



Original Research Article

Determination of dietary copper requirement by the monoamine oxidase activity in kidney of broilers from 1 to 21 days of age

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ABSTRACT

The current dietary copper (Cu) requirement (8 mg/kg) of broilers is mainly based on growth, hemoglobin concentration, or hematocrit, which might not be the most sensitive indices to evaluate dietary Cu requirements of broilers. The present study was carried out to estimate dietary Cu requirements of broilers fed a conventional corn-soybean meal diet from 1 to 21 d of age using biochemical or molecular biomarkers. A total of 384 1-d-old Arbor Acres male broilers were randomly allocated to 1 of 6 treatments with 8 replicates and fed a Cu-unsupplemented corn-soybean meal basal diet containing 5.17 mg Cu/kg by analysis and the basal diet supplemented with 3, 6, 9, 12 or 15 mg Cu/kg as CuSO₄·5H₂O for 21 d. Regression analysis was performed to estimate the optimal dietary Cu level using the broken-line model. Dietary supplemental Cu level affected ($P < 0.05$) Cu contents in serum and liver and kidney monoamine oxidase (MAO) activity, but had no effects ($P > 0.05$) on the growth performance, Cu contents in heart, kidney, pancreas and spleen, Cu- and zinc-containing superoxide dismutase (CuZnSOD) activity and ceruloplasmin content in serum, CuZnSOD and cytochrome *c* oxidase (COX) activities and ceruloplasmin, CuZnSOD, MAO A, MAO B, COX 4I1 and COX 1 mRNA and protein expressions in the above tissues of broilers. As dietary supplemental Cu levels increased, Cu contents in serum and liver increased linearly ($P < 0.05$), but kidney MAO activity decreased linearly and quadratically ($P < 0.05$). The estimated dietary Cu requirement based on the fitted broken-line model ($P = 0.035$) of kidney MAO activity was 11.30 mg/kg. In conclusion, kidney MAO activity is a new and sensitive criterion to evaluate the dietary Cu requirement of broilers, and the dietary Cu requirement was 11.30 mg/kg for broilers fed the conventional corn-soybean meal diet from 1 to 21 d of age, which is higher than the current National Research Council (NRC) Cu requirement (8 mg/kg) of broilers.

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1. Introduction

As an essential trace element, copper (Cu) is fundamental to the growth, development, and function of living organisms (Myint et al., 2018). The importance of dietary Cu for broilers has been well documented (Ognik et al., 2018). As Cu concentrations in common feedstuffs are low, Cu additives are usually supplemented in diets for optimal growth. The current National Research Council (NRC 1994) recommends that the dietary Cu requirement for broiler chicks from 1 to 21 d of age is 8 mg/kg, but this requirement is based on a previous study (McNaughton and Day 1979), which was carried out using semi-purified diets with growth performance

as the main criteria for estimating dietary Cu requirements. However, the Cu requirements estimated with purified or semi-purified diets might be not applicable to those birds fed conventional corn-soybean meal diets because of the absence of phytate and fiber (Wedekind et al., 1992) as well as the abnormal growth of birds due to the reduced feed intake. Phytate and fiber in diets of broiler chicks could inhibit Cu absorption by binding Cu in the intestinal tract, thus decreasing Cu bioavailability. Therefore, it is necessary to find new and more sensitive criteria to evaluate dietary Cu requirements so as to meet the metabolic needs of broiler chicks for Cu in conventional diets.

Our previous studies have demonstrated that the activities and gene expressions of key metalloenzymes were new and sensitive criteria to evaluate dietary manganese, zinc, iron and selenium requirements of broilers (Huang et al., 2007; Li et al., 2011a; Liao et al., 2013, 2017, 2021; Lu et al., 2016; Ma et al., 2016). In fact, Cu functions in the body as a metal cofactor for a variety of enzymes, including Cu- and zinc-containing superoxide dismutase (CuZn-SOD), cytochrome *c* oxidase (COX), ceruloplasmin and monoamine oxidase (MAO). The CuZnSOD has been considered a good marker of Cu status (Harris 1992). Besides, CuZnSOD is a reactive oxygen species (ROS) scavenger and plays an important role in the protection of cells against oxidative stress (Fang et al., 2013). The COX is the terminal enzyme of the mitochondrial respiratory chain and plays a central role in the oxidative production of cellular energy (Rak et al., 2016). The ceruloplasmin is a multi-Cu oxidase and participates in defense mechanisms against oxidative stress (Calabrese et al., 1988; Harris and Gitlin 1996). The MAO serves an essential role in catalyzing the degradation of different monoamines by oxidative deamination (Yeung et al., 2019). During this process, hydrogen peroxide, a major source of ROS, is formed as a side-product. The ROS can predispose cellular DNA to damage (Srinivas et al., 2019). It has been reported that the activities of Cu-containing enzymes CuZnSOD, COX, ceruloplasmin and MAO in animals could be affected by dietary Cu level (Mills et al., 1976; Sharma et al., 2005). However, it remains unclear whether the activities and gene expressions of Cu-containing enzymes could be used to evaluate the dietary Cu requirements of broilers from 1 to 21 d of age.

Therefore, the objective of this study was to investigate the effect of various dietary Cu levels on the growth performance, serum markers, tissue Cu concentrations, as well as the activities and gene expressions of Cu-containing enzymes in various tissues, in order to find new and sensitive criteria and estimate Cu requirements of broilers fed a conventional corn-soybean meal diet from 1 to 21 d of age.

2. Materials and methods

2.1. Animals, diets and experimental design

All experimental procedures were approved by the Animal Management Committee (in charge of animal welfare issue) of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS, Beijing, China) and performed in accordance with the guidelines. Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS.

A total of 384 1-d-old Arbor Acres (AA) male broiler chicks (Huadu Broiler Breeding Co., Ltd., Beijing, China) were used in the 21 d experiment. The broilers were randomly allotted by body weight to 1 of 6 treatments with 8 replicate cages of 8 chicks each in a completely randomized design. All of the broiler chicks were housed in electrically heated, thermostatically controlled, stainless steel cages (160 cm × 90 cm × 75 cm) equipped with fibreglass feeders and waterers. They were maintained on a 24 h constant

Table 1

Composition and nutrient levels of the basal diet for 1- to 21-d-old broilers (% as-fed basis).

Item	Content
Ingredients	
Corn	52.37
Soybean meal	38.73
Soybean oil	4.83
CaHPO ₄ ¹	1.69
CaCO ₃ ¹	1.25
NaCl ¹	0.30
DL-Met	0.29
Premix ²	0.34
Corn starch + Cu ³	0.20
Nutrient levels	
ME, MJ/kg	3.00
Crude protein ⁴	21.56
Lys	1.15
Met	0.58
Met + Cys	0.90
Ca ⁴	1.02
Nonphytate P	0.39
Cu ⁴ , mg/kg	5.17

¹ Reagent grade.

² The premix provided following per kilogram of the basal diet: vitamin A (as all-trans retinol acetate) 15,000 IU, cholecalciferol 4,500 IU, vitamin E (as all-rac- α -tocopherolacetate) 24 IU, vitamin K (as menadione sodium bisulfate) 3 mg, thiamin (as thiamin mononitrate) 3 mg, riboflavin 9.6 mg, vitamin B₆ 3 mg, vitamin B₁₂ 0.018 mg, calcium pantothenate 15 mg, niacin 39 mg, folic acid 1.5 mg, biotin 0.15 mg, choline (as choline chloride) 700 mg, Zn (ZnSO₄•7H₂O) 60 mg, Mn (MnSO₄•H₂O) 110 mg, Fe (FeSO₄•7H₂O) 40 mg, I (KI) 0.35 mg, Se (Na₂SeO₃) 0.35 mg.

³ Cu sulfate supplement added in place of equivalent weights of cornstarch.

⁴ Values determined by analysis; each value is based on triplicate determinations.

light schedule, and allowed ad libitum access to experimental diets and tap water containing no detectable Cu. Dead birds were recorded daily, and chick body weight and feed intake per cage were measured at the beginning and 21 d of age to calculate the average daily gain, average daily feed intake, feed-to-gain ratio, and mortality from 1 to 21 d of age.

The Cu-unsupplemented corn-soybean meal basal diet was formulated to meet or exceed the current NRC (1994) requirements and Chinese Feeding Standard of Chicken (2004) for starter broilers for all nutrients except for Cu, manganese, zinc, iron and selenium (Table 1). However, the NRC (1994) requirement recommendations for trace minerals of broilers are too old, and thus, dietary supplemental manganese, zinc, iron and selenium levels in Table 1 were based on the recent research results on dietary manganese, zinc, iron and selenium requirements of broilers fed the conventional corn-soybean meal diets from 1 to 21 d of age from our laboratory (Huang et al., 2007; Li et al., 2011a; Liao et al., 2021; Ma et al., 2016). Dietary treatments included the Cu-unsupplemented corn-soybean meal basal diet (control) and the basal diet supplemented with 3, 6, 9, 12 or 15 mg Cu/kg in the form of CuSO₄•5H₂O. The dietary Cu concentrations for the 6 treatments by analysis on an as-fed basis were 5.17, 8.23, 11.30, 14.40, 16.50 and 20.20 mg/kg, respectively (Table 2). All of these diets were fed to broilers in mash.

2.2. Sample collections and preparations

The feed ingredient and diet samples were taken for analyses of calcium, Cu, or dietary crude protein contents. The tap water was collected for analysis of Cu content. Four birds from each cage were selected based on the mean body weight of the birds within the cage. Blood samples were taken from each bird via wing vein and then immediately centrifuged for 15 min at 3,000×g at 4 °C to harvest serum samples for analyses of serum Cu and ceruloplasmin content and CuZnSOD activity. Then the selected birds in each cage

Table 2
Analyzed Cu contents in diets of broilers from 1 to 21 d of age.

Supplemental Cu, mg/kg	Analyzed Cu contents ¹ , mg/kg
0	5.17
3	8.23
6	11.3
9	14.4
12	16.5
15	20.2

¹ Values of analyzed Cu contents are based on triplicate determinations.

were killed by cervical dislocation, and heart, liver, kidney, pancreas and spleen samples were collected. One subsample was snap-frozen in liquid N₂ and then stored at –80 °C for analyses of mRNA and protein expressions, and another subsample was kept on ice and stored at –20 °C for determinations of Cu contents and CuZnSOD, COX and MAO activities. To reduce individual biological variation, samples from 4 broiler chicks in each replicate cage were pooled into 1 sample before analyses, and thus there were a total of 8 replicate samples for each treatment.

2.3. Measurements of Cu, Ca and crude protein concentrations

The Cu concentrations in tap water, diets and tissues were determined by inductively coupled plasma emission spectroscopy (model 9000, Thermo Jarrell Ash, Waltham, MA) as described previously (Li et al., 2011b; Zhang et al., 2017). Validation of the mineral analysis was conducted using bovine liver powder (GBW [E] 080193, National Institute of Standards and Technology, Beijing, China) as a standard reference material. The crude protein and Ca contents in feed ingredients and diet samples were determined using Association of Official Analytical Chemists methods (1990).

2.4. Measurements of enzyme activities and ceruloplasmin content

The ceruloplasmin content in serum was measured using a microplate reader with ceruloplasmin assay kit (Shanghai Jiang Lai Biological Technology Co., Ltd., Shanghai, China) according to the instructions of the manufacturer. The CuZnSOD, MAO and COX activities were measured using a microplate reader with CuZnSOD, MAO or COX assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions of the manufacturer, respectively.

2.5. Total RNA isolation and real-time PCR

Total RNA was isolated from heart, liver, kidney, pancreas and spleen samples (50 mg) using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's protocol, and then treated with RNase-free DNase and reverse-transcribed to cDNA using SuperScript III First-Strand Synthesis for RT-PCR kit (cat no. 18080-051,

Table 3
Primer sequences for real-time PCR amplification.

Genes	GenBank ID	Primer sequences	Product length, bp
Ceruloplasmin	XM_015291853.2	F:GTTATCAAGGCGGAAGTGGG R:TGGGAGGCTGGAGATTCAGTA	158
CuZnSOD	NM_205064.1	F:ATTACCGGCTTGTCTGATGG R:TCCTCCCTTGCAGTCACAT	174
MAO A	XM_015300467.2	F:GCTCCAAGTTGCTGTATGA R:TCTCAATGCCAGCTCTTTT	180
MAO B	XM_416766.6	F:ACCAGCTCATCAACCGAATC R:CACAGCTCGTCTTCTTTC	247
COX 1	JX_160009.1	F:GCAGGTGTTTCTCCAT R:GGTTGCGGTCGGTAAAT	187
COX 4I1	XM_015292558.1	F:CITTCACCTCCATCTGTGTGA R:GCTGGATGGCTGAAATCG	174
β-actin	NM_205518.1	F:ACCTGAGCGCAAGTACTCTGTCT R:CATCGTACTCTGCTTGTGAT	95
GAPDH	NM_204305.1	F:CTTTGGCATTGTGGAGGTC R:ACCGTGGGATGATGTTCTGG	128

ID = identity; CuZnSOD = Cu- and zinc-containing superoxide dismutase; F = forward; R = reverse; MAO A = monoamine oxidase a; MAO B = monoamine oxidase b; COX 1 = cytochrome c oxidase subunit 1; COX 4I1 = cytochrome c oxidase subunit 4I1; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

Invitrogen). Two microliters of diluted cDNA (1:10, vol/vol) were used for real-time PCR with an ABI PRISM 7500 Sequence Detection System (Applied Biosystems). All primers (Table 3) were synthesized by Generay Biotech (Shanghai, China). The technical variations were normalized using β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal reference genes. Data were analyzed using the method of 2^{–ΔΔCt}.

2.6. Tissue preparations and Western blotting

Total protein was extracted from 50 mg samples of frozen heart, liver, kidney, pancreas and spleen, as previously described (Liao et al., 2019a, b). The protein concentration was determined using a Pierce BCA Protein Assay kit according to the manufacturer's instructions. Either a 40- or 60-μg sample of protein was used for electrophoresis on a 7.5% or 10.0% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Western blotting analyses for CuZnSOD (ABclonal, Wuhan, China, catalog number A12537; 1:1,000), ceruloplasmin (ABclonal, Wuhan, China, catalog number A7658; 1:1,000), MAO A (ABclonal, Wuhan, China, catalog number A1354; 1:500), COX 4I1 (ABclonal, Wuhan, China, catalog number A6564; 1:500), and COX 1 (ABclonal, Wuhan, China, catalog number A7531; 1:1,000) were performed. The bands were visualized by enhanced chemiluminescence using a High-sig ECL Western blotting Substrate Kit (Tanon, Shanghai, China, catalog number 180–5001). The signals were recorded with a chemiluminescence image scanner (Tanon, Shanghai, China) and analyzed with Tanon Gis 1D software (Tanon, Shanghai, China). Data were presented as the ratio of CuZnSOD, ceruloplasmin, MAO A, COX 4I1 or COX 1 protein band intensity to GAPDH or β-tubulin protein band intensity. The GAPDH or β-tubulin protein was used to normalize the expression level of CuZnSOD, ceruloplasmin, MAO A, COX 4I1 or COX 1.

2.7. Statistical analyses

Data from the present study were subjected to one-way ANOVA with the use of the general linear model procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). Differences among means were tested with the LSD method. The cage was the experimental unit. The percentage mortality was transformed to arcsine for analysis. 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, and the best fit model between responsive criteria and analyzed dietary Cu contents was used to estimate the dietary Cu requirement (the inflection point from a broken-line model) for broiler chicks (Liao et al., 2017; Lu et al., 2016; Ma et al., 2016). The level of statistical significance was set at P < 0.05.

Table 4
Effect of dietary supplemental Cu level on the growth performance and mortality of broilers from 1 to 21 d of age¹.

Supplemental Cu, mg/kg	Average daily gain, g/d	Average daily feed intake, g/d	Feed-to-gain ratio	Mortality ² , %
0	31.90	41.85	1.31	0.00
3	30.51	40.06	1.28	1.56
6	31.70	40.24	1.28	1.56
9	30.65	39.82	1.29	0.00
12	31.60	40.17	1.27	0.00
15	32.30	41.04	1.27	0.00
Pooled SE	0.44	0.50	0.02	0.90
P-value	0.053	0.091	0.733	0.556

¹ Values are the means of 8 replicate cages of 8 birds per replicate cage (n = 8).

² The percentage of mortality was transformed to arcsine for analysis.

Table 5
Effect of dietary supplemental Cu level on Cu contents in serum and tissues of broilers at 21 d of age¹.

Supplemental Cu, mg/kg	Serum Cu, µg/L	Heart Cu, µg/g fresh tissue	Liver Cu, µg/g fresh tissue	Kidney Cu, µg/g fresh tissue	Pancreas Cu, µg/g fresh tissue	Spleen Cu, µg/g fresh tissue
0	84.57 ^d	2.61	2.81 ^b	1.97	1.10	0.49
3	113.28 ^c	2.75	3.02 ^{ab}	1.97	1.05	0.50
6	111.87 ^c	2.77	2.92 ^{ab}	1.94	1.07	0.48
9	123.32 ^{bc}	2.65	3.02 ^{ab}	1.95	1.16	0.48
12	141.42 ^b	2.68	3.04 ^a	1.93	1.03	0.52
15	184.49 ^a	2.74	3.14 ^a	2.01	1.12	0.49
Pooled SE	8.13	0.07	0.08	0.03	0.37	0.02
P-value						
Dietary Cu	<0.001	0.491	0.048	0.402	0.158	0.824
Linear	<0.001	–	0.004	–	–	–
Quadratic	0.054	–	0.946	–	–	–

^{a-d} Values in a column with different letter superscripts differ significantly (P < 0.05).

¹ Values are the means of 8 replicate cages of 4 birds per replicate cage (n = 8).

Table 6
Effect of dietary supplemental Cu level on serum CuZnSOD activity and ceruloplasmin content of broilers at 21 d of age¹.

Supplemental Cu, mg/kg	CuZnSOD, U/mL	Ceruloplasmin, µg/mL
0	73.42	21.03
3	79.03	21.41
6	80.32	25.43
9	82.91	24.68
12	84.80	19.24
15	91.95	22.33
Pooled SE	5.09	3.01
P-value	0.215	0.721

CuZnSOD = Cu- and zinc-containing superoxide dismutase.

¹ Values are the means of 8 replicate cages of 4 birds per replicate cage (n = 8).

Table 7
Effect of dietary supplemental Cu level on activities of Cu-containing enzymes in tissues of broilers at 21 d of age¹.

Supplemental Cu, mg/kg	Heart			Liver			Kidney		Pancreas		Spleen	
	CuZnSOD, U/mg ·prot	MAO, U/mg ·prot	COX, mU/mg ·prot	CuZnSOD, U/mg ·prot	MAO, U/mg ·prot	COX, mU/mg ·prot	CuZnSOD, U/mg ·prot	MAO, U/mg ·prot	CuZnSOD, U/mg ·prot	MAO, U/mg ·prot	CuZnSOD, U/mg ·prot	MAO, U/mg ·prot
0	178	6.15	12.42	278	10.86	49.09	191	16.79 ^a	43.70	2.28	53.44	25.11
3	166	6.23	14.64	271	11.68	48.87	199	15.80 ^{ab}	47.74	2.28	52.80	23.76
6	167	5.94	13.89	270	13.23	52.29	194	13.94 ^b	42.51	2.40	50.04	22.71
9	169	6.76	13.93	275	10.95	63.66	194	15.60 ^{ab}	44.83	2.43	48.28	23.49
12	169	6.55	14.98	278	11.01	62.90	194	13.82 ^b	44.05	2.37	46.88	24.12
15	179	7.63	12.99	270	13.03	67.31	194	15.21 ^{ab}	44.25	2.33	48.46	26.64
Pooled SE	6	0.41	1.24	5	1.21	9.14	3	0.65	3.07	0.19	2.60	1.48
P-value												
Dietary Cu	0.606	0.101	0.692	0.806	0.559	0.575	0.705	0.023	0.894	0.989	0.431	0.506
Linear	–	–	–	–	–	–	–	0.033	–	–	–	–
Quadratic	–	–	–	–	–	–	–	0.048	–	–	–	–

CuZnSOD = Cu- and zinc-containing superoxide dismutase; MAO = monoamine oxidase; COX = cytochrome c oxidase.

^{a, b} Values in a column with different letter superscripts differ significantly (P < 0.05).

¹ Values are the means of 8 replicate cages of 4 birds per replicate cage (n = 8).

3. Results

3.1. Growth performance and mortality

Dietary supplemental Cu level did not affect (P > 0.05) the average daily gain, average daily feed intake, feed-to-gain ratio, and mortality of broiler chicks during 1 to 21 d (Table 4).

3.2. The Cu concentrations

The Cu concentrations in serum and liver were affected (P < 0.05) by dietary supplemental Cu level, and increased linearly (P < 0.05) as dietary supplemental Cu levels increased (Table 5). However, Cu concentrations in heart, kidney, pancreas and spleen were not affected (P > 0.05) by dietary supplemental Cu level.

Table 8
Effect of dietary supplemental Cu level on mRNA expressions of Cu-containing enzymes in tissues of broilers at 21 d of age¹.

Supplemental Cu, mg/kg	Heart			Liver			Kidney			Pancreas			Spleen		
	CuZnSOD, RQ ²	MAO A, RQ ²	MAO B, RQ ²	CuZnSOD, RQ ²	MAO A, RQ ²	MAO B, RQ ²	CuZnSOD, RQ ²	MAO A, RQ ²	MAO B, RQ ²	COX1, RQ ²	COX4I1, RQ ²	COX1, RQ ²	CuZnSOD, RQ ²	MAO A, RQ ²	MAO B, RQ ²
0	1.00	0.90	0.95	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3	0.96	0.94	0.89	1.02	1.08	0.99	0.99	0.99	0.99	1.00	1.05	1.21	1.03	0.88	1.00
6	1.03	0.86	1.03	0.93	1.03	1.32	1.08	0.86	1.18	0.91	1.12	0.88	0.94	0.81	1.02
9	0.96	0.84	0.76	0.96	1.14	1.37	0.99	0.99	1.29	0.92	0.98	0.96	1.04	0.98	0.95
12	0.99	0.86	0.92	0.99	1.06	1.06	0.99	0.84	1.03	0.89	1.06	0.96	1.01	0.82	1.07
15	0.98	0.92	1.23	1.03	1.01	1.36	0.97	1.00	1.19	1.01	0.89	0.99	1.02	0.94	1.06
Pooled SE	0.06	0.07	0.14	0.05	0.08	0.20	0.03	0.10	0.14	0.04	0.06	0.05	0.06	0.08	0.19
P-value	0.958	0.901	0.344	0.722	0.755	0.559	0.342	0.778	0.372	0.202	0.113	0.307	0.929	0.647	0.522

CuZnSOD = Cu- and zinc-containing superoxide dismutase; MAO A = monoamine oxidase a; MAO B = monoamine oxidase b; COX 4I1 = cytochrome c oxidase subunit 4I1; COX 1 = cytochrome c oxidase subunit 1.

¹ Values are the means of 8 replicate cages of 4 birds per replicate cage (n = 8).

² The mRNA expressions were calculated as the relative quantities (RQ) of the target gene mRNA to the geometric mean of β-actin and GAPDH mRNA using the 2^{-ΔΔCt} method.

Further, the quadratic responses for serum and liver Cu concentrations were not significant ($P > 0.05$). Therefore, all of the above indices were not suitable for assessing dietary Cu requirements of broilers.

3.3. Enzyme activities and ceruloplasmin content

Dietary supplemental Cu level did not affect ($P > 0.05$) CuZnSOD activity and ceruloplasmin content in serum (Table 6). Supplemental Cu level affected ($P < 0.05$) MAO activity in kidney, but had no effect ($P > 0.05$) on MAO activities in heart, liver, pancreas and spleen (Table 7). The MAO activity in kidney decreased linearly and quadratically ($P < 0.05$) as dietary supplemental Cu levels increased. No significant alterations ($P > 0.05$) were detected for CuZnSOD activities in heart, liver, kidney, pancreas and spleen, and COX activities in heart and liver with the increase of dietary supplemental Cu levels (Table 7). Based on these results, the MAO activity in kidney was sensitive and suitable for assessing the dietary Cu requirement of broilers.

3.4. mRNA and protein expressions

Dietary supplemental Cu level did not affect ($P > 0.05$) CuZnSOD, MAO A and MAO B mRNA expressions in heart, liver, kidney, pancreas and spleen, and COX 4I1 and COX 1 mRNA expressions in heart, liver and kidney, and ceruloplasmin mRNA expression in liver (Table 8). Consistent with the mRNA expressions, CuZnSOD, MAO A, COX 4I1 and COX 1 protein expressions in heart, liver and kidney and ceruloplasmin protein expression in liver, and CuZnSOD protein expressions in pancreas and spleen were not affected ($P > 0.05$) by dietary supplemental Cu level either (Table 9 and Fig. 1). Therefore, all of these indices were not sensitive biomarkers for assessing dietary Cu requirements of broilers.

3.5. Estimation of the dietary Cu requirement of broilers

The result of the dietary Cu requirement of broilers as estimated by the broken-line model of regression analysis is shown in Table 10. On the basis of the broken-line model ($P = 0.035$) of kidney MAO activity with the analyzed dietary Cu content, the optimal dietary Cu requirement was estimated to be 11.30 mg/kg for broiler chicks fed the conventional corn-soybean meal diet from 1 to 21 d of age.

4. Discussion

It is critically important to find more sensitive criteria to assess Cu requirements and meet the metabolic needs of broiler chicks for Cu from 1 to 21 d of age. The results from the present study indicate that the activity of MAO in kidney is a new and sensitive criterion for estimating the dietary Cu requirement of broilers fed the corn-soybean meal diet, which has not been reported before.

Growth performance parameters were the early and commonly used criteria to assess the Cu requirements of broilers fed with purified or semi-purified diets (Aoyagi and Baker 1993; McNaughton and Day 1979). The current NRC Cu requirement for broilers is 8 mg/kg, which is based on the minimum dietary Cu required for maximum performance (NRC 1994). However, the growth performance indices are usually not sensitive for evaluating Cu requirements of birds when fed with conventional corn-soybean meal diets (Samanta et al., 2011). In our current study, supplemental Cu level did not affect the growth performance of broilers fed a conventional diet, which is in agreement with a previous report (Samanta et al., 2011), indicating that the background Cu level (5.17 mg/kg by analysis) in a Cu-unsupplemented corn-

Table 9
Effect of dietary supplemental Cu level on the protein expressions of Cu-containing enzymes in tissues of broilers at 21 d of age¹.

Supplemental Cu, mg/kg	Heart				Liver					Kidney				Pancreas	Spleen
	CuZn SOD, RQ ²	MAO A, RQ ²	COX 411, RQ ²	COX 1, RQ ²	Ceruloplasmin, RQ ²	CuZn SOD, RQ ³	MAO A, RQ ²	COX 411, RQ ²	COX 1, RQ ²	CuZn SOD, RQ ²	MAO A, RQ ²	COX 411, RQ ²	COX 1, RQ ²	CuZn SOD, RQ ²	CuZn SOD, RQ ²
0	1.35	0.86	1.38	1.00	0.97	1.31	0.92	1.15	0.87	1.45	1.11	1.52	0.94	1.46	1.48
3	1.24	0.94	1.27	1.04	0.93	1.41	1.00	1.27	0.86	1.57	1.29	1.52	1.06	1.46	1.41
6	1.21	0.95	1.40	1.28	0.98	1.31	0.81	1.21	0.82	1.52	1.19	1.60	1.08	1.53	1.52
9	1.54	1.21	1.51	1.26	0.91	1.35	0.91	1.36	0.78	1.53	0.92	1.45	1.04	1.26	1.55
12	1.33	0.96	1.38	1.17	0.83	1.44	0.99	1.23	1.07	1.58	1.05	1.49	1.12	1.63	1.62
15	1.11	0.91	1.66	1.49	0.93	1.40	0.87	1.40	1.03	1.64	1.26	1.54	1.04	1.78	1.65
Pooled SE	0.11	0.14	0.16	0.12	0.07	0.11	0.06	0.09	0.08	0.12	0.12	0.11	0.07	0.13	0.22
P-value	0.121	0.596	0.652	0.067	0.717	0.936	0.310	0.393	0.111	0.927	0.260	0.969	0.593	0.157	0.977

CuZnSOD = copper- and zinc-containing superoxide dismutase; MAO A = monoamine oxidase a; COX 411 = cytochrome c oxidase subunit 411; COX 1 = cytochrome c oxidase subunit 1.

¹ Values are the means of 8 replicate cages of 4 birds per replicate cage (n = 8).

² The protein expressions were calculated as the relative quantities (RQ) of the target gene protein to the GAPDH protein.

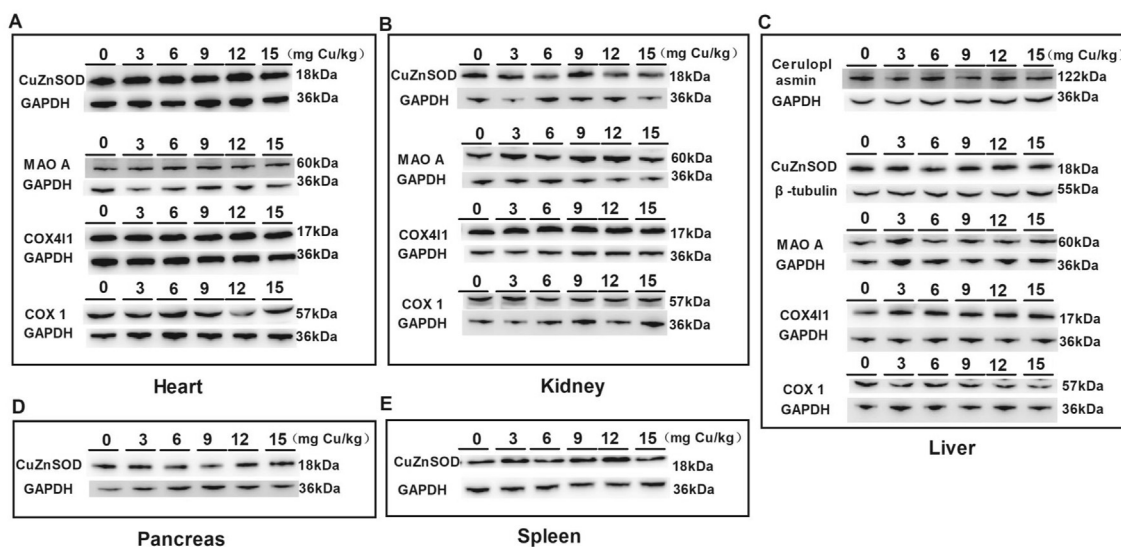


Fig. 1. Representative immunoblots of Cu-containing enzymes in heart (A), kidney (B), liver (C), pancreas (D) and spleen (E) of broilers at 21 d of age. CuZnSOD = Cu- and zinc-containing superoxide dismutase; MAO A = monoamine oxidase a; COX 411 = cytochrome c oxidase subunit 411; COX 1 = cytochrome c oxidase 1.

Table 10
Estimation of dietary Cu requirement based on the broken-line model of kidney MAO activity on the analyzed dietary Cu content.

Dependent variable	Regression equation ¹	R ²	P-value	Dietary Cu requirement, mg/kg
Kidney MAO activity	Y = 18.952,7 - 0.402,7X (5.17 ≤ X < 11.30); Y = 13.601,1 + 0.070,9X (11.30 ≤ X ≤ 20.20)	0.180	0.035	11.30

¹ Y is the kidney MAO activity (U/mg·prot), and X is the analyzed dietary Cu content (mg/kg), which is defined as the analyzed Cu from both the basal diet and supplemental Cu as CuSO₄·5H₂O.

soybean meal basal diet and the current NRC Cu requirement are enough for the growth performance of broilers, and that growth performance is not sufficiently sensitive for assessing dietary Cu requirements of broilers when a conventional corn-soybean meal diet is used.

Numerous studies have demonstrated that serum and liver Cu concentrations are sensitive indices of Cu deficiency in animals (Hambidge 2003; Stemmer et al., 1985; Yang et al., 2018). In a previous study in pigs, liver Cu content could reflect the bioavailability of Cu sources because it showed a good linear response to dietary supplemental Cu level (5 to 160 mg/kg) (Lin et al., 2020). Similarly, in the present study, linear responses of serum and liver

Cu contents to supplemental Cu were observed, indicating that serum and liver Cu contents are sensitive for assessing Cu bioavailability, but not suitable for the assessment of dietary Cu requirements of broilers.

Assays of the activities and gene expressions of selected Cu metalloenzymes might provide sensitive functional biomarkers for assessing the Cu status in the body (Failla and Hopkins 1998; Hambidge 2003). Cu is required for the functions of numerous Cu-containing enzymes, such as CuZnSOD, COX, ceruloplasmin and MAO. The CuZnSOD is a ROS scavenger and plays an important role in the protection of cells against oxidative stress (Fang et al., 2013). A previous study has shown that CuZnSOD activity may be a

sensitive and useful indicator for assessing the Zn status in Chinese yellow-feathered chickens (Li et al., 2019). The COX plays a central role in the oxidative production of cellular energy within mitochondria (Rak et al., 2016). It was reported in our previous study that liver COX activity and mRNA expression are new and sensitive criteria that can be used to evaluate the dietary iron requirements of broilers (Ma et al., 2016). Ceruloplasmin is a multi-Cu oxidase, and contains about 90% of the serum Cu, and also participates in iron homeostasis and in defense mechanisms against oxidative stress (Calabrese et al., 1988; Harris and Gitlin 1996). Plasma ceruloplasmin content has been regarded as an indicator of Cu status in human (Arredondo et al., 2008). However, in our present study, no significant alterations were detected for CuZnSOD and COX activities and *CuZnSOD*, *COX* and ceruloplasmin mRNA and protein expressions in tissues and ceruloplasmin content in serum with dietary supplemental Cu levels. Previous studies have demonstrated that dietary severe Cu deficiency could decrease CuZnSOD activity and expressions, whereas mild or marginal Cu deficiency had little effect on these indices in tissues (Chung et al., 1988; Dameron and Harris 1987), suggesting that the effect of Cu on CuZnSOD activity and mRNA and protein expressions would depend on the degree of dietary Cu deficiency. In the present study, the Cu content in the Cu-unsupplemented corn-soybean meal basal diet was 5.17 mg/kg by analysis, which belongs to a mild or marginal Cu deficiency based on the current NRC's Cu requirement (8 mg/kg) of broilers. Therefore, it is not surprising that the supplemental Cu levels in the present Cu-unsupplemented corn-soybean meal basal diet didn't affect the CuZnSOD activity and mRNA and protein expressions as well as the above other indices in serum and various tissues. These results indicate that CuZnSOD and COX activities and *CuZnSOD*, *COX* and ceruloplasmin mRNA and protein expressions in tissues and ceruloplasmin content in serum were not sensitive and useful criteria for Cu requirement estimation for broiler chicks fed a conventional corn-soybean meal diet from 1 to 21 d of age.

Monoamine oxidase is a Cu-dependent enzyme, which employs a flavin adenine dinucleotide (FAD) cofactor to catalyze the oxidative deamination of several monoamines, including not only neurotransmitters, but also exogenous amines ingested with normal diets (tyramine), generating ROS and the corresponding aldehydes as by-products (Wang et al., 2013). In animals, there are two isoforms (MAO-A and MAO-B) that can be distinguished on the basis of their substrate specificity (Wang et al., 2013). A previous study demonstrated that Cu deficiency in sheep resulted in low Cu concentration and low MAO activity in plasma (Mills et al., 1966). In the present study, no significant alterations were detected for *MAO A* and *MAO B* mRNA and protein expressions in tissues with dietary supplemental Cu levels. However, we found that the MAO activity in kidney decreased linearly and quadratically with increasing dietary supplemental Cu levels. The Cu could regulate MAO expressions and activity at transcriptional, translational or post-translational modification levels. The results from the present study suggested that Cu might modulate MAO activity in kidney at a post-translational modification level based on other related reports (Liao et al., 2019a, b; Prohaska and Brokate 2001). This result is inconsistent with a previous result obtained from Mills et al. (1966). It appears that the effect of Cu on MAO activity is dependent on the dose of supplementation, species, tissues, duration, as well as the dietary types, but the exact reasons are unknown. In the present study, the Cu addition to diets decreased MAO activity in kidney, so as to decrease the production of ROS to reduce oxidative damage. In general, the MAO activity in kidney was well fitted to the broken-line model, indicating that, to our knowledge, this is a new and sensitive criterion for assessing the dietary Cu requirement of broiler chicks. The dietary Cu requirement estimated on the

basis of the fitted broken-line model of kidney MAO activity was 11.30 mg/kg for broiler chicks fed a conventional corn-soybean meal diet from 1 to 21 d of age, which is higher than the current NRC (1994) Cu requirement (8 mg/kg) of broiler chicks based on early results obtained with purified or semi-purified diets and the growth performance of birds (Aoyagi and Baker 1993; McNaughton and Day 1979). The above new findings would be of important theoretical and practical significances for decreasing the production of ROS in the body in order to reduce oxidative damage and potentially improve the health of broilers.

5. Conclusions

The results from the present study indicate that the MAO activity in kidney is a new and sensitive criterion for estimating the dietary Cu requirement of broilers, and 11.30 mg/kg Cu was required by broiler chicks fed a conventional corn-soybean meal diet from 1 to 21 d of age based on the broken-line model of this parameter, which is higher than the current NRC requirement (8 mg/kg).

Author contributions

Yun Hu: Data curation, Writing original draft preparation. **Zhiyong Chen and Liyang Zhang:** Investigation. **Lin Lu:** Formal analysis. **Zhiyong Chen and Tao Liu:** Conceptualization. **Tao Liu and Xiudong Liao:** Methodology. **Xugang Luo:** Supervision, Writing review & editing.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, 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.

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References

- Aoyagi S, Baker DH. Bioavailability of copper in analytical-grade and feed-grade inorganic copper sources when fed to provide copper at levels below the chick's requirement. *Poultry Sci* 1993;72(6):1075–83.
- Arredondo M, González M, Olivares M, Pizarro F, Araya M. Ceruloplasmin, an indicator of copper status. *Biol Trace Elem Res* 2008;123(1–3):261–9.
- Calabrese L, Carbonaro M, Musci G. Chicken ceruloplasmin. Evidence in support of a trinuclear cluster involving type 2 and 3 copper centers. *J Biol Chem* 1988;263(14):6480–3.
- Chung K, Romero N, Tinker D, Keen CL, Amemiya K, Rucker R. Role of copper in the regulation and accumulation of superoxide dismutase and metallothionein in rat liver. *J Nutr* 1988;118(7):859–64.
- Dameron CT, Harris ED. Regulation of aortic Cu/Zn-superoxide dismutase with copper. Effects in vivo. *Biochem J* 1987;248(3):663–8.
- Failla ML, Hopkins RG. Is low copper status immunosuppressive? *Nutr Rev* 1998;56(1 Pt 2):S59–64.
- Fang YC, Chen YP, Chen CT, Lin TS, Mou CY. Protection of HeLa cells against ROS stress by CuZnSOD mimic system. *J Mater Chem B* 2013;1(44):6042–52.
- Feeding standard of chicken. NY/T 33-2004. Agricultural Press of China; 2004.

- Hambidge M. Biomarkers of trace mineral intake and status. *J Nutr* 2003;133(3):948s–55s.
- Harris ED. Copper as a cofactor and regulator of copper, zinc superoxide dismutase. *J Nutr* 1992;122(3 Suppl):636–40.
- Harris ZL, Gitlin JD. Genetic and molecular basis for copper toxicity. *Am J Clin Nutr* 1996;63(5):836s–41s.
- Huang YL, Lu L, Luo XG, Liu B. An optimal dietary zinc level of broiler chicks fed a corn-soybean meal diet. *Poultry Sci* 2007;86(12):2582–9.
- Li L, Abouelezz KFM, Gou Z, Lin X, Wang Y, Fan Q, et al. Optimization of dietary zinc requirement for broiler breeder hens of Chinese yellow-feathered chicken. *Animals* 2019;9(7):472.
- Li SF, Lin YX, Lu L, Xi L, Wang ZY, Hao SF, et al. An estimation of the manganese requirement for broilers from 1 to 21 days of age. *Biol Trace Elem Res* 2011a;143(2):939–48.
- Li SF, Lu L, Hao SF, Wang YP, Zhang L, Liu SB, et al. Dietary manganese modulates expression of the manganese-containing superoxide dismutase gene in chickens. *J Nutr* 2011b;141(2):189–94.
- Liao JZ, Yang F, Chen HL, Yu WL, Han QY, Li Y, et al. Effects of copper on oxidative stress and autophagy in hypothalamus of broilers. *Ecotoxicol Environ Saf* 2019a;185:109710.
- Liao XD, Li A, Lu L, Liu SB, Li SF, Zhang LY, et al. Optimal dietary zinc levels of broiler chicks fed a corn–soybean meal diet from 22 to 42 days of age. *Anim Prod Sci* 2013;53(3):388–94.
- Liao XD, Ma CY, Lu L, Zhang LY, Luo XG. Determination of dietary iron requirements by full expression of iron-containing cytochrome c oxidase in the heart of broilers from 22 to 42 d of age. *Br J Nutr* 2017;118(7):493–9.
- Liao XD, Liu GQ, Sun GM, Sun XM, Liu T, Lu L, et al. Determination of optimal dietary selenium levels by full expression of selenoproteins in various tissues of broilers from 1 to 21 d of age. *Anim Nutr* 2021;7(4):1133–44.
- Liao XD, Wang G, Lu L, Zhang LY, Lan YX, Li SF, et al. Effect of manganese source on manganese absorption and expression of related transporters in the small intestine of broilers. *Poultry Sci* 2019b;98(10):4994–5004.
- Lin G, Guo Y, Liu B, Wang RG, Su XO, Yu DY, et al. Optimal dietary copper requirements and relative bioavailability for weanling pigs fed either copper proteinate or tribasic copper chloride. *J Anim Sci Biotechnol* 2020;11:54.
- Lu L, Chang B, Liao XD, Wang RL, Zhang LY, Luo XG. Use of molecular biomarkers to estimate manganese requirements for broiler chickens from 22 to 42 d of age. *Br J Nutr* 2016;116(9):1512–8.
- Ma XY, Liao XD, Lu L, Li SF, Zhang LY, Luo XG. Determination of dietary iron requirements by full expression of iron-containing enzymes in various tissues of broilers. *J Nutr* 2016;146(11):2267–73.
- McNaughton JL, Day EJ. Effect of dietary Fe to Cu ratios on hematological and growth responses of broiler chickens. *J Nutr* 1979;109(4):559–64.
- Mills CF, Dalgarno AC, Wenham G. Biochemical and pathological changes in tissues of Friesian cattle during the experimental induction of copper deficiency. *Br J Nutr* 1976;35(3):309–31.
- Mills CF, Dalgarno AC, Williams RB. Monoamine oxidase in ovine plasma of normal and low copper content. *Biochem Biophys Res Commun* 1966;24(4):537–40.
- Myint ZW, Oo TH, Thein KZ, Tun AM, Saeed H. Copper deficiency anemia: review article. *Ann Hematol* 2018;97(9):1527–34.
- NRC. Nutrition requirements of poultry. 9th ed. Washington D.C.: National Academy Press; 1994.
- Ognik K, Sembratowicz I, Cholewińska E, Jankowski J, Kozłowski K, Juśkiewicz J, et al. The effect of administration of copper nanoparticles to chickens in their drinking water on the immune and antioxidant status of the blood. *Anim Sci J* 2018;89(3):579–88.
- Prohaska JR, Brokate B. Lower copper, zinc-superoxide dismutase protein but not mRNA in organs of copper-deficient rats. *Arch Biochem Biophys* 2001;393:170–6.
- Rak M, Bénéit P, Chrétien D, Bouchereau J, Schiff M, El-Khoury R, et al. Mitochondrial cytochrome c oxidase deficiency. *Clin Sci (Lond)* 2016;130(6):393–407.
- Samanta B, Ghosh PR, Biswas A, Das SK. The effects of copper supplementation on the performance and hematological parameters of broiler chickens. *Asian Austral J Anim* 2011;24(7):1001–6.
- Sharma MC, Joshi C, Pathak NN, Kaur H. Copper status and enzyme, hormone, vitamin and immune function in heifers. *Res Vet Sci* 2005;79(2):113–23.
- Srinivas US, Tan BWQ, Vellayappan BA, Jeyasekharan AD. ROS and the DNA damage response in cancer. *Redox Biol* 2019;25:101084.
- Stemmer KL, Petering HG, Murthy L, Finelli VN, Menden EE. Copper deficiency effects on cardiovascular system and lipid metabolism in the rat; the role of dietary proteins and excessive zinc. *Ann Nutr Metab* 1985;29(6):332–47.
- Wang CC, Billett E, Borchert A, Kuhn H, Ufer C. Monoamine oxidases in development. *Cell Mol Life Sci* 2013;70(4):599–630.
- Wedekind KJ, Hortin AE, Baker DH. Methodology for assessing zinc bioavailability: efficacy estimates for zinc-methionine, zinc sulfate, and zinc oxide. *J Anim Sci* 1992;70(1):178–87.
- Yang HJ, Ralle M, Wolfgang MJ, Dhawan N, Burkhead JL, Rodriguez S, et al. Copper-dependent amino oxidase 3 governs selection of metabolic fuels in adipocytes. *PLoS Biol* 2018;16(9):e2006519.
- Yeung AWK, Georgieva MG, Atanasov AG, Tzvetkov NT. Monoamine oxidases (MAOs) as privileged molecular targets in neuroscience: research literature analysis. *Front Mol Neurosci* 2019;12:143.
- Zhang LY, Li XF, Liao XD, Zhang LY, Lu L, Luo XG. Effect of iron source on iron absorption and gene expression of iron transporters in the ligated duodenal loops of broilers. *J Anim Sci* 2017;95(4):1587–97.