



Original Research Article

Determination of optimal dietary selenium levels by full expression of selenoproteins in various tissues of broilers from 22 to 42 d of age



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ARTICLE INFO

Article history:

Received 14 February 2021

Received in revised form

29 April 2021

Accepted 11 May 2021

Available online 23 September 2021

Keywords:

Selenium

Selenoprotein

Gene expression

Requirement

Broiler

ABSTRACT

The current NRC dietary selenium (Se) requirement (0.15 mg/kg) of broilers from 22 to 42 d of age is primarily based on a previous study reported in 1986, which might not be applicable to modern classes of rapidly growing broilers. The present experiment was conducted to determine the optimal dietary Se level for meeting metabolic and functional Se requirements of broilers fed a corn-soybean meal diet from 22 to 42 d of age. A total of 336 Arbor Acres male broilers at 22 d old were randomly assigned to 1 of 6 treatments with 7 replicates and fed a basal corn-soybean meal diet (control, containing 0.014 mg Se/kg) and the basal diet supplemented with 0.10, 0.20, 0.30, 0.40, or 0.50 mg Se/kg from Na₂SeO₃ for 21 d. The results showed that the Se concentrations in plasma, liver, kidney, pancreas, breast and thigh muscles, the activity of glutathione peroxidase (GPX) in plasma, liver and kidney, the mRNA expression levels of *Gpx4*, selenoprotein (*Seleno*) *h* and *Selenou* in liver, *Selenop* and *Selenoh* in kidney, and the protein expression levels of GPX4 in the liver and kidney of broilers were affected ($P < 0.05$) by supplemental Se level, and increased quadratically ($P < 0.05$) with the increase of supplemental Se level. The estimates of optimal dietary Se levels were 0.10 to 0.49 mg/kg based on the fitted broken-line or asymptotic models ($P < 0.0001$) of the above Se concentration indices, and 0.08 to 0.37 mg/kg based on the fitted broken-line, quadratic or asymptotic models ($P < 0.007$) of the above selenoprotein expression indices. These results indicate that the optimal dietary Se levels would be 0.49 mg/kg to support the maximum Se concentrations and 0.37 mg/kg to support the full expression of selenoproteins in plasma and various tissues of broilers fed a corn-soybean meal diet from 22 to 42 d of age.

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1. Introduction

Selenium (Se) is an essential micronutrient for humans and animals, and it plays an important role in antioxidation, immunity and thyroid hormone metabolism (Edens and Sefton, 2016; Lei and Burk, 2018; Rayman, 2000; Roman et al., 2014; Wang et al., 2021; Yuan et al., 2011; Zhang et al., 2018). Broilers are fast-growing birds and susceptible to dietary Se deficiency, and thus supplementing Se in diets prevent clinical defects and ensure optimal growth is a common technique in the broiler industry (Jensen et al., 1986; Sun et al., 2018; Wang and Xu, 2008). The current NRC dietary Se requirements for broilers from 1 to 21 and 22 to 42 d of age are 0.15 mg/kg (NRC, 1994), which is primarily based on growth

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.



performance data from a previous study (Jensen et al., 1986). However, growth performance might be not the most sensitive of indices to reflect the metabolic and functional Se requirements for broilers (Sunde et al., 2016). Therefore, it is necessary to evaluate dietary Se requirements based on Se metabolic and functional indices of modern broilers strains.

The many metabolic functions of Se are carried out by selenoproteins. There are 24 confirmed selenoproteins in chickens, including glutathione peroxidase (GPX), iodothyronine deiodinase (DIO) and thioredoxin reductase (TXNRD) families, selenoprotein H (SELENOH), selenoprotein P (SELENOP), selenoprotein U (SELENOU), etc (Kryukov et al., 2003; Labunsky et al., 2014; Li et al., 2018). Li and Sunde (2016) found that the minimum Se requirements were 0.15 to 0.20 mg/kg based on enzyme activity and transcript level of selenoproteins in different tissues of broilers fed a semi-purified diet from 1 to 29 d of age. Furthermore, in our laboratory, it has been found that the optimal dietary Se levels were 0.36 to 0.46 mg/kg to meet selenoprotein expressions in plasma, liver and kidney or in the pancreas of broilers fed a practical corn-soybean meal diet from 1 to 21 d of age (Liao et al., 2021). However, dietary Se requirements for meeting all the selenoprotein expressions in various tissues of broilers from 22 to 42 d of age have not been investigated before. Furthermore, the dietary Se requirements of broilers might be different between 1 to 21 and 22 to 42 d. Therefore, the present study was a continuation of our above study, and it was hypothesized that the dietary Se requirements for meeting the metabolic and functional Se requirements in modern classes of rapidly growing broilers might be higher than the current NRC Se requirement for optimum growth performance. The objective of the current study is to investigate the influence of dietary Se levels on growth performance, Se concentrations, and enzyme activity as well as mRNA and protein expression levels of selenoproteins, so as to evaluate the optimal dietary Se level of broilers fed a corn-soybean meal diet from 22 to 42 d of age.

2. Materials and methods

All experimental procedures were approved by the Animal Management Committee of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS, Beijing, China), and performed in accordance with the guidelines. We have followed the ARRIVE guidelines for reporting animal research (Kilkenny et al., 2012).

2.1. Animals, diets and experimental design

A total of 400 1-d-old Arbor Acres male broilers (Huadu Broiler Breeding Co., Beijing, China) were housed in electrically heated, thermostatically controlled stainless steel cages equipped with fibreglass feeders and a watering system, and were allowed ad libitum access to the experimental diets and tap water containing no detectable Se. Broilers were vaccinated with Newcastle disease vaccine at 7 and 21 d of age, and with infectious bursal disease virus vaccine and bronchitis virus vaccine at 14 and 28 d of age, respectively. During 1–21 d, the broilers were fed with a corn-soybean meal-based diet, containing 0.46 mg Se/kg of diet by analysis, which was formulated to meet the requirements of starter broilers for all nutrients (Table 1), and the dietary Se level was based on the result of our previous study in broilers from 1 to 21 d of age (Liao et al., 2021). At 22 d of age, 336 broilers with similar body weights were randomly divided into 1 of 6 treatment groups with 7 replicate cages (8 chickens/cage) for each treatment in a completely randomized design. Broilers were fed a corn-soybean based diet supplemented with 0.00, 0.10, 0.20, 0.30, 0.40 or 0.50 mg Se/kg (reagent grade sodium selenite, Sinopharm Chemical

Table 1
Ingredients and nutrient levels of basal diets for broilers (% as-fed basis).

Item	Day 1 to 21	Day 22 to 42
Ingredients		
Corn	53.41	55.15
Soybean meal	38.10	36.00
Soybean oil	4.42	5.30
CaHPO ₄ ¹	1.82	1.50
CaCO ₃ ¹	1.32	1.22
NaCl ¹	0.30	0.30
DL-Methionine	0.30	0.11
Premix ^{2,3}	0.33	0.22
Cornstarch + Se	0.00	0.2
Total	100	100
Nutrient composition		
Metabolizable energy, kcal/kg	3,000	3,065
Crude protein ⁴	21.51	19.75
Lysine	1.14	1.00
Methionine	0.59	0.40
Methionine + Cysteine	0.90	0.74
Calcium ⁴	1.00	0.90
Non-phytate phosphorus	0.45	0.35
Selenium ⁴ , mg/kg	0.46	0.014

¹ Feed grade before d 21 of age and reagent grade after d 21 of age.

² Provided per kilogram of diet from d 1 to 21: vitamin A (all-trans retinol acetate), 15,000 IU; cholecalciferol, 4,500 IU; vitamin E (all-rac- α -tocopherol acetate), 24 IU; vitamin K₃ (menadione sodium bisulfate), 3 mg; thiamin (thiamin mononitrate), 3 mg; riboflavin, 9.6 mg; pyridoxine, 3 mg; vitamin B₁₂, 0.018 mg; calcium pantothenate, 15 mg; niacin, 39 mg; folic acid, 1.5 mg; biotin, 0.15 mg; choline, 700 mg; Zn (ZnSO₄•7H₂O), 60 mg; Cu (CuSO₄•5H₂O), 8 mg; Mn (MnSO₄•H₂O), 110 mg; Fe (FeSO₄•7H₂O), 40 mg; Se (Na₂SeO₃) 0.35 mg; I (KI), 0.35 mg; chlortetracycline, 50 mg.

³ Provided per kilogram of diet from d 22 to 42: vitamin A (all-trans retinol acetate), 10,000 IU; cholecalciferol, 3,000 IU; vitamin E (all-rac- α -tocopherol acetate), 16 IU; vitamin K₃ (menadione sodium bisulfate), 2 mg; thiamin (thiamin mononitrate), 2 mg; riboflavin, 6.4 mg; pyridoxine, 2 mg; vitamin B₁₂, 0.012 mg; calcium pantothenate, 10 mg; niacin, 26 mg; folic acid, 1 mg; biotin, 0.1 mg; choline, 500 mg; Zn (ZnSO₄•7H₂O), 40 mg; Cu (CuSO₄•5H₂O), 8 mg; Mn (MnSO₄•H₂O), 80 mg; Fe (FeSO₄•7H₂O), 30 mg; I (KI), 0.35 mg.

⁴ Values determined by analysis. Each value based on triplicate determinations.

Reagent Co., Ltd, Shanghai, China) for 21 d, and the basal diet met the requirements for all nutrients except for Se (Table 1). The dietary Se concentrations for the 6 treatments were 0.014, 0.125, 0.221, 0.324, 0.427 and 0.521 mg/kg by analysis on an as-fed basis, respectively. At 42 d of age, broiler weight and feed intake were recorded to calculate average daily weight gain, average daily feed intake, and gain-to-feed ratio during the study period.

2.2. Sample collections and preparations

At 42 d of age, 14 chickens (2 chickens/cage) from each treatment group were selected according to average body weight of each cage after a 12-h fast, respectively. Blood samples were taken from each broiler via cardiac puncture and then centrifuged to harvest Se concentrations and the activity of GPX, DIO and TXNRD. Liver, pancreas, kidney, thigh and breast muscle samples were collected immediately, and a set of tissue samples were snap frozen in liquid N₂ and then stored at –80 °C for determinations of *Gpx1*, *Gpx4*, *Dio1*, *Txnrd1*, *Txnrd2*, *Selenoh*, *Selenop* and *Selenou* mRNA expression levels as well as GPX1 and GPX4 protein expression levels. Another set of sub-samples were kept on ice and stored at –20 °C for subsequent measurements of Se concentrations, GPX, DIO and TXNRD activity. All samples from two birds in each replicate were pooled into one sample in equal ratios before analysis.

2.3. Se concentrations and enzyme activity

The Se concentrations in feed ingredients, water, diets, blood and all tissues were measured using the fluorescence method with

a Hitachi 850 fluorescence spectrophotometer (Liao et al., 2012; Zhang et al., 2018). Bovine liver powder (GBW (E) 080,193, National Institute of Standards and Technology, Beijing, China) was used as a standard reference material for validation of the Se analysis. The GPX and TXNRD activity (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as well as DIO activity (Shanghai Jianglaibio Company Ltd, Shanghai, China) were measured by a colorimetric method with the use of specific commercial assay kits, respectively. All procedures were carried out according to the manufacturers' instructions.

2.4. RNA extraction and quantitative RT-PCR

Total RNA in all tissues was isolated using a Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The purity, concentration, and integrity of the RNA were checked using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and an Agilent 2100 Bio-analyzer (Agilent Technologies, Palo Alto, Calif), respectively. Single-strand cDNA was synthesized using the PrimeScript RT Master Mix kit (Takara, Otsu, Japan). Real-time quantitative PCR was performed using an Applied Biosystems 7500 Real-Time PCR System using SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Primer sequences for *Gpx1*, *Gpx4*, *Dio1*, *Txnrd1*, *Txnrd2*, *Selenop*, *Selenoh* and *Selenou*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β -actin (Table 2) were used for amplification reactions according to their gene sequences published in GenBank, respectively. Each gene was amplified independently in triplicate within a single instrument run. The mRNA level of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and the geometric mean of internal references, β -actin and *GAPDH*, was used to normalize the expression of the target gene.

2.5. Western blotting assay

The GPX1 and GPX4 protein levels in the liver, kidney and pancreas were determined using the Western blot technique. Frozen liver, pancreas and kidney samples were homogenized in ice-cold Radio Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with protease inhibitor (Biotool, Houston, TX, USA). The homogenate was centrifuged at $10,000 \times g$ for 10 min at 4 °C, and the supernatant was collected for total protein determination using a BCA protein assay kit (Pierce, Rockford, IL, USA). Total proteins (30 μ g) were separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride membrane (Merck-Millipore, Munich, Germany). After being blocked in 5% BSA blocking solution for 1 h at

room temperature, the membrane was incubated overnight at 4 °C with primary antibodies against GPX1, GPX4 (Abcam, Cambridge, MA, USA) and GAPDH (Huaxingbio, Beijing, China). After 5 washes for 5 min in Tris-buffered saline with Tween, the strips with GPX1 and GPX4 were incubated with rabbit anti-goat horseradish peroxidase-conjugated antibody (Huaxingbio, Beijing, China) and the strip with GAPDH was incubated with goat anti-mouse horseradish peroxidase-conjugated antibody (Huaxingbio, Beijing, China). The GAPDH protein was used to normalize the expression levels of target proteins.

2.6. Statistical analyses

The effect of dietary Se treatment was analyzed by one-way ANOVA using the general liner model procedure of SAS (version 9.4, SAS Institute Inc.). The least significant difference method was used to test the differences among means. The cage was the experimental unit. Orthogonal comparisons were applied for linear and quadratic responses of dependent variables to independent variables. Regression analyses of broken-line, quadratic and asymptotic models were performed, respectively, and the best fit model between responsive criteria and supplemental Se concentration was used to estimate the optimal supplemental Se level (the break point from the broken-line model, the maximum response from the quadratic model, or 95% of the maximum response from the asymptotic model) for broiler chicks (Corzo et al., 2006; Ma et al., 2016; Robbins et al., 2007). The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Growth performance

Dietary Se level did not affect average daily weight gain, average daily feed intake, and gain-to-feed ratio of broilers from 22 to 42 d of age as described in our previous study (Wang et al., 2020).

3.2. Se concentration

Dietary Se level affected ($P < 0.0001$) Se concentrations in blood and tissues (Table 3). The Se concentrations of blood and tissues increased linearly ($P < 0.0001$) and quadratically ($P < 0.0001$) with the increase of dietary Se level. The Se concentration of breast muscle reached a plateau at supplemental 0.30 mg Se/kg; the Se concentration of liver reached a plateau at supplemental 0.40 mg Se/kg; and the Se concentrations of blood, kidney, pancreas and thigh muscle reached the highest point at supplemental 0.50 mg Se/kg.

Table 2
Primer sequences for real-time PCR amplification.

Gene	GenBank identity	Product length, bp	Primer sequences
β -actin	NM205518.1	95	Forward:5'-ACCTGAGCGCAAGTACTCTGTCT-3' Reverse:5'-CATCGTACTCCTGCTTCTGAT-3'
<i>GAPDH</i>	NM204305.1	128	Forward:5'-CTTTGGCATTGTGGAGGGTC-3' Reverse:5'-ACGCTGGGATGTGTTCTGG-3'
<i>Gpx1</i>	NM001277853.2	73	Forward:5'-ACGGCGCATCTTCCAAAG-3' Reverse:5'-TGTTCCCCAACCAATTCTC-3'
<i>Gpx4</i>	NM001346448.1	72	Forward:5'-CTTCGTCTGCATCATACCAA-3' Reverse:5'-TCGACGAGCTGAGTGTAAATTCAC-3'
<i>Txnrd1</i>	NM001352023.1	107	Forward:5'-TACGCCTCTGGGAAATTCGT-3' Reverse:5'-CTTGCAAGCTTGTCCAGTA-3'
<i>Txnrd2</i>	NM001122691.2	82	Forward:5'-GCTCTTAAAGATGCCAGCACTAC-3' Reverse:5'-GAACAGCTTGAGCCATCACAGA-3'
<i>Selenop</i>	NM001031609.2	81	Forward:5'-CCAAGTGGTCAGCATTACATC-3' Reverse:5'-ATGACGACCACCCTCACGAT-3'
<i>Selenoh</i>	NM001193519.2	133	Forward:5'-GATGCTTTCAGGCTTCTTCC-3' Reverse:5'-CTGTCTCCTGCTCCAATCA-3'
<i>Selenou</i>	NM001277865.1	135	Forward:5'-CATCGAGCACTGCCGTAG-3' Reverse:5'-GACACTCGAAGCTGTCTCT-3'
<i>Dio1</i>	NM001097614.1	128	Forward:5'-GCGCTATACCAACAGCGAGTA-3' Reverse:5'-GGTCTTGCAAATGTCCACCAC-3'

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase; *Gpx1* = glutathione peroxidase 1; *Gpx4* = glutathione peroxidase 4; *Txnrd1* = thioredoxin reductase 1; *Txnrd2* = thioredoxin reductase 2; *Selenop* = selenoprotein P; *Selenoh* = selenoprotein H; *Selenou* = selenoprotein U; *Dio1* = iodothyronine deiodinase 1.

Table 3
Effect of supplemental Se level on Se concentrations in plasma and tissues of broilers at 42 d of age.¹

Supplemental Se, mg/kg	Plasma Se, µg/mL	Liver Se, fresh basis, µg/g	Kidney Se, fresh basis, µg/g	Pancreas Se, fresh basis, µg/g	Breast muscle Se, fresh basis, µg/g	Thigh muscle Se, fresh basis, µg/g
0.00	0.013 ^d	0.099 ^d	0.219 ^e	0.078 ^d	0.045 ^d	0.051 ^e
0.10	0.122 ^c	0.394 ^c	0.521 ^d	0.184 ^c	0.082 ^c	0.097 ^d
0.20	0.142 ^b	0.423 ^{bc}	0.599 ^c	0.205 ^c	0.096 ^b	0.100 ^{dc}
0.30	0.143 ^b	0.444 ^{ab}	0.633 ^{bc}	0.237 ^b	0.107 ^a	0.107 ^{bc}
0.40	0.148 ^b	0.482 ^a	0.695 ^{ab}	0.262 ^a	0.105 ^a	0.115 ^{ab}
0.50	0.166 ^a	0.477 ^a	0.756 ^a	0.275 ^a	0.108 ^a	0.122 ^a
Pooled SE	0.005	0.015	0.025	0.007	0.002	0.003
P-value						
Se level	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Linear	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Quadratic	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Se = selenium.
^{a,b,c,d,e} Means with different superscripts within a column differ ($P < 0.05$).

¹ Each datum represents the mean of 7 replicate cages ($n = 7$).

3.3. Enzyme activity

Dietary Se level did not affect ($P > 0.05$) TXNRD activity in the blood and pancreas, or DIO activity in the blood, liver and pancreas; however, it affected ($P < 0.05$) GPX activity in the blood, liver, kidney and pancreas, TXNRD activity in the liver and kidney, and DIO activity in the kidney (Table 4). Apart from GPX activity in the pancreas, DIO activity in the kidney, and TXNRD activity in the liver and kidney increased linearly ($P < 0.01$) with the increase of dietary Se level; the other indicators increased linearly ($P < 0.001$) and quadratically ($P < 0.001$). The GPX and TXNRD activity in the liver and TXNRD activity in the kidney reached plateaus at supplemental 0.20 mg Se/kg; DIO activity in the kidney reached a plateau at supplemental 0.30 mg Se/kg; GPX activity in the blood, kidney and pancreas reached the highest point at supplemental 0.50 mg Se/kg.

3.4. mRNA expression levels

Dietary Se level did not affect ($P > 0.05$) the mRNA expression levels of *Gpx1*, *Txnrd1*, *Txnrd2* and *Dio1* in the liver, *Txnrd1*, *Txnrd2* and *Dio1* in the kidney or *Gpx4*, *Txnrd1*, *Txnrd2*, *Selenop* and *Selenoh* in the pancreas; however, it affected ($P < 0.05$) the mRNA expression levels of *Gpx4*, *Selenop*, *Selenou* and *Selenoh* in the liver and kidney, *Gpx1* in the kidney and pancreas, and *Dio1* in the pancreas (Tables 5–7). The mRNA expression levels of *Selenop* in the liver, and *Gpx4* and *Selenou* in the kidney increased linearly ($P < 0.05$) with the increase of dietary Se level; the mRNA levels of

Selenou in the liver; and *Gpx1* in the kidney increased quadratically ($P < 0.05$) with the increase of dietary Se level; the mRNA expression levels of *Gpx4* and *Selenoh* in the liver, and *Selenop* and *Selenoh* in the kidney increased linearly ($P < 0.05$) and quadratically ($P < 0.05$) with the increase of dietary Se level. The mRNA expression levels of *Selenop* and *Selenoh* in the kidney reached plateaus and the mRNA expression levels of *Gpx1* in the liver reached the highest point at supplemental 0.10 mg Se/kg; the mRNA expression levels of *Selenou* in the liver reached a plateau at 0.20 mg Se/kg; the mRNA expression level of *Selenoh* in the liver reached the highest point at supplemental 0.30 mg Se/kg; the mRNA expression level of *Gpx4* in the liver reached a plateau at supplemental 0.40 mg Se/kg.

3.5. Protein expression levels

Dietary Se level did not affect ($P > 0.05$) the protein expression levels of GPX1 in the liver, kidney and pancreas; however, it affected ($P < 0.0001$) the protein expression levels of GPX4 in the liver, kidney and pancreas (Table 8). The protein expression level of GPX4 in the pancreas increased linearly ($P < 0.0001$) with the increase of dietary Se level; the protein expression levels of GPX4 in the liver and kidney increased linearly ($P < 0.0001$) and quadratically ($P < 0.001$) with the increase of dietary Se level. The protein expression levels of GPX4 in the liver and kidney reached plateaus at supplemental 0.20 mg Se/kg.

Table 4
Effect of supplemental Se level on the activity of selenoproteins in plasma and tissues of broilers at 42 d of age.¹

Supplemental Se, mg/kg	Plasma			Liver			Kidney			Pancreas		
	GPX, U/mL	TXNRD, U/mL	DIO, U/L	GPX, U/mg prot	TXNRD, U/mg prot	DIO, U/g prot	GPX, U/mg prot	TXNRD, U/mg prot	DIO, U/g prot	GPX, U/mg prot	TXNRD, U/mg prot	DIO, U/g prot
0.00	91 ^c	1.029	25.9	11.1 ^c	2.50 ^b	0.548	9.0 ^d	2.51 ^b	0.683 ^a	3.9 ^c	0.455	0.449
0.10	695 ^b	0.686	19.4	31.3 ^b	3.13 ^a	0.568	23.0 ^c	3.20 ^a	0.674 ^a	5.4 ^{bc}	0.468	0.491
0.20	765 ^{ab}	0.788	23.9	34.3 ^{ab}	3.27 ^a	0.534	27.7 ^b	3.25 ^a	0.671 ^a	5.8 ^{bc}	0.416	0.457
0.30	773 ^a	0.641	21.0	32.2 ^{ab}	3.05 ^a	0.518	29.5 ^{ab}	3.27 ^a	0.556 ^b	7.0 ^{ab}	0.517	0.498
0.40	783 ^a	0.766	35.2	35.8 ^a	3.15 ^a	0.561	31.6 ^a	3.59 ^a	0.629 ^{ab}	7.9 ^a	0.517	0.417
0.50	796 ^a	0.625	16.6	34.6 ^{ab}	3.28 ^a	0.556	32.8 ^a	3.43 ^a	0.612 ^{ab}	8.9 ^a	0.555	0.388
Pooled SE	24.82	0.133	4.02	1.42	0.159	0.02	1.15	0.213	0.03	0.66	0.052	0.04
P-value												
Se level	<0.0001	0.1475	0.0718	<0.0001	0.0149	0.7332	<0.0001	0.0208	0.0088	<0.0001	0.4650	0.2668
Linear	<0.0001			<0.0001	0.0080		<0.0001	0.0024	0.0077	<0.0001		
Quadratic	<0.0001			<0.0001	0.0762		<0.0001	0.1087	0.2602	0.9133		

Se = selenium; GPX = glutathione peroxidase; TXNRD = thioredoxin reductase; DIO = iodothyronine deiodinase.

^{a,b,c,d} Means with different superscripts within a column differ ($P < 0.05$).

¹ Each datum represents the mean of 7 replicate cages ($n = 7$).

Table 5
Effect of supplemental Se level on mRNA expression levels of selenoproteins in liver of broilers at 42 d of age.¹

Supplemental Se, mg/kg	<i>Gpx1</i>	<i>Gpx4</i>	<i>Txnrd1</i>	<i>Txnrd2</i>	<i>Selenop</i>	<i>Selenou</i>	<i>Selenoh</i>	<i>Dio1</i>
0.00	1.00	1.00 ^b	1.00	1.00	1.00 ^b	1.00 ^b	1.00 ^b	1.00
0.10	2.08	1.82 ^a	0.61	1.07	1.94 ^a	1.93 ^a	4.76 ^a	1.28
0.20	1.72	1.95 ^a	0.61	1.24	1.99 ^a	2.24 ^a	6.01 ^a	1.66
0.30	1.90	1.97 ^a	0.52	1.00	2.03 ^a	1.75 ^a	7.33 ^a	1.74
0.40	2.22	2.27 ^a	0.74	1.15	2.12 ^a	2.05 ^a	7.18 ^a	2.04
0.50	1.79	2.15 ^a	0.61	1.17	2.21 ^a	1.84 ^a	6.37 ^a	1.80
Pooled SE	0.279	0.201	0.124	0.078	0.278	0.243	1.248	0.251
<i>P</i> -value								
Se level	0.0626	0.0014	0.121	0.1988	0.0460	0.0197	0.0083	0.0715
Linear		0.0002			0.0071	0.0514	0.0012	
Quadratic		0.0361			0.1192	0.0158	0.0131	

Se = selenium; *Gpx1* = glutathione peroxidase 1; *Gpx4* = glutathione peroxidase 4; *Txnrd1* = thioredoxin reductase 1; *Txnrd2* = thioredoxin reductase 2; *Selenop* = selenoprotein P; *Selenou* = selenoprotein U; *Selenoh* = selenoprotein H; *Dio1* = iodothyronine deiodinase 1.

^{a,b} Means with different superscripts within a column differ (*P* < 0.05).

¹ Each datum represents the mean of 7 replicate cages (*n* = 7). The relative quantity (RQ) is calculated based on target gene mRNA level to β-actin and *GAPDH* mRNA (RQ = 2^{-ΔΔCt}).

Table 6
Effect of supplemental Se level on mRNA expression levels of selenoproteins in kidney of broilers at 42 d of age.¹

Supplemental Se, mg/kg	<i>Gpx1</i>	<i>Gpx4</i>	<i>Txnrd1</i>	<i>Txnrd2</i>	<i>Selenop</i>	<i>Selenou</i>	<i>Selenoh</i>	<i>Dio1</i>
0.00	1.00 ^b	1.00 ^b	1.00	1.00	1.00 ^c	1.00 ^c	1.00 ^b	1.00
0.10	1.79 ^a	1.61 ^a	1.03	1.16	1.74 ^{ab}	2.19 ^a	2.49 ^a	1.36
0.20	1.26 ^b	1.61 ^a	0.90	1.06	1.59 ^b	1.63 ^b	2.47 ^a	1.33
0.30	1.29 ^b	1.39 ^a	0.98	1.16	1.96 ^a	1.85 ^{ab}	2.55 ^a	1.42
0.40	1.15 ^b	1.49 ^a	0.66	1.10	1.81 ^{ab}	1.93 ^{ab}	2.07 ^a	1.28
0.50	1.08 ^b	1.62 ^a	0.93	1.08	1.82 ^{ab}	2.07 ^{ab}	2.57 ^a	1.40
Pooled SE	0.148	0.135	0.098	0.079	0.108	0.153	0.290	0.101
<i>P</i> -value								
Se level	0.0091	0.0188	0.1800	0.6833	<0.0001	<0.0001	0.0028	0.062
Linear	0.2219	0.0319			<0.0001	0.0007	0.0090	
Quadratic	0.0495	0.1142			0.0011	0.0629	0.0147	

Se = selenium; *Gpx1* = glutathione peroxidase 1; *Gpx4* = glutathione peroxidase 4; *Txnrd1* = thioredoxin reductase 1; *Txnrd2* = thioredoxin reductase 2; *Selenop* = selenoprotein P; *Selenou* = selenoprotein U; *Selenoh* = selenoprotein H; *Dio1* = iodothyronine deiodinase 1.

^{a,b,c} Means with different superscripts within a column differ (*P* < 0.05).

¹ Each datum represents the mean of 7 replicate cages (*n* = 7). The relative quantity (RQ) is calculated based on target gene mRNA level to β-actin and *GAPDH* mRNA (RQ = 2^{-ΔΔCt}).

Table 7
Effect of supplemental Se level on mRNA expression levels of selenoproteins in pancreas of broilers at 42 d of age.¹

Supplemental Se, mg/kg	<i>Gpx1</i> , RQ	<i>Gpx4</i> , RQ	<i>Txnrd1</i> , RQ	<i>Txnrd2</i> , RQ	<i>Selenop</i> , RQ	<i>Selenou</i> , RQ	<i>Selenoh</i> , RQ	<i>Dio1</i> , RQ
0.00	1.00 ^b	1.00	1.00	1.00	1.00	1.00	1.00	1.00 ^c
0.10	1.76 ^a	1.41	1.19	1.18	1.67	1.67	2.09	1.49 ^a
0.20	1.14 ^b	1.43	0.91	1.07	1.53	1.57	2.07	1.06 ^{bc}
0.30	1.37 ^{ab}	1.32	1.08	1.15	1.51	1.47	2.27	1.23 ^{abc}
0.40	1.07 ^b	1.37	0.89	1.04	1.86	1.64	2.13	1.13 ^{bc}
0.50	1.11 ^b	1.47	0.90	1.15	1.82	1.53	2.32	1.37 ^{ab}
Pooled SE	0.163	0.145	0.135	0.131	0.220	0.216	0.357	0.106
<i>P</i> -value								
Se level	0.0240	0.2581	0.5607	0.9090	0.1016	0.2889	0.1191	0.0211
Linear	0.3518							0.2879
Quadratic	0.1261							0.9402

Se = selenium; *Gpx1* = glutathione peroxidase 1; *Gpx4* = glutathione peroxidase 4; *Txnrd1* = thioredoxin reductase 1; *Txnrd2* = thioredoxin reductase 2; *Selenop* = selenoprotein P; *Selenou* = selenoprotein U; *Selenoh* = selenoprotein H; *Dio1* = iodothyronine deiodinase 1.

^{a,b,c} Means with different superscripts within a column differ (*P* < 0.05).

¹ Each datum represents the mean of 7 replicate cages (*n* = 7). The relative quantity (RQ) is calculated based on target gene mRNA level to β-actin and *GAPDH* mRNA (RQ = 2^{-ΔΔCt}).

3.6. Optimal dietary Se levels

The optimal dietary Se levels of broilers from 22 to 42 d of age as estimated by the nonlinear regression analyses were showed in Table 9. Based on fitted broken-line or asymptotic models (*P* < 0.0001) of the Se concentrations in plasma, liver, kidney, pancreas, breast and thigh muscle, the optimal dietary Se levels

were 0.10–0.49 mg/kg; and based on fitted broken-line, quadratic or asymptotic models (*P* < 0.007) of the GPX activity in plasma, liver and kidney, the mRNA expression levels of *Gpx4*, *Selenou* and *Selenoh* in the liver, *Gpx1*, *Selenop* and *Selenoh* in the kidney, and the protein expression levels of GPX4 in the liver and kidney, the optimal dietary Se levels were 0.08–0.37 mg/kg for broilers fed a corn-soybean meal diet from 22 to 42 d of age.

Table 8
Effect of supplemental Se level on GPX1 and GPX4 protein expression levels of broilers at 42 d of age.¹

Supplemental Se, mg/kg	Liver		Kidney		Pancreas	
	GPX1	GPX4	GPX1	GPX4	GPX1	GPX4
0	0.361	0.07 ^b	0.408	0.15 ^b	1.50	0.38 ^c
0.10	0.373	1.05 ^a	0.394	1.31 ^a	1.15	0.50 ^{bc}
0.20	0.324	1.22 ^a	0.393	1.41 ^a	1.30	0.74 ^{bc}
0.30	0.356	1.03 ^a	0.394	1.24 ^a	1.07	0.82 ^b
0.40	0.345	1.27 ^a	0.508	1.39 ^a	1.11	0.85 ^b
0.50	0.394	1.36 ^a	0.437	1.28 ^a	1.21	1.37 ^a
Pooled SE	0.072	0.130	0.063	0.121	0.209	0.132
Se level	0.9905	<0.0001	0.7748	<0.0001	0.7289	0.0001
Linear		<0.0001		<0.0001		<0.0001
Quadratic		0.0007		<0.0001		0.3344

Se = selenium; GPX1 = glutathione peroxidase 1; GPX4 = glutathione peroxidase 4.

^{a,b,c} Means with different superscripts within a column differ ($P < 0.05$).

¹ Each datum represents the mean of 7 replicate cages ($n = 7$). The GAPDH protein was used to normalize the expression levels of the target protein in each tissue.

Table 9
The optimal dietary Se level of broilers from 22 to 42 d of age as estimated based on fitted models.

Dependent variable	Regression equation ¹	Coefficient of determination (R^2)	P-value	Optimal supplemental Se Level, mg/kg	Optimal dietary Se level ² , mg/kg
Plasma Se	$Y = 0.0131 + 1.086X (0 \leq X \leq 0.1082);$ $Y = 0.1220 + 0.0794X (0.1082 < X \leq 0.5)$	0.94	<0.0001	0.108	0.12
Liver Se	$Y = 0.0987 + 2.953X (0 \leq X \leq 0.1045);$ $Y = 0.3863 + 0.2005X (0.1045 < X \leq 0.5)$	0.93	<0.0001	0.105	0.12
Kidney Se	$Y = 0.2188 + 3.026X (0 \leq X \leq 0.1065);$ $Y = 0.4845 + 0.5319X (0.1065 < X \leq 0.5)$	0.89	<0.0001	0.107	0.12
Pancreas Se	$Y = 0.2819 - 0.1988 \times e^{-5.554X}$	0.91	<0.0001	0.476	0.49
Breast muscle Se	$Y = 0.1086 - 0.0635 \times e^{-8.786X}$	0.92	<0.0001	0.280	0.29
Thigh muscle Se muscle	$Y = 0.0509 + 0.5309X (0 \leq X \leq 0.0810);$ $Y = 0.0885 + 0.0661X (0.0810 < X \leq 0.5)$	0.92	<0.0001	0.081	0.10
Plasma GPX activity	$Y = 90.90 + 6040X (0 \leq X \leq 0.1099);$ $Y = 743 + 102X (0.1099 < X \leq 0.5)$	0.95	<0.0001	0.110	0.12
Liver GPX activity	$Y = 34.36 - 23.29 \times e^{-20.24X}$	0.85	<0.0001	0.129	0.14
Kidney GPX activity	$Y = 8.994 + 140X (0 \leq X \leq 0.1246);$ $Y = 24.29 + 17.47X (0.1246 < X \leq 0.5)$	0.89	<0.0001	0.125	0.14
Liver Gpx4 mRNA	$Y = 2.154 - 1.141 \times e^{-10.50X}$	0.39	<0.0001	0.225	0.24
Liver Selenou mRNA	$Y = 1.000 + 9.273X (0 \leq X \leq 0.1282);$ $Y = 2.309 - 0.934X (0.1282 < X \leq 0.5)$	0.28	0.0068	0.128	0.14
Liver Selenoh mRNA	$Y = 1.221 + 35.32X - 50.35X^2$	0.35	0.0003	0.351	0.37
Kidney Selenop mRNA	$Y = 1.811 - 0.8073 \times e^{-18.78X}$	0.52	<0.0001	0.117	0.13
Kidney Selenoh mRNA	$Y = 0.9969 + 20.97X (0 \leq X \leq 0.0709);$ $Y = 2.500 - 0.2340X (0.0709 < X \leq 0.5)$	0.35	0.0009	0.071	0.09
Liver GPX4 protein	$Y = 1.232 - 1.160 \times e^{-17.85X}$	0.63	<0.0001	0.164	0.18
Kidney GPX4 protein	$Y = 1.330 - 1.180 \times e^{-44.56X}$	0.68	<0.0001	0.065	0.08

Se = selenium; GPX = glutathione peroxidase; GPX4 = glutathione peroxidase 4; Selenoh = selenoprotein H; Selenop = selenoprotein P; Selenou = selenoprotein U.

¹ Y: measurement of index; X: supplemental Se level. Regression equations based on supplemental Se level.

² Optimal dietary Se level = optimal supplemental Se level + Se in the basal diet (0.014 mg/kg).

4. Discussion

The hypothesis that the dietary Se requirements for meeting metabolic and functional Se requirements in modern commercial broilers might be higher than the current NRC Se requirement for the optimum growth performance has been supported by the results of the present study. The present study demonstrated that the optimal dietary Se levels were 0.49 mg/kg to support the maximum Se concentrations and 0.37 mg/kg to support the full expression of selenoproteins in plasma and tissues of broilers fed a corn-soybean meal diet from 22 to 42 d of age, which are higher than the current NRC Se requirement (0.15 mg/kg). These findings could better characterize requirements and meet the Se metabolic functions of broilers in which Se functions either as selenoenzymes or other selenoproteins.

The current NRC dietary Se requirements are 0.15 mg/kg for broilers from 1 to 21 and 22 to 42 d of age, which is primarily based on optimum body weight and feed intake (Jensen et al., 1986).

Furthermore, it was found that supplemental 0.1–0.2 mg Se/kg was necessary to maintain normal growth and feed intake for broilers up to 21 d of age from our laboratory (Liao et al., 2021). Additionally, Zhou and Wang (2011) reported that final body weight, daily weight gain and gain-to-feed ratio of Guangxi yellow chickens reached plateaus at dietary Se level of 0.3 mg/kg. Nevertheless, Yoon et al. (2007) found no significant linear or quadratic response on daily feed intake and daily gain of broiler chickens from 22 to 42 d of age with the increase of dietary Se level. The results from our previous study also demonstrated that dietary Se levels did not affect the growth performance (Wang et al., 2020). These above different results may be related to different types of birds, basal diets, growth periods, or Se forms.

Se concentration is another of several commonly-used indices for evaluating Se status or requirements in broilers. Zhou and Wang (2011) reported that Se concentrations in the liver and muscle of broilers increased linearly with the increase of dietary Se level (0.1 to 0.3 mg/kg), although it is not a criterion for evaluating the Se

requirement. However, Cai et al. (2012) reported that Se concentration in liver and muscle increased quadratically as dietary Se level increased. Furthermore, Yoon et al. (2007) also found that the Se concentration in the whole blood could reflect the dietary Se level, and increased quadratically as dietary Se levels increased. In addition, the results from the current study showed that the Se concentrations in plasma, liver, kidney, pancreas, breast and thigh muscles of broilers from 22 to 42 d of age increased both linearly and quadratically with the increase of dietary Se level. Therefore, the present study and the above studies indicate that the Se concentrations in various tissues are sensitive criteria for evaluating dietary Se requirements of broilers in different growth periods.

A previous study showed that the minimum dietary Se level was 0.12 mg/kg for the optimum plasma GPX activity in chicks (Omaye and Tappel, 1974). Furthermore, Jiang et al. (2009) reported that the addition of Se in diets elevated GPX activity in plasma, and that supplemental 0.225 mg Se/kg had the highest enzymatic activity. Plenty of recent studies have demonstrated that GPX activity is a molecular biomarker to evaluate the Se requirement or Se deficiency in animals (Heindl et al., 2010; Zhou and Wang, 2011). Furthermore, the mRNA expression of *Gpx* as a biomarker was used to evaluate rat Se requirement (Weiss et al., 1997; Weiss and Sunde, 2001). Knight and Sunde (1988) reported that the protein expression of GPX was a sensitive indicator for evaluating rat Se requirement. In addition, the results from the current study indicate that the full expression levels of GPX in different tissues are sensitive criteria for estimating Se requirements of broilers from 22 to 42 d of age. The above studies collectively demonstrate that full expressions of GPX are sensitive for reflecting the Se status in different species, and the optimal GPX expression could better improve the antioxidation in tissues of animals. It is worth noting that the mRNA level of the *Gpx* in different isoforms is not completely consistent with its protein expression in the same tissue, and the exact reasons need to be further studied.

The researchers have subsequently expanded these biomarkers to include full expressions of other selenoproteins (Evangelos et al., 2018). Barnes et al. (2009) reported that the Se requirements could be evaluated by the liver TXNRD activity and the mRNA expression of *Txnrd1*, *Txnrd4* and *Dio1* in the liver or kidney of rats. Li and Sunde (2016) found that TXNRD activity in the liver, and the mRNA expression levels of 25 selenoprotein transcripts (*Dio1*, *Selenoh*, *Selenop*, *Selenou*, etc.) in the liver, gizzard and pancreas, were sensitive biomarkers for assessing Se requirements of chicks fed a semi-purified diet. In a recent study from our laboratory, it was found that the mRNA expression levels of *Dio1*, *Selenoh*, *Selenop* and *Selenou* in the liver, *Dio1*, *Txnrd1*, *Txnrd2*, *Selenoh*, *Selenop* and *Selenou* in the kidney, and *Selenoh* and *Selenou* in the pancreas could be used to evaluate the Se requirements of broilers fed a corn-soybean meal diet from 1 to 21 d of age (Liao et al., 2021). In addition, the results from the current study indicated that the mRNA expression levels of *Selenou* and *Selenoh* in the liver, and *Selenop* and *Selenoh* in the kidney are sensitive criteria for estimating Se requirement of broilers from 22 to 42 d of age. Therefore, the above results demonstrate that the full expression of selenoproteins could be used as sensitive biomarkers to reflect the Se requirements of broilers from 22 to 42 d of age. Additionally, the higher mRNA expressions of these selenoproteins might better represent Se nutritional status and metabolic functions, but their protein expressions and the specific functions need to be further studied.

The current study showed that the optimal dietary Se levels were 0.08–0.49 mg/kg based on Se concentrations and selenoprotein expression in plasma and various tissues of broilers, and the maximum response was 0.49 mg Se/kg based on the Se concentration in the pancreas. Li and Sunde (2016) reported that the minimum dietary Se requirement of chickens was 0.34 mg/kg based on the

GPX4 activity in the pancreas. It was also found that the highest plateau break point was 0.46 mg Se/kg based on the mRNA expression of *Selenoh* in the pancreas in a recent study from our laboratory (Liao et al., 2021). Those results indicate that the pancreas could be the most important metabolic and targeted tissue for Se. Another of the highest plateaus in our current study was 0.37 mg Se/kg, based on the mRNA expression of *Selenoh* in the liver, possibly due to the high Se retention and selenoprotein synthesis in the liver (Burk and Hill, 2015). Although the optimal dietary Se levels were different for different sensitive criteria, the maximum dietary Se level of 0.49 mg/kg could meet all the maximum Se concentrations in plasma and various tissues, and 0.37 mg/kg could meet all of the full expression of selenoproteins in plasma and various tissues of broilers according to the different best fit models.

5. Conclusions

The results from the present study indicate that the Se concentrations in plasma, liver, kidney, pancreas breast and thigh muscle, the activity of GPX in plasma, liver and kidney, the mRNA levels of *Gpx4*, *Selenou* and *Selenoh* in the liver, *Selenop* and *Selenoh* in the kidney, and the protein levels of GPX4 in the liver and kidney are sensitive criteria for estimating optimal dietary Se levels, and the optimal dietary Se levels would be 0.49 mg/kg to support the maximum Se concentrations, and 0.37 mg/kg to support the full expression of selenoproteins in plasma and various tissues of broilers fed a corn-soybean meal diet from 22 to 42 d of age. These levels are higher than the dietary Se requirement (0.15 mg/kg) as recommended by the current NRC.

Author contributions

X. Luo and X. Liao designed the research; C. Wang, L. Wang, T. Liu, L. Zhang, L. Lu and S. Li conducted the research; C. Wang and L. Wang analyzed the data; C. Wang, X. Luo and X. Liao wrote the paper; X. Luo and X. Liao had the primary responsibility for final content. All authors read and approved the final manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (project no. 31601956), the Agricultural Science and Technology Innovation Program (project no. ASTIP-IAS09), and the China Agriculture Research System of MOF and MARA (project no. CARS-41).

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