Antagonistic effects of different selenium sources on growth inhibition, oxidative damage, and apoptosis induced by fluorine in broilers

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ABSTRACT Fluorosis can induce oxidative stress through leading to reactive oxygen species (ROS) generation. Selenium (Se) can eliminate ROS by direct and indirect manners. In this study, therefore, we investigated the possible protective effects of sodium selenite (SS) and selenomethionine (Se-Met) on fluorine (F)-induced oxidative stress in broilers. A total of 720 1-day-old Lingnan Yellow broilers were allotted to 4 groups (6 replicates of 30 birds each group) and fed with basal diet (control group), 800 mg/kg F (high F group), 800 mg/kg F+0.15 mg Se/kg as SS (SS group), or Se-Met (Se-Met group), respectively. The experiment lasted 50 d. High F group significantly decreased ($P < 0.05$) the average daily gain (ADG) and feed efficiency (FE) in comparison with control group. The contents of ROS, malondialdehyde, 8-hydroxydeoxyguanosine, protein carbonyl, and cysteinyl aspartate specific proteinases 3 in serum, liver, and kidney were higher ($P < 0.05$) in high F group than those in control group. Compared with control group, the decreased ($P < 0.05$) activities of glutathione peroxidase (GSH-Px) and cytoplasmic thioredoxin reductase (TrxR1) as well as contents of selenoprotein P (SelP), total protein (TP), and B-cell lymphoma-2 in serum and tissues were observed in high F group. Moreover, the pathological lesions of liver and kidney in high F group were more than those in control group. However, supplementation with SS and Se-Met could improve ADG and FE, increase SelP and TP concentrations, elevate GSH-Px and TrxR1 activities, minimize the changes of oxidative stress and apoptosis parameters as well as ultrastructure of liver and kidney, whereas the effects of Se-Met were better than those of SS. The results indicated that excess F could result in growth inhibition of broilers through inducing oxidative stress and subsequently caused oxidative damage to biological macromolecules and soft tissues as well as apoptosis, whereas dietary SS and Se-Met supplementation could antagonize high F induced growth retardation by inhibiting oxidative stress and a mechanism of apoptosis regulation and the impact was more with Se-Met.

Key words: selenomethionine, sodium selenite, fluorine, oxidative stress, broiler

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INTRODUCTION

It has been well established that fluorosis is endemic in many regions of the world (Ho et al., 2004; Meenakshi and Maheshwari, 2006). Besides dental and skeletal fluorosis (Liu et al., 2013), fluorine (**F**) intoxication can also cause metabolic disorders (Liu et al., 2012a), immunotoxicity (Deng et al., 2013; Liu et al., 2013), and damages in soft tissues including the liver (Chinoy et al., 2004), kidney (Bai et al., 2010; Nabavi et al., 2012), intestines (Shashi, 2002; Luo et al., 2012), and lymphoid organs (Chen et al., 2011a,b; Liu et al., 2012b) in chickens, rats, or rabbits.

F is the most reactive ionized element with a rather small molecular weight, and hence it can readily pass through cell membranes by simple diffusion. Then, it attack oxygen and disturb oxygen metabolism, leading to the generation of reactive oxygen species (**ROS**) which causes oxidative stress (García-Montalvo et al., 2009). Oxidative stress is a condition generated due to the imbalance between the production of ROS and antioxidative defense systems and results in superfluous ROS formation (Halliwell, 2007). Excessive levels of ROS can lead to cellular injury, apoptosis, and oxidation of proteins, lipids, and DNA (Brenneisen et al., 2005), which ultimately cause decreased weight gain and meat production, negative feed conversions, high mortality rate, immunosuppression, increase susceptibility to other

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diseases, and other important traits governing the poultry industry earnings (Yang et al., 2010; Fisinin et al., 2016; Surai and Fisinin, 2016). It has been confirmed in humans, rats, and chickens that oxidative stress was the key mechanism in F-induced toxic effects (Sharma and Chinoy, 1998; Chlubek, 2003; Liu et al., 2012b; Luo et al., 2012). In China, fluorosis causes great economic losses for the poultry industry due to the use of nondefluorinated calcium monohydrogen phosphate as a mineral supplement (average dietary F level reaches about 300 mg/kg and in some cases as high as 2,000 mg/kg) (Liu et al., 2003). Therefore, seeking an effective approach against F-induced oxidative stress may provide us with a convenient and available method for improving poultry health and production.

It was reported that selenium (**Se**) could eliminate ROS through antioxidant selenoproteins such as glutathione peroxidases (**GSH-Px**), cytoplasmic thioredoxin reductase (**TrxR1**), and selenoprotein P (**SelP**) by acting at their active sites (Tapiero et al., 2003). Furthermore, Se could also directly scavenge ROS (Halliwell et al., 1995). Previous studies suggested that Se could relieve the destructive oxidative stress in rats caused by heroin and adriamycin (Cemek et al., 2011; Taskin and Dursun, 2012).

It is well known that the form of supplemental Se includes inorganic Se sodium selenite (**SS**) and organic Se such as selenized yeast and selenomethionine (**Se-Met**). A series of previous studies from other and our laboratories have established that Se-Met has higher bioavailability and rates of tissue Se retention than SS in broilers (Jiang et al., 2009; Wang et al., 2011a,b,c,2016; Zhan et al., 2014; Zhang et al., 2014). Our data implied that Se-Met might be superior to SS in alleviating F-induced oxidative stress in broilers via detoxification of ROS. Therefore, the protective effects of SS and Se-Met against F-induced oxidative stress in broilers need to be elucidated. Thus, the purpose of this experiment was to examine the effects of dietary SS and Se-Met supplementation on F-induced growth inhibition, oxidative damage, and apoptosis in broilers to determine whether dietary supplementation with Se, especially Se-Met, can alleviate the negative effects of high F on broilers.

MATERIALS AND METHODS

All procedures of the experiment adhered to the guidelines of, and were approved by, the Animal Care and Use Committee of Zhejiang A and F University, which has adopted Animal Care and Use Guidelines governing all animal use in experimental procedures.

Animals, Diets, and Feeding Protocol

A total of 720 1-day-old healthy Lingnan Yellow broilers were purchased from a commercial rearing farm (Zhejiang Guangda Poultry Industry Co., Ltd., Jiaxing, Zhejiang, China). The birds were randomly divided into 4 treatments of 6 replicates each with 30 birds per replicate (15 males and 15 females). Briefly, the control group was offered the basal diet; the high F group was given the basal diet supplemented with 800 mg F/kg as sodium fluoride (**NaF**) (Sigma-Aldrich Chemical Co., St. Louis, MO; Cat: S7920); the groups SS and Se-Met were given the high F diet supplemented with 0.15 mg Se/kg as SS (Sigma-Aldrich Chemical Co.; Cat: S5261) or Se-Met (Beijing InnoChem Science & Technology Co., Ltd., Beijing, China; Cat: 259,960,000), respectively. The optimum doses of Se and F were chosen according to the previous results from our (Wang et al., 2016) and other (Bai et al., 2010; Luo et al., 2012; Deng et al., 2014) laboratories, respectively. Nutritional requirements of broilers during starter (day 1 to 21) and grower (day 22 to 50) phases in the basal diets were adequate according to NRC (1994) except for Se (Table 1). The Se and F concentrations in the basal diets and experimental diets were analyzed by hydride generation atomic fluorescence spectrometry and fluorine ion-selective electrodes, respectively. Feed samples were analyzed in triplicate. The analyzed dietary Se and F concentrations were given in Table 2.

The broilers were housed in an environmentally controlled experimental building with 24 plastic-wire-floor pens (width 150 cm, length 150 cm, height 200 cm). Birds were maintained at a brooding temperature of 34 ± 1°C for the first week, and then it was gradually reduced by 2 to 3°C per week until maintained at 22°C. The birds were supplied with fresh water as well as abovementioned diets ad libitum with a 24 h constant light schedule. Daily observations were made to record mortality and temperature.

Growth Performance

The feed intake of broilers was recorded weekly. At 21 and 50 d of age, chickens were weighed after a 12 h overnight fast, considering pen as replicate. Average daily gain (**ADG**), average daily feed intake (**ADFI**), and feed conversion ratio (**FCR**) were calculated. ADFI and FCR were adjusted appropriately when there were mortalities.

Sample Collection and Preparation

At 50 d of age, blood samples were randomly collected from 12 male broilers in each treatment (2 birds per replicate) by main wing vein puncture after birds were weighed. Non-anticoagulative blood samples were allowed to clot at room temperature for 1 h. Serum was isolated by centrifugation for 10 min at 4°C and 3,000 rpm and then transferred to 1.5 mL centrifuge tubes respectively. After blood collection, chickens were anesthetized with sodium pentobarbital and necropsied. The fresh liver and kidney samples were quickly removed manually from carcasses on an ice-cold

Table 1. Ingredients and nutrient levels of the basal diets (as fed basis).[1]

Items	Starter (day 1 to 21)	Grower (day 22 to 50)
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], %		
Metabolisable energy[4], kcal/kg	2915	3020
Crude protein	21.39	19.62
Crude fat	3.86	4.54
Crude ash	6.92	6.23
Lysine	1.01	0.95
Methionine	0.46	0.34
Methionine + cysteine	0.84	0.69
Calcium	1.07	0.89
Total phosphorus	0.66	0.57
Nonphytate phosphorus	0.44	0.36

[1]Sodium fluoride (Sigma-Aldrich Chemical Co.; Cat: S7920), sodium selenite (Sigma-Aldrich Chemical Co.; Cat: S5261), and selenomethionine (Beijing InnoChem Science & Technology Co., Ltd; Cat: 259,960,000) were added to the diets at 800 mg fluorine/kg and 0.15 mg selenium/kg to achieve the appropriate treatment levels at the expense of corn.

[2]The premix contained the following vitamins and minerals (per kilogram 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, 1000 mg choline (choline chloride), 80 mg iron ($FeSO_4 \cdot 7H_2O$), 8 mg copper ($CuSO_4 \cdot 5H_2O$), 80 mg manganese ($MnSO_4 \cdot H_2O$), 60 mg zinc ($ZnSO_4 \cdot 7H_2O$), 0.35 mg iodine (KI).

[3]Analyzed values were based on triplicate determinations.

[4]Calculated data.

Table 2. Analyses of fluorine and selenium contents in starter and grower diets for broilers (as fed basis).[1]

	Analyzed F level[2] (mg/kg)		Analyzed Se level[2] (mg/kg)	
	Starter (day 1 to 21)	Grower (day 22 to 50)	Starter (day 1 to 21)	Grower (day 22 to 50)
Control group	21.93	16.38	0.049	0.052
High F group	812.98	825.32	0.047	0.049
SS group	829.26	817.98	0.202	0.204
Se-Met group	820.47	810.65	0.199	0.203

[1]Abbreviation: F, fluorine; Se, selenium; SS, sodium selenite; Se-Met, selenomethionine.

[2]Values are based on triplicate determinations and presented as means.

surface. The samples of serum, liver, and kidney were used for further analysis.

Ultrastructural Observation in the Liver and Kidney

The representative samples were taken from the fresh liver and kidney tissues of birds. The samples were fixed in 2.5% glutaraldehyde for 48 h, washed 3 times with 0.1 M phosphate at pH 7.2, postfixed in 1% osmic acid for 2 h at 4°C, washed 3 times with 0.1 M phosphate at pH 7.2, dehydrated in ascending grades of alcohol and acetone, and then embedded in epoxy resin at 60°C for 24 h. Ultrathin sections, obtained by Leica EM UC7 Ultramicrotome (Leica Microsystems, Inc., Buffalo Grove), were stained with uranyl acetate and lead citrate. Then, the samples were examined on a TEM H-7650 (Hitachi Ltd., Tokyo, Japan) electron microscope.

Oxidative Stress Parameters Determinations

Fresh samples of liver and kidney were rinsed with icy normal saline buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.25 M sucrose, and 0.1% peroxide-free Triton X-100) before being weighed. Then, 1 g of liver and kidney tissues in 9 mL of above isotonic saline was homogenized for 10 s in an ice bath with an Ultra-Turrax (T8, IKA-Labortechnik, Staufen, Germany) at

8,000 rpm. The homogenate was divided into 2 fractions. In 1 fraction, the homogenate was centrifuged at 4,000 rpm for 15 min at 4°C to provide a clear supernatant, which was used for analysis of ROS, malondialdehyde (**MDA**), 8-hydroxydeoxyguanosine (**8-OHdG**), protein carbonyl, SelP, and total protein (**TP**) contents as well as GSH-Px activity. ROS contents in serum, liver, and kidney were detected using a chick ROS-HRP conjugate ELISA kit (Cusabio Biotech Co., Ltd., Wuhan, Hubei, China; Cat: CSB-EQ027740CH) following the manufacturer's protocols. SelP concentrations in serum, liver, and kidney were also assayed using commercial ELISA kits (Abebio Science Co., Ltd., Wuhan, Hubei, China; Cat: AE56582CH) according to the manufacturer's introductions. The activity of GSH-Px (Cat: A005) and concentrations of MDA (Cat: A003–1), 8-OHdG (Cat: H165), protein carbonyl (Cat: A087–2), and TP (Cat: A045–2) in serum, liver, and kidney were assayed by the corresponding assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) in accordance with the manufacturer's introductions. In another fraction, the homogenate was centrifuged at 4°C and 12,000 rpm for 15 min to obtain a supernatant fraction for TrxR1 activity assay. Serum, liver, and kidney TrxR1 activities were determined according to the procedures of our previous study (Yuan et al., 2012). All samples were measured in triplicate in a single assay to avoid interassay variation. Data were expressed as U per mg protein (U/mgprot), U/gprot, and mgprot/g in tissues and as U/mL, U/mL, and gprot/L in serum for GSH-Px, TrxR1, and TP, respectively. The concentrations of ROS, MDA, 8-OHdG, SelP, and protein carbonyl were expressed as U/L, nmol/mgprot, nmol/mgprot, ng/mgprot, and nmol/mgprot in tissues and U/L, nmol/mL, nmol/mL, ng/mL, and nmol/mgprot in serum, respectively.

Determination Apoptosis Parameters

According to the methods mentioned above for ROS assay, liver and kidney homogenates were prepared. The contents of B-cell lymphoma-2 (**Bcl-2**) (Cat: MBS260943) and cysteinyl aspartate specific proteinases 3 (**Caspase-3**) (Cat: MBS9390231) in serum, liver, and kidney were examined by ELISA using detection kits of MyBioSource (San Diego, CA) for chicks according to the kit's introduction. Each sample was measured in triplicate. The Bcl-2 and Caspase-3 concentrations were expressed as ng/mL.

Statistical Analysis

All the experimental data were statistically evaluated with SPSS software for windows (version 16.0; SPSS Inc., Chicago, IL). Data comparisons were conducted using one-way analysis of variance followed by Turkey's post hoc test to compare means between the different treatment groups. A level less than 0.05 ($P < 0.05$) was taken as significant unless indicated otherwise. Replicate was generally accepted as the experiment unit. The results were presented as means ± standard deviation.

RESULTS

Oxidative Stress Parameters

Table 3 shows the effects of F and different Se sources on oxidative stress parameters of broilers. The contents of ROS, protein carbonyl, 8-OHdG, and MDA in serum, liver, and kidney were significantly increased ($P < 0.05$) by high F group in comparison with control group. Compared with high F group, SS group significantly decreased ($P < 0.05$) the contents of ROS and 8-OHdG in serum and kidney, protein carbonyl in kidney, and MDA in serum and liver, and Se-Met group significantly decreased ($P < 0.05$) the contents of ROS, protein carbonyl, 8-OHdG, and MDA in serum, liver, and kidney. The ROS content in kidney, MDA content in liver, and protein carbonyl and 8-OHdG contents in serum, liver, and kidney in Se-Met group were lower ($P < 0.05$) than those in SS group. Compared with those in control group, the contents of ROS, protein carbonyl, and 8-OHdG in serum, liver, and kidney as well as MDA in liver and kidney in SS group were increased ($P < 0.05$), and the content of kidney 8-OHdG in Se-Met group was increased ($P < 0.05$).

Changes in GSH-Px and TrxR1 Activities and SelP Concentration

In Table 4, changes of the GSH-Px and TrxR1 activities and SelP concentrations in serum, liver, and kidney are shown. Serum and tissues GSH-Px and TrxR1 activities and SelP concentrations were decreased ($P < 0.05$) in broilers fed the high F supplemented diets as compared with broilers fed the basal diet. SS and Se-Met groups led to a significantly higher ($P < 0.05$) increase in serum and tissues GSH-Px and TrxR1 activities and SelP concentrations than high F group. Compared with that in SS group, the kidney TrxR1 activity in Se-Met group was decreased ($P < 0.05$), but the differences were not statistically significant in serum and tissues GSH-Px activities and SelP concentrations as well as serum and liver TrxR1 activities ($P > 0.05$). The serum and kidney GSH-Px activities and SelP concentrations in SS group were evidently lower ($P < 0.05$) than those in control group, whereas the kidney GSH-Px and TrxR1 activities and serum SelP concentration in Se-Met group were significantly lower ($P < 0.05$) than those in control group.

Ultrastructural Changes

Ultrastructural changes of liver and kidney are shown in Figures 1–3. The shorter, sparser, and swollen

Table 3. Effects of fluorine and different selenium sources on oxidative stress parameters of broilers.[1]

Items	Control group	High F group	SS group	Se-Met group
ROS				
Serum (U/L)	1244.45 ± 35.64^{c}	1642.65 ± 69.42^{a}	1421.75 ± 132.46^{b}	1331.67 ± 124.27^{b,c}
Liver (U/L)	3900.07 ± 336.37^{c}	4623.05 ± 424.72^{a}	4498.87 ± 327.88^{a,b}	4007.87 ± 360.37^{b,c}
Kidney (U/L)	3179.83 ± 311.94^{c}	4585.16 ± 371.86^{a}	3988.44 ± 354.41^{b}	3476.93 ± 288.72^{c}
Protein carbonyl				
Serum (nmol/mgprot)	0.69 ± 0.02^{b}	1.00 ± 0.09^{a}	0.95 ± 0.09^{a}	0.76 ± 0.05^{b}
Liver (nmol/mgprot)	3.25 ± 0.27^{b}	4.08 ± 0.39^{a}	3.82 ± 0.27^{a}	3.67 ± 0.23^{b}
Kidney (nmol/mgprot)	2.43 ± 0.14^{c}	3.43 ± 0.34^{a}	2.99 ± 0.22^{b}	2.56 ± 0.21^{c}
8-OHdG				
Serum (nmol/mL)	39.00 ± 0.32^{c}	51.98 ± 1.57^{a}	46.63 ± 3.14^{b}	39.02 ± 3.62^{c}
Liver (nmol/mgprot)	22.03 ± 1.75^{b}	27.69 ± 2.42^{a}	26.16 ± 1.59^{a}	23.27 ± 1.69^{b}
Kidney (nmol/mgprot)	16.11 ± 1.51^{d}	31.13 ± 2.37^{a}	26.39 ± 2.39^{b}	21.04 ± 1.53^{c}
MDA				
Serum (nmol/ml)	3.54 ± 0.17^{b}	4.60 ± 0.51^{a}	3.98 ± 0.39^{b}	3.76 ± 0.41^{b}
Liver (nmol/mgprot)	0.57 ± 0.07^{c}	0.99 ± 0.05^{a}	0.85 ± 0.05^{b}	0.60 ± 0.07^{c}
Kidney (nmol/mgprot)	0.79 ± 0.10^{c}	1.01 ± 0.07^{a}	0.92 ± 0.03^{a,b}	0.83 ± 0.10^{b,c}

[1]Abbreviation: F, fluorine; SS, sodium selenite; Se-Met, selenomethionine; ROS, reactive oxygen species; 8-OHdG, 8-hydroxydeoxyguanosine; MDA, malondialdehyde.

Data are presented with the means ± standard deviation (n = 6).

[a,b,c,d]Means within rows with different superscript letters differ significantly ($P < 0.05$).

Table 4. Effects of fluorine and different selenium sources on glutathione peroxidase and cytoplasmic thioredoxin reductase activities and selenoprotein P concentrations in serum, liver and kidney of broilers.[1]

Items	Control group	High F group	SS group	Se-Met group
GSH-Px				
Serum (U/mL)	3086.22 ± 210.77^{a}	2097.73 ± 240.17^{c}	2748.30 ± 201.25^{b}	2916.88 ± 288.28^{a,b}
Liver (U/mgprot)	31.52 ± 2.37^{a}	17.42 ± 0.87^{b}	30.41 ± 2.36^{a}	30.74 ± 2.94^{a}
Kidney (U/mgprot)	49.30 ± 5.94^{a}	34.30 ± 3.58^{c}	42.51 ± 2.69^{b}	40.59 ± 4.55^{b}
TrxR1				
Serum (U/mL)	2533.18 ± 176.62^{a}	2058.02 ± 159.25^{b}	2450.31 ± 247.83^{a}	2550.92 ± 118.32^{a}
Liver (U/gprot)	7302.40 ± 407.21^{a}	5968.67 ± 538.75^{b}	7294.95 ± 522.77^{a}	6889.65 ± 814.37^{a}
Kidney (U/gprot)	5926.64 ± 591.03^{a}	4315.15 ± 603.70^{c}	5900.65 ± 440.93^{a}	5040.07 ± 480.33^{b}
SelP				
Serum (ng/mL)	169.20 ± 14.31^{a}	120.30 ± 12.75^{c}	144.71 ± 13.19^{b}	147.14 ± 12.47^{b}
Liver (ng/mgprot)	44.68 ± 4.36^{a}	32.88 ± 3.14^{b}	42.63 ± 5.05^{a}	40.06 ± 3.22^{a}
Kidney (ng/mgprot)	42.51 ± 4.46^{a}	27.62 ± 2.40^{c}	35.34 ± 3.20^{b}	39.28 ± 4.55^{a,b}

[1]Abbreviation: F, fluorine; SS, sodium selenite; Se-Met, selenomethionine; GSH-Px, glutathione peroxidase; TrxR1, cytoplasmic thioredoxin reductase; SelP, selenoprotein P.

Data are presented with the means ± standard deviation (n = 6).

[a,b,c]Means within rows with different superscript letters differ significantly ($P < 0.05$).

microvilli in renal proximal convoluted tubule epithelial cells were observed in high F group in comparision with control group. Compared with control group, the mitochondria of renal proximal convoluted tubule epithelial cells and hepatocytes were further enlarged and their cristae were further fractured or disappeared or both as well as the endoplasmic reticulum was further dilated or fractured or both in high F group. However, the structures of mitochondria, endoplasmic reticulum, and microvilli were better maintained in SS and Se-Met groups than those in high F group. Furthermore, the structures of mitochondria, endoplasmic reticulum, and microvilli were more complete and clear in Se-Met group than those in SS group. The pathological lesions in SS group were less than those in control group, whereas the major lesion in Se-Met group was similar with but less than that in control group.

Apoptosis Parameters

The contents of Bcl-2 and Caspase-3 in serum, liver, and kidney are presented in Table 5. The Caspase-3 contents in serum, liver, and kidney were increased ($P < 0.05$), and the serum, liver, and kidney Bcl-2 contents were decreased ($P < 0.05$) in high F group when compared with those in control group. The contents of Bcl-2 in serum, liver, and kidney were significantly higher ($P < 0.05$) in Se-Met group than those in high F group, whereas the Caspase-3 contents in serum, liver,

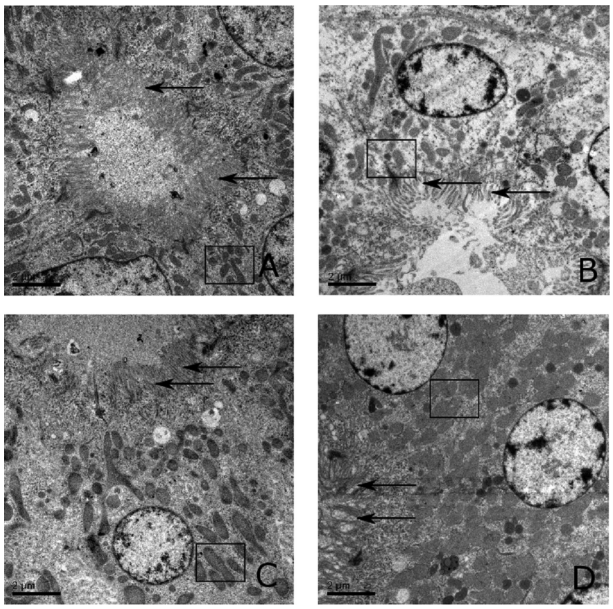

Figure 1. Effects of fluorine and different selenium sources on ultrastructural changes of renal proximal convoluted tubule epithelial cells in broilers. (A) The control group. The microvilli (TEM, ×10,000; arrows). (B) The high fluorine group. The shorter, sparser, and swollen microvilli (TEM, ×10,000; arrows) are observed in high F group in comparision with control group. (C) The sodium selenite group. The microvilli are more thinner and denser (TEM, ×10,000; arrows) than those in high F group. (D) The selenomethionine group. The microvilli are more complete and longer (TEM, ×10,000; arrows) than those in sodium selenite group and are similar with those in control group. The framed areas in (A, B, C, D) are magnified in (A, B, C, D) in Figure 2 respectively. Treatments were as follows: the control group was offered the basal diet; the high fluorine group was given the basal diet supplemented with 800 mg fluorine/kg as sodium fluoride; the groups sodium selenite and selenomethionine were given the high fluorine diet supplemented with 0.15 mg selenium/kg as sodium selenite or selenomethionine, respectively.

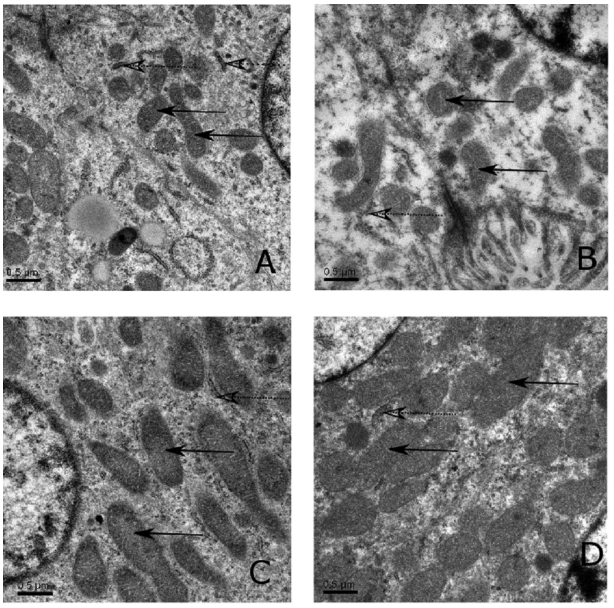

Figure 2. Effects of fluorine and different selenium sources on ultrastructural changes of renal proximal convoluted tubule epithelial cells in broilers. (A) The control group. The structures of endoplasmic reticulum (TEM, ×30,000; dotted arrows) and mitochondria (TEM, ×30,000; solid arrows). (B) The high fluorine group. The endoplasmic reticulum are further dilated or fractured or both (TEM, ×30,000; dotted arrow) and the cristae of mitochondria are further fractured or vanished or both (TEM, ×30,000; solid arrows) than those in control group. (C) The sodium selenite group. The structures of endoplasmic reticulum (TEM, ×30,000; dotted arrow) and mitochondria (TEM, ×30,000; solid arrows) are better maintained than those in high F group. (D) The selenomethionine group. The structures of endoplasmic reticulum (TEM, ×30,000; dotted arrow) and mitochondria (TEM, × 30,000; solid arrows) are more complete and clear than those in sodium selenite group and are restored to be close to those in control group. Treatments were as follows: the control group was offered the basal diet; the high fluorine group was given the basal diet supplemented with 800 mg fluorine/kg as sodium fluoride; the groups sodium selenite and selenomethionine were given the high fluorine diet supplemented with 0.15 mg selenium/kg as sodium selenite or selenomethionine, respectively.

and kidney were significantly dectreased ($P < 0.05$) in Se-Met group in comparison with high F group. Compared with high F group, SS group significantly increased ($P < 0.05$) serum and kidney Bcl-2 contents and decreased ($P < 0.05$) serum and liver Caspase-3 contents. Broilers consuming the Se-Met-supplemented diets had higher ($P < 0.05$) serum and tissues Bcl-2 contents and lower ($P < 0.05$) serum and kidney Caspase-3 contents than those receiving the SS-supplemented diets. Compared with control group, the Caspase-3 contents in serum and kidney were increased ($P < 0.05$), and the liver and kidney Bcl-2 contents were decreased ($P < 0.05$) in SS group. There were no significant differences in the serum and tissues Caspase-3 and Bcl-2 contents between Se-Met group and control group ($P > 0.05$).

TP Concentrations

The TP concentrations of serum, liver, and kidney were significantly decreased ($P < 0.05$) in high F group when compared with those in control group. Compared with high F group, Se-Met group significantly increased ($P < 0.05$) the TP concentrations in serum, liver, and kidney, and SS group significantly increased ($P < 0.05$) the serum and liver TP concentrations. In addition, the TP concentrations in serum, liver, and kidney were higher ($P < 0.05$) in Se-Met group than those in SS group. The concentrations of TP in serum, liver, and kidney were markedly decreased ($P < 0.05$) by SS group in comparison with control group. But there were no obvious differences between Se-Met group and control group ($P > 0.05$) (Table 6).

Growth Performance

As shown in Table 7, high F group significantly decreased ($P < 0.05$) the ADG and feed efficiency (**FE**) during growing phase (22 to 50 d of age) and cumulative period (1 to 50 d of age) but not starting phase (1 to 21 d of age) ($P > 0.05$) when compared with control group. The ADG and FE in 2 Se supplement groups were increased ($P < 0.05$) from 22 to 50 d and 1 to 50 d of age when compared with those in high F group.

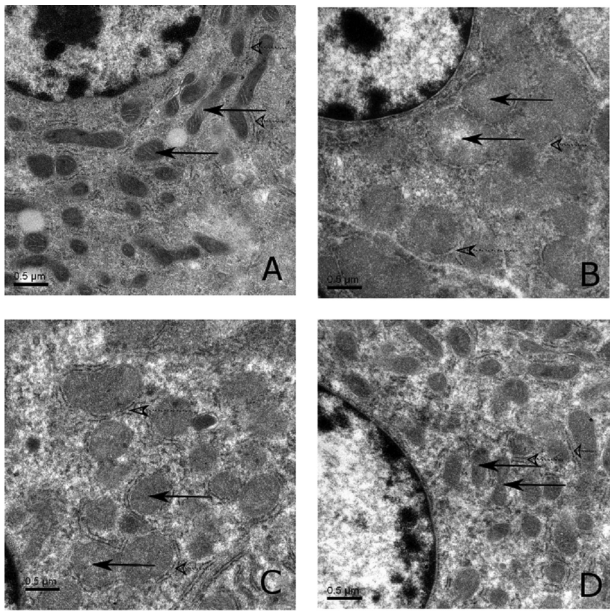

Figure 3. Effects of fluorine and different selenium sources on ultrastructural changes of hepatocytes in broilers. (A) The control group. The structures of endoplasmic reticulum (TEM, ×30,000; dotted arrows) and mitochondria (TEM, ×30,000; solid arrows). (B) The high fluorine group. The endoplasmic reticulum is further dilated or fractured or both (TEM, ×30,000; dotted arrows) and the mitochondria is further enlarged and their cristae are further fractured or disappeared or both (TEM, ×30,000; solid arrows) than those in control group. (C) The sodium selenite group. The structures of endoplasmic reticulum (TEM, ×30,000; dotted arrows) and mitochondria (TEM, ×30,000; solid arrows) are better maintained than those in high F group. (D) The selenomethionine group. The structures of endoplasmic reticulum (TEM, ×30,000; dotted arrows) and mitochondria (TEM, × 30,000; solid arrows) are more complete and clear than those in sodium selenite group and are restored to be close to those in control group. Treatments were as follows: the control group was offered the basal diet; the high fluorine group was given the basal diet supplemented with 800 mg fluorine/kg as sodium fluoride; the groups sodium selenite and selenomethionine were given the high fluorine diet supplemented with 0.15 mg selenium/kg as sodium selenite or selenomethionine, respectively.

Besides, the ADG and FE in group of Se-Met were higher ($P < 0.05$) than those in group of SS from 22 to 50 d and 1 to 50 d of age. No differences among all groups were observed in ADFI in any period of growth or in the overall period ($P > 0.05$). Compared with those in control group, the ADG and FE in SS group were decreased ($P < 0.05$) from 22 to 50 d and 1 to 50 d of age, whereas the ADG and FE from 22 to 50 d and 1 to 50 d of age in Se-Met group were approximate to those in control group ($P > 0.05$).

DISCUSSION

Fluorine is a redox-active metal; it was reported to induce the formation of ROS (García-Montalvo et al., 2009). The relationships between F and ROS have been investigated by numerous authors (Wang et al., 1997; Shanthakumari et al., 2004; Chouhan and Flora, 2008). As expected, ROS levels in serum, liver, and kidney were significantly higher for broilers in high F group than those for broilers in control group in the current study, which were consistent with previous studies.

GSH-Px, TrxR1, and SelP play an important role in the elimination of ROS (Steinbrenner and Sies, 2009). The data from our present trial demonstrated that high F group could decrease the SelP concentrations as well as GSH-Px and TrxR1 activities in serum, liver, and kidney when compared with control group, which were consistent with those of previous reports (Hassan and Yousef, 2009; Liu et al., 2012b; Deng et al., 2014). The decreased GSH-Px and TrxR1 activities as well as SelP concentration in high F group might be due to the overproduction of ROS and consumption of the GSH-Px, TrxR1, and SelP, thus making excessive ROS accumulation.

Excessive levels of ROS can lead to lipid, protein, and DNA oxidation and subsequently produce MDA, protein carbonyl, and 8-OHdG respectively, which are reliable biomarkers for oxidative stress (Chevion et al., 2000; Gaweł et al., 2004). In this research, our data showed that birds from high F group had higher MDA, protein carbonyl, and 8-OHdG contents in serum, liver, and kidney than those from control group, which were similar to previous reports of Jia et al. (2008), Chen et al. (2011a), and Deng et al. (2014), and indicating that oxidative stress may be induced by high F in the present study.

It has been well established that oxidative stress induced by F could cause damage to liver and kidney (Chinoy et al., 2004; Bai et al., 2010; Nabavi et al., 2012). In the present study, we observed the mitochondria were swollen and their cristae were fragmented or disappeared or both as well as the dilation or fragmentation of endoplasmic reticulum in hepatocytes and renal proximal convoluted tubule epithelial cells in high F group in comparision with control group, indicating that high F may cause cell oxidative damage to liver and kidney, which were in accordance with the results of previous studies. Besides, our results aslo showed that high F group had shorter, sparser, and swollen microvilli in renal proximal convoluted tubule epithelial cells than control group, implying that the renew absorptive function of renal proximal convoluted tubule epithelial cells may be impaired by high F.

Oxidative damage that occur in mitochondria can lead to apoptosis through a mitochondria-mediated pathway (Chatterjee et al., 2008), and Bcl-2 and Caspase-3 are the proteins that associated with apoptosis mediated by mitochondria. Bcl-2 is an anti-apoptotic protein, and Caspase-3 activation plays the great role in the execution phase of cell apoptosis (Bruce-Keller et al., 1998; Chen et al., 2001). In the present study, compared with those in control group, a significant increase in Caspase-3 contents along with the marked decrease in Bcl-2 contents in serum, liver, and kidney was observed in high F group, which were in concordance with earlier studies of broilers (Chen et al., 2009) and rats (Wang et al., 2009). Our results implied that high F may induce apoptosis by controling some regulatory

Table 5. Effects of fluorine and different selenium sources on apoptosis parameters of broilers.[1]

Items	Control group	High F group	SS group	Se-Met group
Bcl-2				
Serum (ng/mL)	21.06 ± 1.29[a,b]	15.73 ± 0.60[c]	18.95 ± 0.91[b]	22.91 ± 2.41[a]
Liver (ng/mL)	152.67 ± 14.73[a]	113.84 ± 7.65[b]	121.00 ± 10.49[b]	142.32 ± 10.76[a]
Kidney (ng/mL)	188.56 ± 17.42[a]	111.98 ± 17.94[c]	138.73 ± 10.92[b]	181.54 ± 8.77[a]
Caspase-3				
Serum (ng/mL)	30.23 ± 3.37[c]	51.42 ± 5.33[a]	40.56 ± 2.57[b]	31.05 ± 1.25[c]
Liver (ng/mL)	34.76 ± 2.24[b]	44.15 ± 4.43[a]	38.07 ± 2.94[b]	35.45 ± 3.06[b]
Kidney (ng/mL)	38.32 ± 2.95[b]	46.34 ± 3.68[a]	44.08 ± 3.17[a]	38.85 ± 2.61[b]

[1]Abbreviation: F, fluorine; SS, sodium selenite; Se-Met, selenomethionine; Bcl-2, B-cell lymphoma-2; Caspase-3, cysteinyl aspartate specific proteinases 3.

Data are presented with the means ± standard deviation (n = 6).

[a,b,c]Means within rows with different superscript letters differ significantly ($P < 0.05$).

Table 6. Effects of fluorine and different selenium sources on total protein concentrations in serum, liver, and kidney of broilers.[1]

Items	Control group	High F group	SS group	Se-Met group
Serum (gprot/L)	35.04 ± 4.30[a]	25.89 ± 2.88[c]	31.03 ± 3.88[b]	35.15 ± 3.98[a]
Liver (mgprot/g)	100.20 ± 12.33[a]	72.50 ± 7.20[c]	84.56 ± 8.10[b]	97.19 ± 12.31[a]
Kidney (mgprot/g)	119.26 ± 9.78[a]	88.37 ± 9.57[b]	100.55 ± 11.00[b]	115.55 ± 11.67[a]

[1]Abbreviation: F, fluorine; SS, sodium selenite; Se-Met, selenomethionine; Bcl-2, B-cell lymphoma-2; Caspase-3, cysteinyl aspartate specific proteinases 3.

Data are presented with the means ± standard deviation (n = 6).

[a,b,c]Means within rows with different superscript letters differ significantly ($P < 0.05$).

Table 7. Effects of fluorine and different selenium sources on growth performance and feed efficiency of broilers.[1]

Items	Control group	High F group	SS group	Se-Met group
1 to 21 d of age				
Initial weight (g)	38.03 ± 0.27	38.39 ± 0.21	38.36 ± 0.32	38.36 ± 0.39
ADG (g/d)	21.30 ± 0.90	20.67 ± 0.47	20.86 ± 0.80	21.14 ± 0.54
ADFI (g/d)	33.78 ± 0.73	34.89 ± 0.96	34.64 ± 2.73	34.27 ± 0.70
FCR (g/g)	1.61 ± 0.08	1.70 ± 0.03	1.67 ± 0.07	1.62 ± 0.05
22 to 50 d of age				
ADG (g/d)	46.83 ± 0.92[a]	41.21 ± 0.86[c]	43.29 ± 1.08[b]	46.03 ± 1.49[a]
ADFI (g/d)	117.15 ± 3.73	111.83 ± 3.00	117.58 ± 3.74	113.59 ± 1.15
FCR (g/g)	2.52 ± 0.05[c]	2.99 ± 0.07[a]	2.80 ± 0.02[b]	2.55 ± 0.09[c]
1 to 50 d of age				
ADG (g/d)	36.11 ± 0.18[a]	32.58 ± 0.38[c]	33.87 ± 0.31[b]	35.58 ± 0.66[a]
ADFI (g/d)	81.23 ± 1.47	80.00 ± 1.03	82.45 ± 0.78	80.79 ± 0.40
FCR (g/g)	2.29 ± 0.05[c]	2.60 ± 0.05[a]	2.49 ± 0.02[b]	2.31 ± 0.05[c]

[1]Abbreviation: F, fluorine; SS, sodium selenite; Se-Met, selenomethionine; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio (feed/gain).

Data are presented with the means ± standard deviation (n = 6).

[a,b,c]Means within rows with different superscript letters differ significantly ($P < 0.05$).

genes (such as Bcl-2 and Caspase-3), but there is a lot of work which still need to be done before arriving to a final conclusion.

It has been reported that fluorosis can result in decreased tissue and serum TP concentrations in rats (Chinoy et al., 1993; Paul et al., 1998). Our study found that the concentrations of serum, liver, and kidney TP in control group were higher than those in high F group, consistent with previous reports. Serum and tissues TP concentrations were decreased in high F group which may contribute to the decreased activity of Na^+-K^+-activated ATPase, an enzyme that was indispensable to the uptake of amino acids by tissues (Hoertz and McCarty, 1971; Holland, 1979). When NaF suppressed Na^+-K^+-activated ATPase activity by inducing ROS production (Opit et al., 1966), protein synthesis was inhibited and this could ultimately resulted in the reduction of serum and soft tissues TP concentrations. Moreover, in the present study, the decreased ADG and FE were observed in high F group in comparison with control group, which were in agreement with similar findings by Shanthakumari et al. (2004) in rats and Chen et al. (2009) in broilers. A depletion of protein in soft tissues, apoptosis, and oxidative damage to biological macromolecules and soft tissues may explain why high F could inhibit body growth of broilers. However, no difference in ADFI between high F group and control group was observed throughout the experimental

period. But inconsistent results in rats have been reported by Pillai et al. (1988) and Paul et al. (1998). Such discrepancies may result from the variations in animals, diet type, dietary F dosage, experimental conditions, and other parameters.

In the present study, the contents of ROS, protein carbonyl, 8-OHdG, and MDA in SS and Se-Met groups were lower than those in high F group, and the GSH-Px and TrxR1 activities as well as SelP concentration in SS and Se-Met groups were higher than those in high F group. Compared with those in high F group, the contents of Bcl-2 and TP in SS and Se-Met groups were increased, whereas the contents of Caspase-3 were decreased. Moreover, the pathological lesions in liver and kidney in SS and Se-Met groups were less than those in high F group. At the same time, it was also observed in SS and Se-Met groups that the ADG and FE were higher than those in high F group. Besides, SS and Se-Met supplementation could restore some parameters influenced by high F to be close to those in control group. Our results indicated that SS and Se-Met could effectively protect broilers from adverse effects caused by high F, which could be explained by the fact that SS and Se-Met supplementation enhanced Se concentrations in serum and tissues of broilers (Cantor et al., 1975; Payne and Southern, 2005; Wang and Xu, 2008; Wang et al., 2011a,b,c). The increased Se concentrations elevated SelP concentration and activities of GSH-Px and TrxR1, then the abilities of Se-dependent and Se-independent ROS scavenging were enhanced and antioxidative capacity of broilers was restored, thus leading to an increase in TP concentrations and a decrease in oxidative injury to biological macromolecules and soft tissues, which helped to maintain cell structure integrity and repress ROS-mediated apoptosis, and eventually counteracted growth restriction caused by high F in broilers. These results were confirmed by Molina and Garcıa (1997) and Yu et al. (2006). In addition, increased GSH-Px and TrxR1 activities, SelP concentration, and Se deposition may also improve immune function, which also benefited the recovery of growth of stressed broilers.

Data obtained from the present study also exhibited that the broilers feeding Se-Met had higher Bcl-2 content, TP concentration, ADG, and FE as well as lower ROS, Caspase-3, and oxidation products contents than those feeding SS. Meanwhile, Se-Met had better efficacy in maintaining cell structure than SS. Our results suggested that Se-Met was more effective than SS in alleviating the deleterious effects of high F on broilers. However, in the current study, kidney TrxR1 activity was higher in SS group than that in Se-Met group, and no significant changes in serum and tissues GSH-Px activities and SelP concentrations as well as serum and liver TrxR1 activities were detected between SS and Se-Met treatments, which were in line with the reports of Cantor et al. (1975) and Jiang et al. (2009) as well as a series of previous studies from our laboratory (Zhan et al., 2007,2014; Hu et al., 2011; Wang et al., 2011a,b). Therefore, we speculated that the different protective roles of SS and Se-Met on F-induced oxidative stress might have nothing to do with the GSH-Px, TrxR1, and SelP, whereas the GSH-Px and TrxR1 activities and SelP concentration might not be conclusive indexes of bioavailability or Se's ability to influence anti-oxidative stress capacity in broilers. The increased serum and tissues Se depositions in Se-Met group (Wang et al., 2011a,b,c; Yuan et al., 2011) due to the differences in absorption and metabolism of SS and Se-Met (Wolffram et al., 1989; Schrauzer, 2000) and oxidative stress-related signaling pathways may play a vital role in regulating the different protective effects of SS and Se-Met on F-induced oxidative stress. Xiao and Parkin (2006) showed that Se-Met supplementation resulted in increased activities of the Nrf2-target enzymes in Hepa 1c1c7 cells, but SS supplementation was not capable of Nrf2-target enzymes induction. In addtion, Chang et al. (2014) observed that organic Se treatment significantly suppressed asthma-induced NF-κB expression, but the same result did not occur in the SS treatment. Therefore, we inferred that oxidative stress-related signaling pathways Nrf2 and NF-κB may play an important part in regulating the different antagonistic effects of SS and Se-Met on F-induced oxidative stress, but the exact mechanisms need to be further investigated. Our results also suggested that dietary Se-Met supplementation could be considered an effective nutritional solution to decrease negative effects of oxidative stress on broilers.

According to our results and the above discussions, we concluded that high F intake could lead to increase ROS production and depress the activities of GSH-Px and TrxR1 as well as SelP concentration, which resulted in oxidative stress and subsequently caused decreased TP concentrations, oxidative damage to biological macromolecules and soft tissues as well as apoptosis, and ultimately reduced ADG and FE of broilers. However, dietary SS and Se-Met supplementation substantially alleviated the detrimental effects of high F on broilers through elevating the activities of GSH-Px and TrxR1 as well as SelP concentration, blocking ROS overproduction, increasing TP concentrations, decreasing oxidative injury to biological macromolecules and soft tissues, and changing the expression of Bcl-2 and Caspase-3. Our study also showed that the protective effects of Se-Met were better than those of SS. The possible mechanism was that dietary supplementation with Se-Met was more favorable for the improvement of Se deposition and activation of oxidative stress-related signaling pathways. However, the specific mechanism remains to be evaluated rigorously in future studies.

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