}	0.789^{b}
P value	Se	0.005	0.007	0.242	< 0.001
	Diquat	< 0.001	< 0.001	0.001	< 0.001
	Se × Diquat	< 0.001	0.085	0.411	0.037

Chicken embryo (n = 8) received diquat (25 μg) injection on 17th day. Liver samples were collected on 18th day. Data are represented as mean ± SD. *P* value less than 0.05 is considered significant different.
CON = control; SS = sodium selenite; SM = selenomethionine.
MPO = myeloperoxidase; XOD = xanthine oxidase; NOS = total nitric oxide synthase; MAO = monoamine oxidase.

3.4. Liver cell histological examination

There was a small amount of vacuolation in the SS and CON group (Fig. 3). The rapid induction of diquat (CON-DIQ) significantly aggravated the phenomenon. The liver cells of SM group had normal morphology, nuclei clearly, no obvious necrosis and swelling. Furthermore, SS and SM (SS-DIQ and SM-DIQ) had significantly effects on the vacuolation which induced by diquat, especially SM treatment.

Fig. 4 showed moderate electron density, a slight swelling of the mitochondrial inner ridge and the ribosome detachment of the endoplasmic reticulum in liver cells of the CON group and the SM-DIQ group. There was medium electron density, clear mitochondrial inner ridge, the endoplasmic reticulum and ribosome arranged closely in SS and SM groups. The CON-DIQ and SS-DIQ groups showed slight electron density desalination, part of the mitochondrial inner ridges disappeared and the endoplasmic reticulum ribosome shedding.

4. Discussion

During the incubation of eggs, chicken embryos are always in gas exchange with the outside environment. The chicken embryo is

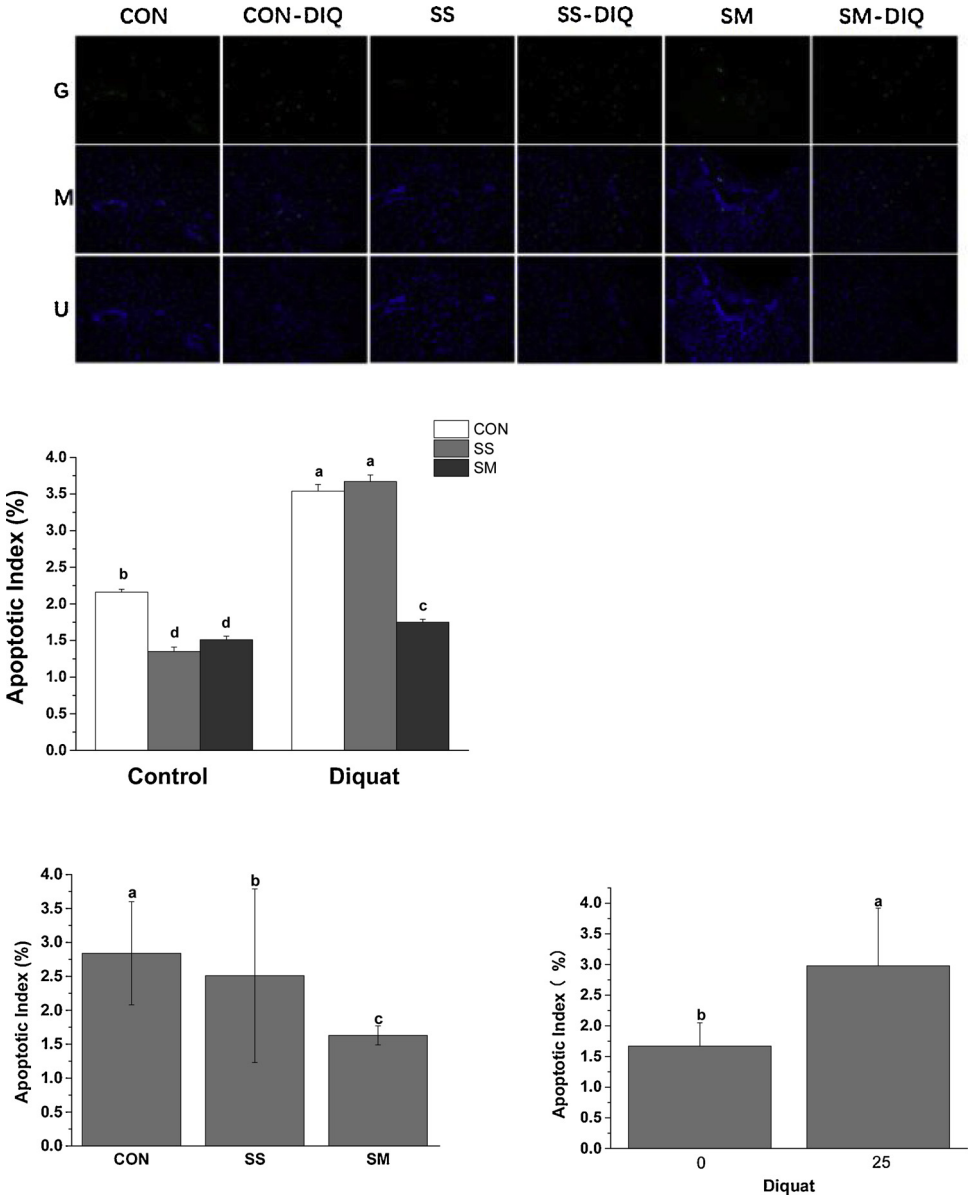

Fig. 2. Effects of maternal dietary supplementation with different selenium sources on apoptotic index of liver cells (TUNEL) against oxidative stress induced by diquat. Chicken embryo (n = 3) received 25 μg diquat injection on 17th day. Liver samples were collected on 18th day. Apoptotic cells were labeled as green, and intact cells were labeled as blue. The apoptosis index was calculated according to the following formula: Apoptosis index (%) = [the number of apoptotic cells/ (the number of apoptotic cells + the number of intact cells)] × 100. Data are represented as mean ± SD. *P* value less than 0.05 is considered significant different. Images of the chicken embryo liver cells morphology were observed at 200× magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
CON = control; SS = sodium selenite; SM = selenomethionine

in a low oxygen environment at the early stage of hatching. After converting to the lung respiration, it is in a stage of high oxygen exposure. Therefore, it induces oxidative stress of chicken embryos (Visschedijk, 1968). Diquat is a common bipyridyl herbicide which could utilize molecular oxygen to produce superoxide anion radical (Fu et al., 1999). Many studies have successfully established oxidative stress models with diquat (Gupta et al., 2000). In this study, there was no significant difference in chicken embryo mortality under normal conditions, which was also observed in our previous research(Cantor and Scott, 1974a,1974b; Stanley et al., 2012). However, some studies showed that organic Se significantly reduced chicken embryo mortality compared to inorganic Se and control. A possible reason is that there was no restriction of Se supplement in rooster diet. But after the rapid of diquat-induced, the mortality of CON-DIQ group was significantly higher than CON group. Mortality in Se supplemented groups were significantly lower compared to CON-DIQ group. There was no significant difference between SS and SM groups. This study indicated that Se has a benefit effect on mortality of chicken embryo hatching in the case of oxidative stress.

Table 6
Effects of maternal dietary supplementation with different selenium sources on bcl-2 content and caspase-3 content against oxidative stress induced by diquat in chicken embryo liver.

Se	Diquat (μg)	Caspase-3 (ng/ml)	Bcl-2 (ng/ml)
CON	0	1.658	6.641^{bcd}
	25	1.893	5.702^{d}
SS	0	1.666	6.031^{cd}
	25	1.661	7.111^{b}
SM	0	1.564	7.028^{bc}
	25	1.585	8.888^{a}
SEM		0.027	0.216
CON		1.775^{a}	6.172^{b}
SS		1.664^{b}	6.571^{b}
SM		1.575^{b}	7.958^{a}
	0	1.629	5.567^{b}
	25	1.713	7.234^{a}
P value	Se	0.002	< 0.001
	Diquat	0.056	0.024
	Se × Diquat	0.052	0.001

Chicken embryo (n = 8) received diquat (25 μg) injection on 17th day. Liver samples were collected on 18th day. Data are represented as mean ± SD. *P* value less than 0.05 is considered significant different.
CON = control; SS = sodium selenite; SM = selenomethionine.
Bcl-2= B-cell LeukemiaLynmphoma 2.

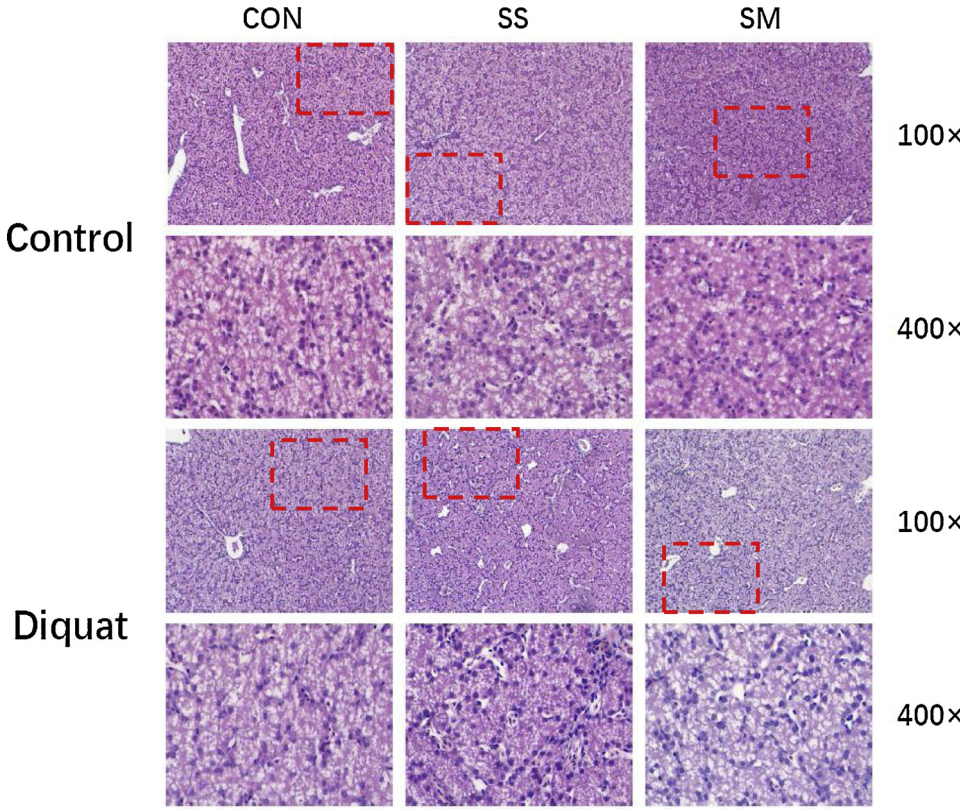

Fig. 3. Comparison of H&E staining in chicken embryo liver cells. Chicken embryo received 25 μg diquat injection on 17th day. Liver samples were collected on 18th day. Images of the chicken embryo liver cells morphology were observed at 100× and 400× magnification. Images of 400× magnification shows a detail of 100× magnification images which marked in the red box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
CON = control; SS = sodium selenite; SM = selenomethionine

Reactive oxygen species (ROS) is the by-products of normal aerobic metabolism, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) (Zhang et al., 2019b). NO is also a kind of free radical in the body, which will react with ROS to form RNS. The increase of ROS content and NO content indicates the destruction of body's redox balance. Nisar et al. reported

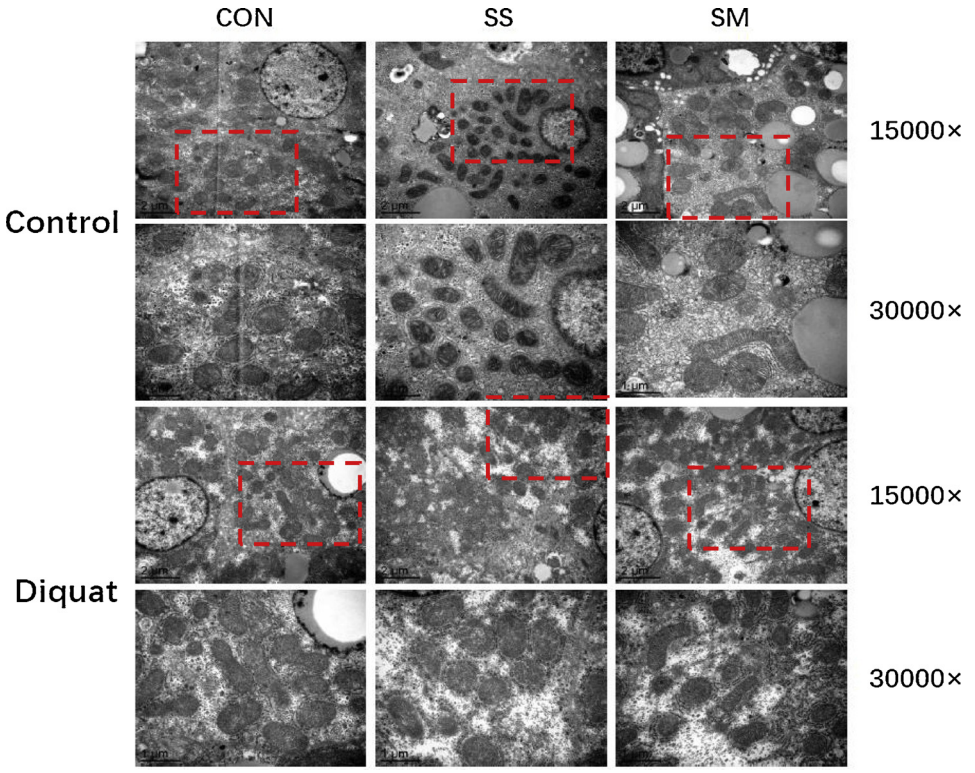

Fig. 4. Comparison of Electron microscopic in chicken embryo liver cells. Chicken embryo received 25 μg diquat injection on 17th day. Liver samples were collected on 18th day. Images of the chicken embryo liver cells morphology were observed at 15,000× and 30,000× magnification. Images of 30,000× magnification shows a detail of 15,000× magnification images which marked in the red box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
CON = control; SS = sodium selenite; SM = selenomethion

that diquat (1 − 500 μM) caused a significant increase in ROS production of SH-SY5Y cells (Nisar et al., 2015b). In this research, after diquat injected, the content of ROS and NO significantly increased in the CON-DIQ group compared with the CON group. It indicated that injection of diquat induces free radicals in the liver of chicken embryos. SS and SM both reduced the ROS content induced by diquat, but there was no significant difference between them. In addition, SM was significantly more efficient than SS in reducing NO content. These results indicated that Se can effectively reduce the content of free radicals induced by diquat. SM has better protection than SS in oxidative status. This was supported by the finding of Liu et al., who found Se supplementation during dietary cadmium reduced the production of nitric oxide in chickens (Liu et al., 2014). Xiao et al. also reported that Se supplementation reduced the ROS content which induced by high temperature in chicken embryos (Xiao et al., 2016).

Total antioxidant capacity (T-AOC) is an important indicator for evaluating the function of antioxidant systems. In this study, diquat significantly decreased T-AOC activity, which indicated that the rapid injection of diquat reduced the body's capacity of anti-oxidative stress by inducing acute oxidative stress. After the diquat-induced, the SM-DIQ group had the highest T-AOC activity compared with the CON-DIQ group and the SS-DIQ group. This was similar to the findings of Pan et al. that dietary supplementation of Se significantly improved the activity of T-AOC in hens (Chen, 2010). GPx plays an important role in antioxidative defense in poultry. In our experiment, SM increased the GPx activity in liver compared with the CON groups. The results were in accordance with our previous studies (Wang et al., 2011; Yuan et al., 2011; Xiao et al., 2016). Furthermore, SOD and CAT are essential for removing free radicals and maintaining cell energy metabolism. They are the first oxidative barrier of against free radicals in the body. SOD can catalyze the disproportionation reaction of superoxide anion (O^{2-}) ($2O^{2-} + 2H^{+} \rightarrow H_2O_2 + O_2$) to repair and restore cells damaged by ROS. CAT has the biological function of catalyzing hydrogen peroxide to water and oxygen. The activity of T-SOD and CAT in Se supplemented groups (SS and SM) was higher than that in CON group in our experiment. SS group had significant difference on CAT activity compared with CON group and SM group, which was similar with the report of Xiao et al. (Xiao et al., 2016). Interestingly, in this report, we found that the activity of CAT and T-SOD in the SS-DIQ group and SM-DIQ group decreased after diquat-induced compared with the CON-DIQ group. The SM-DIQ group was significantly lower than the CON-DIQ group. Lee et al. reported that the CAT and T-SOD activity of chicken which injected 10 μg Se at 10d of embryo age was significantly lower than the control group after Eimeria-infected at 14th day (Lee et al., 2014). In 2009, Zoidis et al. found that the expression of CAT mRNA decreased with the supplementation of Se in chicken liver (Zoidis et al., 2010). But there were also some opposite reports. Jiang et al. found that SM significantly increased SOD activity in broiler breast muscles (Jiang et al., 2009).

Malondialdehyde (MDA) is an important indicator of lipid peroxidation, which produced by lipid oxidation reaction induced by

ROS. The protein carbonyl is a marker of ROS-mediated protein oxidation in proteins. 8-OHDG is a predominant from of oxidative lesions which induced by free radical in nuclear and mitochondrial DNA. It is used as a biomarker for oxidative stress widely, too. Mitochondria are important organelles for maintaining the normal function in the cell. MMP changes are the earliest changes in apoptosis. Thus, the changes of MMP could reflect the oxidative stress in the body. We found that the injection of diquat caused the oxidation of proteins in the chicken embryo liver cells in this experiment. But there was no significant effect on the MDA, 8-OHDG and MMP. In addition, SM significantly reduced the content of protein carbonyl, MDA and MMP on normal conditions and oxidative stress. It was supported by previous reports that the injection of diquat caused oxidation of lipids and proteins (Mao et al., 2014; Kohen and Nyska, 2016). Song et al. reported that SM reduced the MDA content on oxidative stress induced by diquat (Song et al., 2017b). The same results were obtained by Ahmad et al., who found that the diet supplemented organic Se had less MDA content compared with inorganic Se (Ahmad et al., 2012). These indicated that free radicals induced by diquat caused cells damage in chicken embryo liver. SM had better protective effect against oxidative stress induced by diquat than SS.

Monoamine oxidase (MAO) is a mitochondrial enzyme for the oxidative deamination of the exogenous mono-amines in all tissues. MPO is an enzyme in activated PMN leukocytes, which presented in neutrophils and monocytes to protect against microbial infection. XOD is the enzyme which is an important biological source of oxygen derived free radicals by forming hypoxanthine and xanthine to uric acid. It is benefit for many disease pathological processes of oxidative damage induced by ROS in tissues. NO is produced by NOS in the enzymatic reaction of biological systems ($NADPH_2$ + arginine + $O_2 \Rightarrow$ citrulline + H_2O + NO). But it is worth noting that the activity of MPO and MAO in the SS-DIQ and SM-DIQ groups increased significantly compared with the CON-DIQ group. It was similar to the report of Zhou et al. that Se increased MAO activity in liver, kidney, skeletal muscle and myocardium of rats compared with Se-deficient (Fan and C.L.H., 2010). This may be due to acute oxidative stress induced by diquat, causing massive apoptosis in mitochondria and chicken embryo liver cells.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (UTP)-biotin nick end-labeling (TUNEL) is a common staining method used to assist cells in tissue sections. Bcl-2 is localized to intracellular sites of oxygen free radical generation including mitochondria, endoplasmic reticula, and nuclear membranes. It is a key regulator of apoptosis, and the cell suicide program is critical for development, tissue homeostasis, and protection. Caspase are crucial mediators of apoptosis. Among them, caspase-3 is frequently activated by death protease. Our results showed that the apoptosis index of the CON group was significantly higher than the SS group and SM group under normal conditions. But there were no significant differences on caspase-3 and bcl-2 among the CON, SS and SM groups. After the rapid injection of diquat, apoptosis index and caspase-3 content in the CON-DIQ group were significantly increased compared with the CON group. These indicated that diquat induced apoptosis of chicken embryo liver cells. The SM-DIQ group was significantly better than the CON-DIQ group and SS-DIQ group on apoptosis index and bcl-2 content. But SS had no effect on hepatocyte apoptosis induced by diquat. These indicated that SM can protect cells by up-regulating the expression of bcl-2. These were the same as our histological results. It can prove that SM has a significant protective effect on chicken embryo liver cells. It was similar with the report by Cure et al. that Se had the protection effect on liver damage caused by monocrotaline (MCT) (Cuce et al., 2017). In 2008, Sarada et al., found that Se can inhibit the up-regulation of caspase-3 protein and increase the expression of Bcl-2 protein induced by hypoxia in neuroblastoma cell line (Sarada et al., 2008). Wang et al. also reported similar results in 2013 (Wang et al., 2013).

Through histological examination, we found that the chick embryo liver cells of the CON group were in a mild oxidative stress state, and dietary supplemented Se can protected liver cells. The rapid induction of diquat significantly caused changes in liver cells of chicken embryo. The oxidative stress in the SM-DIQ group was similar with the CON group. However, SS had no significant protective effect on oxidative stress induced by diquat. These showed that SM had better protection than SS on oxidative stress induced by diquat. The changes in mitochondrial morphology were also consistent with previous experimental results. Wang et al. reported that Se had protective effects on pathological damage of mice kidney cells caused by Cd in 2013 (Wang et al., 2013).

5. Conclusions

In summary, our research showed that diquat increased the content of free radicals in chicken embryos, which induced lipid and protein oxidation and oxidative stress. Furthermore, it caused the damage of mitochondrial structure and cell apoptosis in liver cells. Se had a protective effect on chicken embryo liver cells. It can reduce the mortality rate in the late incubation period by improving the ability of antioxidant. Under oxidative stress, SM had better protective effect compared with SS. These results suggested that SM might have a bright perspective as feed additives used in the maternal dietary of broiler breeders.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

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