

Dietary chloride levels affect performance and eggshell quality of laying hens by substitution of sodium sulfate for sodium chloride

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ABSTRACT The objective of this study was to evaluate the effect of dietary chloride (Cl) levels on performance, eggshell mechanical quality, and ultrastructure in layers based on the substitution of sodium chloride (NaCl) by sodium sulfate (Na₂SO₄). Three hundred sixty Jing Brown laying hens aged 43 wk were randomly divided into 5 groups and fed with corn–soybean meal diets containing 0.06, 0.10, 0.15, 0.20, and 0.25% total Cl inclusion. Every group had 8 replicates of 9 birds each. The feeding trial lasted for 12 wk. The results showed dietary 0.06% Cl due to complete substitution of NaCl by Na₂SO₄, depressed performance ($P < 0.05$) from 45 to 54 wk of age, increased serum creatinine level ($P < 0.05$), and caused visible renal tubular atrophy. Dietary Cl levels quadratically affected breaking strength, thickness, and weight of eggshell ($P < 0.05$). Better eggshell quality could be obtained when NaCl was partly replaced by Na₂SO₄ in laying hen diets

maintaining Cl level at 0.10 or 0.15%. Moreover, the eggshell ash content was affected by Cl levels in a quadratic ($P < 0.001$) manner, with higher values observed in the 0.10 and 0.15% Cl groups ($P < 0.05$). Besides, the eggshell ultrastructural data showed that the total thickness and effective thickness significantly increased ($P < 0.05$) and mammillary thickness decreased ($P < 0.05$) in the group of dietary 0.15% Cl compared with the groups of 0.06 and 0.25% Cl. In conclusion, the complete substitution of dietary NaCl by Na₂SO₄ may induce Cl deficiency and depress laying performance and eggshell quality. Na₂SO₄ could partly replace NaCl in diets for laying hens (43–54 wk of age) without adverse effects on performance at the dietary Cl level from 0.10 to 0.25%. Better eggshell quality could be obtained when NaCl was partly replaced by Na₂SO₄ in laying hen diets maintaining Cl level at ~0.15%.

Key words: sodium chloride, sodium sulfate, egg performance, eggshell quality, laying hen

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INTRODUCTION

It is well known that chloride (Cl) is one of the essential minerals for poultry. Cl plays an important role in the maintenance of fluid and electrolyte balance in the kidney. The recommended requirement of laying hens for dietary Cl is 0.15% (NRC, 1994), and the negative effect of Cl deficiency on performance was mostly observed at total dietary level less than 0.09% of intake (Christmas and Harms, 1982; Harms and Wilson, 1984; Pimentel and Cook, 1987; Harms, 1991). Similarly,

when an excess of Cl was fed, birds also showed poor performance (Christmas and Harms, 1982). In addition, eggshell quality is sensitive to Cl levels in diets and water for laying hens. Eggshell calcification is an ion transport process by which multiple ion transport, regulate, and collaborate to make the calcium and bicarbonate ion flow through the uterine apical membrane into uterine fluid to synthesize calcium carbonate (Eastin et al., 1978a, b; Nys et al., 1991; Jonchère et al., 2012). Nys (1999) reported that the dietary level of Cl below 0.2% was unfavorable to eggshell quality. In the model of avian uterine ion transport, the concentration of Cl⁻ in the blood had an effect on the transport of HCO₃⁻ (Jonchère et al., 2012), which may be the reason that the elevated Cl level affected the eggshell quality.

In commercial production, about 0.3% sodium chloride (NaCl) is usually added to the diets to meet the laying hens' needs of Cl and Na. It is to note that the contents of Cl and Na of NaCl are 59 and 39.5%, while

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sodium sulfate (Na_2SO_4) is an alternative and cost-effective Cl-free source of Na for poultry. Therefore, Na_2SO_4 could effectively maintain the optimum dietary electrolyte balance in growing broiler diets (Jarule et al., 2009). We also previously evaluated the biological safety of Na_2SO_4 for laying hens and observed the positive effects of dietary Na_2SO_4 on laying performance and eggshell quality (Wei, 2015). The substitution of Na_2SO_4 for NaCl in diets, which reduced the dietary Cl levels and increased the ratio of Na to Cl, has been reported to improve eggshell quality of laying hens (Faria et al., 2000). With the increasing substitution ratio of Na_2SO_4 for NaCl, dietary level of Cl decreased sequentially and may be in turn limiting the substitution. To test this hypothesis, this study was carried out to examine the effect of dietary Cl levels on the performance, eggshell mechanical quality, and ultrastructure when Na_2SO_4 was supplemented at varying levels as a Na source substitute. Besides, the serum biochemical parameters and renal histopathology were also examined. These findings would contribute to the application of Na_2SO_4 as an alternative of NaCl for laying hen production.

MATERIALS AND METHODS

Experimental Design and Diets

This study was approved by the Animal Care and Use Committee of the Feed Research Institute of the Chinese Academy of Agricultural Sciences. A total of 360 43-wk-old Jing Brown laying hens were randomly divided into 5 groups that were fed corn–soybean meal diet (Table 1) with different Cl levels. As determined by analysis, the

Table 1. Composition and nutrient content of the basal diet (air-dry basis).

Ingredient	%	Nutrient level ⁴	%
Corn	60.50	AME (MJ/kg)	11.34
Soybean meal	18.50	Crude protein	16.64
Extruded soybean	8.00	Calcium	3.65
Limestone	9.12	Total phosphorus	0.60
Calcium phosphate	1.72	Available phosphorus	0.36
DL-methionine	0.14	Lysine	0.83
L-Lysine HCl	0.10	Methionine + cystine	0.69
Montmorillonite	0.08	Sodium	0.02
Phytase	0.01	Chloride	0.06
Yeast Culture ¹	1.00		
Vitamin and mineral premix ²	0.23		
Experimental premix ³	0.60		
Total	100.00		

¹Yeast culture for white distiller's grains was an additive for laying hens' intestinal healthy.

²Provided the following per kilogram of diet: vitamin A, 12,500 IU; vitamin D₃, 4,125 IU; vitamin E, 15 IU; vitamin K₃, 2 mg; thiamine, 1 mg; riboflavin, 8.5 mg; calcium pantothenate, 50 mg; niacin, 32.5 mg; pyridoxine, 8 mg; biotin, 2 mg; folic acid, 5 mg; vitamin B₁₂, 5 mg; Zn, 66 mg; I, 1 mg; Fe, 60 mg; Cu, 8 mg; Se, 0.3 mg.

³Includes NaCl, Na_2SO_4 , and zeolite.

⁴Experimental diets were formulated in accordance with the Chinese Feeding Standard of Chicken (Ministry of Agriculture of China, 2004) and NRC (1994). The contents of Na and Cl in basal diets were determined by analysis in accordance with the Chinese national standard (Standardization Administration of China, 2017; Standardization Administration of China, 2007). Others are calculated values.

basal diet contained 0.02% Na and 0.06% Cl. The total dietary Cl levels of the 5 groups were 0.06, 0.10, 0.15, 0.20, 0.25%, which were provided by different supplemental levels of NaCl and Na_2SO_4 (Table 2). The Na levels of all diets were set to be 0.15% in accordance with the nutrient requirements of NRC (1994). Each group was replicated 8 times with 9 birds per replicate. The prefeeding period lasted for 1 wk (42–43 wk of age) with corn–soybean meal containing 0.15% Na and 0.15% Cl, and the feeding trial lasted for 12 wk (43–54 wk of age). Three birds were allotted to one cage (40 cm × 40 cm × 35 cm). The NaCl (Shandong Feicheng Refined Salt Plant Co., Ltd, Shandong, China) and Na_2SO_4 (Jiangsu Baimei Chemical Industry Co., Ltd, Jiangsu, China) were used. The water (containing 24.8 mg/L of Na and 17.8 mg/L of Cl) intake was recorded daily.

Performance and Egg Quality

During the feeding trial, egg production and egg weight were recorded daily, and feed consumption was recorded every 2 wks.

At the 48 wks and 54 wks of age, a total of 12 eggs from each replicate collected on 3 successive days were used to determine egg quality. The albumen height, yolk color, and Haugh unit (HU) were determined using an egg analyzer (ORKA Food Technology Ltd, Ramat Hasharon, Israel). Additional 12 eggs per replicate collected on 3 successive days were used to determine eggshell quality at the end of 46 wk, 50 wk, and 54 wk of age. Eggshell thickness and breaking strength were measured using the Egg Shell Thickness Gauge (Israel Orka Food Technology Ltd.) and the Egg Force Reader (Israel Orka Food Technology Ltd.) with membrane, respectively. Shell weight was measured after the eggshell was dried at room temperature for 48 h. The shell ratio was calculated as shell weight/egg weight × 100.

Eggshell Ultrastructure

Four sampled eggs from each replicate in 0.06, 0.15, and 0.25% Cl groups were selected. Eggshell ultrastructure on one piece of eggshell approximately 0.5 cm² from the equatorial section of each sampled egg was visualized by scanning electronic microscopy (FEI Quanta 600, Thermo Fisher Scientific Ltd., Portland, OR). Before scanning electronic microscopy imaging, both inside and outside of the eggshells were washed with distilled water to remove dirt. The effective thickness (combined palisade, vertical crystal layer, and cuticle), mammillary thickness, and the width of the mammillary knobs were measured and calculated in accordance with the method by Zhang et al. (2017). Mammillary thickness was measured as the length from the top of the membrane to the bottom of the palisade. The width of the mammillary knobs was calculated as follows: width = the length of mammillary knobs/the number of mammillary knobs. The total thickness referred to the combined effective

Table 2. Treatments and total Na and Cl levels in experimental diets.

Items	Experimental groups				
Experimental premix ¹					
Total dietary Cl level (%) ²	0.06 (0.05)	0.10 (0.09)	0.15 (0.14)	0.20 (0.20)	0.25 (0.24)
Total dietary Na level (%) ²	0.15 (0.16)	0.15 (0.16)	0.15 (0.16)	0.15 (0.15)	0.15 (0.15)
NaCl supplemental level (%)	0.00	0.07	0.15	0.24	0.33
Na ₂ SO ₄ supplemental level (%)	0.40	0.32	0.22	0.11	0.00
Zeolite powder (%)	0.20	0.21	0.23	0.25	0.27

¹Experimental premix accounted for 0.6% of basal diets.

²The number in parentheses is the analyzed value.

and mammillary thickness. The effective and mammillary layers (%) were the thickness of each layer relative to the total thickness. All of the scanning electronic microscopy photographs were taken using 200× magnification.

Ash Content in Eggshell

At the end of the trial, 4 eggshells from each replicate were collected as a sample to measure the ash content in eggshell. First, these eggshells were washed with distilled water to obliterate dirt and eggshell membranes completely. After drying at room temperature for 48 h, the eggshells were weighted, ground, and calcined to determine the ash content in eggshell.

Serum Biochemical Parameters

At the end of the feeding trial, 8 laying hens from each treatment (one laying hen per replicate) were selected randomly. The blood was collected from the wing vein and set at 37.5°C for 10 h to harvest serum. The serum was stored at -20°C until analysis. Plasma levels of creatinine (CRE), uric acid, albumen, and alkaline

phosphatase were determined using an automatic biochemical analyzer (Model 7020, Hitachi, Tokyo, Japan).

Histopathology

At the end of the feeding trial, 8 laying hens from each treatment (one laying hen per replicate) were selected randomly and executed. Kidneys were collected and stored in 4% formaldehyde solution for the pathological section. The samples were processed into wax sections of 4-μm thickness and then stained with hematoxylin-eosin (H&E) stain for histological analysis, in accordance with the method followed by Jadhav et al. (2007). The histopathological alterations were examined using an Olympus BX43 microscope (Olympus Corp., Tokyo, Japan). Histopathology was assessed by the qualified staff in the Department of Veterinary Pathology in Northeast Agricultural University.

Statistical Analysis

All analyses were performed using SAS, version 9.2, (2001, SAS Institute, NC). The replicate (each replicate in three cages) was taken as an experimental unit for

Table 3. Effect of dietary Cl levels by substitution of Na₂SO₄ for dietary NaCl on performance of laying hens (43-54 wk of age)¹.

Items ²	Time	Cl level (%)					SEM	P-value		
		0.06	0.10	0.15	0.2	0.25		ANOVA ³	Linear ⁴	Quadratic ⁴
Egg weight (g)	Weeks 43 to 44	63.73	64.83	64.73	64.62	63.96	0.26	0.590	0.950	0.290
	Weeks 45 to 46	61.10 ^b	63.38 ^a	63.58 ^a	63.33 ^a	63.38 ^a	0.23	<0.001	0.006	<0.001
	Weeks 47 to 50	60.87 ^b	63.73 ^a	63.92 ^a	63.20 ^a	63.36 ^a	0.24	<0.001	0.013	<0.001
	Weeks 51 to 54	60.55 ^b	65.99 ^a	66.18 ^a	64.99 ^a	65.44 ^a	0.39	<0.001	0.002	<0.001
	Weeks 43 to 54	61.56 ^b	64.48 ^a	64.60 ^a	64.03 ^a	64.03 ^a	0.24	<0.001	0.001	<0.001
Egg production (%)	Weeks 43 to 44	89.19	88.19	89.39	89.88	89.39	0.65	0.950	0.640	0.900
	Weeks 45 to 46	76.89 ^b	85.91 ^a	86.41 ^a	86.91 ^a	87.00 ^a	0.71	<0.001	<0.001	<0.001
	Weeks 47 to 50	78.27 ^b	89.04 ^a	90.18 ^a	88.24 ^a	90.77 ^a	1.00	<0.001	<0.001	<0.001
	Weeks 51 to 54	77.20 ^b	92.07 ^a	91.49 ^a	92.32 ^a	92.90 ^a	1.04	<0.001	<0.001	<0.001
	Weeks 43 to 54	80.39 ^b	88.80 ^a	89.37 ^a	89.34 ^a	90.01 ^a	0.66	<0.001	<0.001	<0.001
ADFI (g)	Weeks 43 to 44	111.27	114.70	116.32	117.23	116.18	0.35	0.310	0.070	0.090
	Weeks 45 to 46	101.14 ^c	107.97 ^{a,b}	108.14 ^{a,b}	105.03 ^b	108.53 ^a	0.63	<0.001	0.010	0.006
	Weeks 47 to 50	114.39 ^b	121.46 ^a	121.94 ^a	120.87 ^a	120.51 ^a	0.69	<0.001	0.020	<0.001
	Weeks 51 to 54	122.79 ^b	132.92 ^a	131.73 ^a	129.44 ^a	133.29 ^a	0.97	<0.001	0.013	0.011
	Weeks 43 to 54	112.40 ^b	119.26 ^a	119.53 ^a	118.14 ^a	119.63 ^a	0.59	<0.001	0.001	<0.001
FCR	Weeks 43 to 44	1.75	1.77	1.8	1.81	1.82	0.01	0.410	0.050	0.130
	Weeks 45 to 46	1.66	1.70	1.70	1.66	1.71	0.01	0.070	0.270	0.520
	Weeks 47 to 50	1.88	1.91	1.91	1.91	1.90	0.01	0.820	0.470	0.500
	Weeks 51 to 54	2.03	2.02	1.99	1.99	2.04	0.01	0.520	0.980	0.250
	Weeks 43 to 54	1.83	1.85	1.85	1.84	1.87	0.01	0.420	0.100	0.270

^{a,b,c} Means within a row with no common superscripts differ significantly ($P < 0.05$).

¹Data are the mean of 8 replicates with 9 birds each.

²ADFI, average daily feed intake; FCR, feed conversion ratio (feed: egg, g: g).

³Comparisons among groups of all variables were evaluated from one-way ANOVA and Duncan test.

⁴Linear and quadratic effects of dietary Cl inclusion were evaluated using regression analysis.

Table 4. Effect of dietary Cl levels by substitution of Na₂SO₄ for dietary NaCl on egg quality of laying hens (43–54 wk of age)¹.

Items ²	Time	Cl level (%)					SEM	P-value		
		0.06	0.10	0.15	0.20	0.25		ANOVA ³	Linear ⁴	Quadratic ⁴
Albumen height (mm)	48 wk	6.36	7.12	6.95	5.99	6.46	0.21	0.460	0.500	0.660
	54 wk	6.31	6.11	6.06	6.18	6.19	0.05	0.690	0.720	0.430
Yolk color	48 wk	5.10	4.83	4.83	4.88	5.08	0.05	0.150	0.920	0.040
	54 wk	4.79	4.59	4.56	4.84	4.67	0.06	0.480	0.960	0.820
HU ³	48 wk	72.91	79.64	80.20	72.09	77.72	1.80	0.490	0.920	0.800
	54 wk	81.29	80.38	78.72	79.42	78.63	0.48	0.350	0.066	0.140

^{a,b,c} Means within a row with no common superscripts differ significantly ($P < 0.05$).

¹Data are the mean of 8 replicates (12 eggs of each replicate).

²HU, Haugh unit.

³Comparisons among groups of all variables were evaluated from one-way ANOVA and Duncan test.

⁴Linear and quadratic effects of dietary Cl inclusion were evaluated using regression analysis.

analysis of performance data. All data were analyzed using a one-way ANOVA, and means were compared using the Duncan multiple range test. The linear and quadratic effects of total Cl dose were assessed using regression analysis. Data were expressed as mean and pooled SEM. A P -value less than 0.05 was declared significant.

The regression model was as follows: $Y_{ij} = \alpha + \beta_1 X_i + e_{ij}$ (linear regression); $Y_{ij} = \alpha + \beta_1 X_i + \beta_2 X_i^2 + e_{ij}$ (quadratic regression).

Y_{ij} was the response variable, α was the intercept (indicators with the basal diet), β_1 and β_2 were regression coefficients, X_i was the studied factor effect as the inclusion of Cl ($i = 0.06, 0.10, 0.15, 0.20, 0.25\%$), and e_{ij} was the observational error for (ij)th observation.

RESULTS

Laying Performance

As showed in Table 3, during 43 to 44 wk of age, the Cl levels in diets had no significant effect ($P > 0.05$) on laying hens' performance, but the egg weight, egg production, and average daily feed intake (ADFI) were linearly and quadratically affected ($P < 0.05$) by the dietary Cl level from 45 to 54 wk of age.

Compared with other treatments, the egg weight, egg production, and ADFI of laying hens fed with the 0.06% Cl significantly decreased ($P < 0.05$). Over the whole 12 wk of the experiment, the water intake of each bird was 230 to 277 mL per day, and there was no significant difference among treatments ($P > 0.05$; data not shown).

Egg and Eggshell Quality

No linear and quadratic effects ($P > 0.05$) were observed in albumen height, yolk color, and HU in response to dietary Cl levels during the entire experimental period (Table 4).

The effect of dietary Cl levels on eggshell quality of laying hens is shown in Table 5. Total Cl levels in diets quadratically affected the breaking strength of eggshell during 47 to 50 wk and 51 to 54 wk ($P < 0.001$). Among all the groups, breaking strength of laying hens fed diets containing 0.10 and 0.15% Cl were much higher. Compared with the 0.10 and 0.15% Cl levels, the breaking strength of laying hens fed diets containing 0.20 and 0.25% Cl significantly decreased ($P < 0.05$) during 47 to 54 wk. The eggshell thickness ($P = 0.002$) and eggshell weight were quadratically affected ($P < 0.001$) by dietary Cl levels. Compared

Table 5. Effect of dietary Cl levels by substitution of Na₂SO₄ for dietary NaCl on eggshell quality of laying hens (43–54 wk of age)¹.

Item	Time	Cl level (%)					SEM	P-value		
		0.06	0.10	0.15	0.20	0.25		ANOVA ²	Linear ³	Quadratic ³
Breaking strength (N)	46 wk	37.95	38.04	38.19	38.03	38.12	0.32	0.990	0.890	0.980
	50 wk	35.16 ^c	38.88 ^a	39.24 ^a	37.00 ^b	36.14 ^{b,c}	0.34	<0.001	0.880	<0.001
	54 wk	33.93 ^c	39.05 ^a	39.01 ^a	36.15 ^b	35.99 ^b	0.38	<0.001	0.810	<0.001
Thickness (μm)	46 wk	42.76	42.75	42.7	42.38	42.14	0.16	0.710	0.190	0.340
	50 wk	42.25	43.23	43.47	42.65	42.29	0.18	0.100	0.620	0.040
	54 wk	42.44 ^c	44.52 ^a	44.54 ^a	43.45 ^b	43.52 ^b	0.18	<0.001	0.500	0.002
Shell weight (g)	46 wk	6.10	6.28	6.28	6.23	6.23	0.03	0.380	0.053	0.150
	50 wk	6.31	6.46	6.48	6.43	6.31	0.03	0.190	0.820	0.046
	54 wk	5.89 ^c	6.82 ^a	6.89 ^a	6.59 ^b	6.62 ^b	0.06	<0.001	0.007	<0.001
Shell ratio (%)	46 wk	10.10	10.04	9.91	10.00	9.94	0.03	0.310	0.120	0.190
	50 wk	10.31	10.05	10.00	10.16	10.07	0.04	0.140	0.240	0.170
	54 wk	9.79	9.88	10.12	10.03	10.00	0.05	0.310	0.130	0.130

^{a,b,c} Means within a row with no common superscripts differ significantly ($P < 0.05$).

¹Data are the mean of 8 replicates (12 eggs of each replicate).

²Comparisons among groups of all variables were evaluated from one-way ANOVA and Duncan test.

³Linear and quadratic effects of dietary Cl inclusion were evaluated using regression analysis.

Table 6. Effect of dietary Cl levels by substitution of Na₂SO₄ for dietary NaCl on serum biochemical parameters of laying hens (43–54 wk of age)¹.

Item ²	Cl level (%)					SEM	P-value		
	0.06	0.1	0.15	0.2	0.25		ANOVA ³	Linear ⁴	Quadratic ⁴
ALP (U/L)	304.90	260.31	310.38	259.58	248.63	17.33	0.720	0.410	0.670
ALB (g/L)	20.90	20.73	20.20	20.14	19.86	0.23	0.590	0.240	0.500
CRE (U/L)	23.00 ^a	13.81 ^b	15.88 ^b	17.38 ^b	15.25 ^b	0.74	<0.001	0.030	0.010
UA (U/L)	316.19	257.69	253.56	268.81	254.69	9.55	0.190	0.110	0.120

^{a,b,c} Means within a row with no common superscripts differ significantly ($P < 0.05$).

¹Data are the mean of 8 replicates (one laying hen per replicate).

²ALP, alkaline phosphatase; ALB, activities of albumen; CRE, creatinine; UA, uric acid.

³Comparisons among groups of all variables were evaluated from one-way ANOVA and Duncan test.

⁴Linear and quadratic effects of dietary Cl inclusion were evaluated using regression analysis.

with the 0.10 and 0.15% Cl groups, 0.20 and 0.25% Cl groups had decreased eggshell thickness ($P < 0.05$) and weight ($P < 0.05$).

Serum Biochemical Parameters and Renal Histopathology

The Cl levels linearly and quadratically decreased ($P = 0.030$, $P = 0.010$, respectively) the concentration of CRE in serum (Table 6). The dietary 0.06% Cl treatment significantly increased ($P < 0.05$) the serum CRE level in birds. Figure 1 showed the renal histopathology images of laying hens fed diets containing 0.06, 0.15, and 0.25% Cl. The laying hens fed diets containing 0.06% Cl showed kidney lesions, characterized by visible tubular atrophy.

Ash Content in Eggshell

As showed in Table 7, the ash content in sampled eggshells was affected by the Cl levels in diets in a quadratic ($P < 0.001$) manner. In comparison with that of 0.10 and 0.15% Cl groups, the ash contents of laying hens fed the diets containing the 0.20 and 0.25% Cl significantly decreased ($P < 0.05$) in sampled eggshells.

Eggshell Ultrastructure

Scanning electron microscopy images in Figure 2 reveal the eggshell ultrastructure of laying hens fed with 0.06, 0.15, and 0.25% Cl levels. Compared with the other two treatments, the effective thickness and

total thickness of eggshell of the 0.15% Cl group significantly increased ($P < 0.05$; Table 8), and the mammillary thickness of that group significantly decreased ($P < 0.05$; Table 8).

DISCUSSION

Na₂SO₄ is an available Cl-free source of Na for laying hens which could be used to reduce NaCl to maintain the ratio between Cl and Na in diets (Wei, 2015; Fu, 2019). However, this replacement, which greatly reduced the dietary level of Cl, would have potentially detrimental effects on performance. In the present study, feed formulation was based on the substitution of NaCl by Na₂SO₄, maintaining total dietary Na level at 0.15% during the phase from 43 to 54 wk of age. In accordance with our hypotheses, there were linear and quadratic decreases in performance data in response to reduced dietary Cl level. In accordance with other studies, the diets with 0.06% Cl, which was caused by complete substitution of NaCl by Na₂SO₄ in the experiment diets, significantly decreased the egg weight, egg production, and ADFI of the laying hens (Harms and Wilson, 1984; Harms, 1991). No significant effect on feed conversion ratio was observed, and the performance depression might be mainly attributed to the reduced ADFI. The deficiency of Cl decreased ADFI may result in the emergence of metabolic alkalosis, which could downregulate the γ -aminobutyric acid release in the brain to reduce feed intake (Pu et al., 1999; Lu et al., 2012). Besides, Cl deficiency also caused an increased reabsorption of

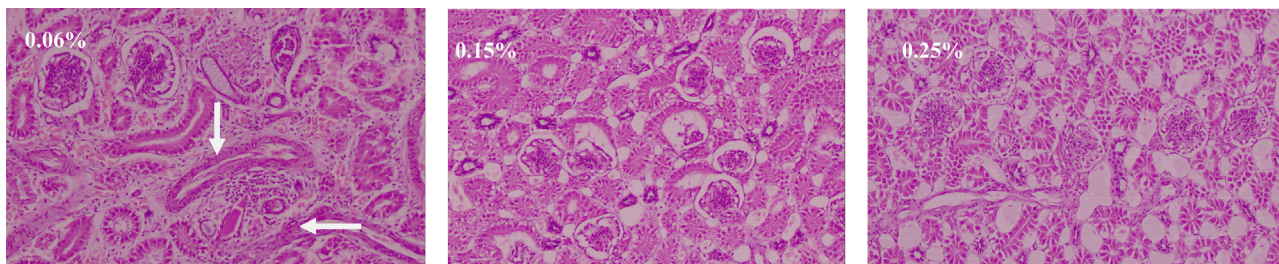


Figure 1. Effect of dietary Cl level by substitution of Na₂SO₄ for dietary NaCl on kidney histopathology of layers fed the experimental diets (hematoxylin–eosin staining, magnification, 200 \times). The 0.06, 0.15, and 0.25% were fed a corn–soybean meal diets with 0.06, 0.15, and 0.25% Cl, respectively. The laying hens fed with 0.06% Cl level in diets showed visible tubular atrophy (arrows).

Table 7. Effect of dietary Cl levels by substitution of Na_2SO_4 for dietary NaCl on eggshell ash content of laying hens (54 wk of age)¹.

Item	Cl level (%)					SEM	P-value		
	0.06	0.1	0.15	0.2	0.25		ANOVA ²	Linear ³	Quadratic ³
Ash content (%)	95.90 ^c	96.83 ^a	97.11 ^a	96.40 ^b	96.17 ^b	0.09	<0.001	0.980	<0.001

^{a,b,c} Means within a row with no common superscripts differ significantly ($P < 0.05$).

¹Data are the mean of 8 replicates (4 eggs of each replicate).

²Comparisons among groups of all variables were evaluated from one-way ANOVA and Duncan test.

³Linear and quadratic effects of dietary Cl inclusion were evaluated using regression analysis.

bicarbonates in the tubular atrophy of rats, followed by the increased angiotensin II (Tank et al., 1998) and then tubular atrophy (Mitani et al., 2002; Kitayama et al., 2006; Ozawa et al., 2007), and finally impaired the renal function. In the present study, the renal histological damage of laying hens characterized by visible tubular atrophy was observed in the group of 0.06% Cl level. Serum levels of CRE, an indicator of kidney function, was also increased by the diets with the level of 0.06% Cl. The observed tubular atrophy and higher CRE level may indicate kidney impairment (Benjamin, 1985; Yadav et al., 2015; Oka and Raka-Widiana, 2015) induced by complete substitution of NaCl by Na_2SO_4 in the experiment diets, which might be responsible for the depression in performance.

In fact, the adverse effects of dietary Cl were mostly reported to be at the level less than 0.09% of intake (Christmas and Harms, 1982; Harms and Wilson, 1984; Pimentel and Cook, 1987; Harms, 1991), and no adverse effects on performance were observed when the Cl level in diets (containing 0.14~0.18% Na) was more than 0.13% (Austic, 1984; Faria et al., 2000). In the present study, there was no significant difference among groups on performance when NaCl was substituted by Na_2SO_4 , maintaining dietary Cl level from 0.10 to 0.25%. Similar results were observed in serum biochemical parameters. These data suggested that Na_2SO_4 could partly replace NaCl in diets for laying hens (43–54 wk of age) without adverse effects on performance at the dietary Cl level from 0.10 to 0.25%.

The substitution of NaCl by Na_2SO_4 in diets had a quadratic effect on eggshell quality in response to the

variation of dietary Cl levels. But no differences were observed in the other egg quality items among the groups during the overall feeding period, except a temporary quadratic change of yolk color on the 48 wk of age. Lowest values were observed in the group of 0.06% Cl level and higher in 0.10 and 0.15% groups for eggshell breaking strength, thickness, and weight. Reduced ADFI of laying hens caused by complete substitution of NaCl by Na_2SO_4 might be the main reason for the decreased eggshell quality in the group of 0.06% Cl level. For example, the intake of Ca was reduced by 6.1% in 0.06% group compared with 0.10% group because of lower ADFI from 45 wk to 54 wk in this study, which would provide less Ca for calcium carbonate synthesis in the eggshell gland. It is evident by the observed decreased eggshell ash content, composed of ~97% calcium carbonate, in the group of dietary 0.06% Cl. On the other hand, the diets with the Cl level of 0.20 and 0.25%, close to the Cl level in commercial diets, also had lower eggshell ash and quality compared with the diets of 0.10 and 0.15% Cl. During eggshell formation, the HCO_3^- flow through the uterine apical membrane was facilitated by output of Cl^- (Jonchère et al., 2012). And elevated Cl level in the diets or water may disturb the balance of Cl^- and HCO_3^- and not be favorable to calcium carbonate synthesis, which needs to be further confirmed. Moreover, Na_2SO_4 supplementation might not be responsible for the decreased eggshell quality in this study. Instead, we observed previously the positive effects of 0.3 and 0.6% Na_2SO_4 supplementation in diets (containing 0.3% NaCl) on eggshell quality (Wei, 2015).

Our results showed that better eggshell quality, that is, higher eggshell breaking strength, thickness, and

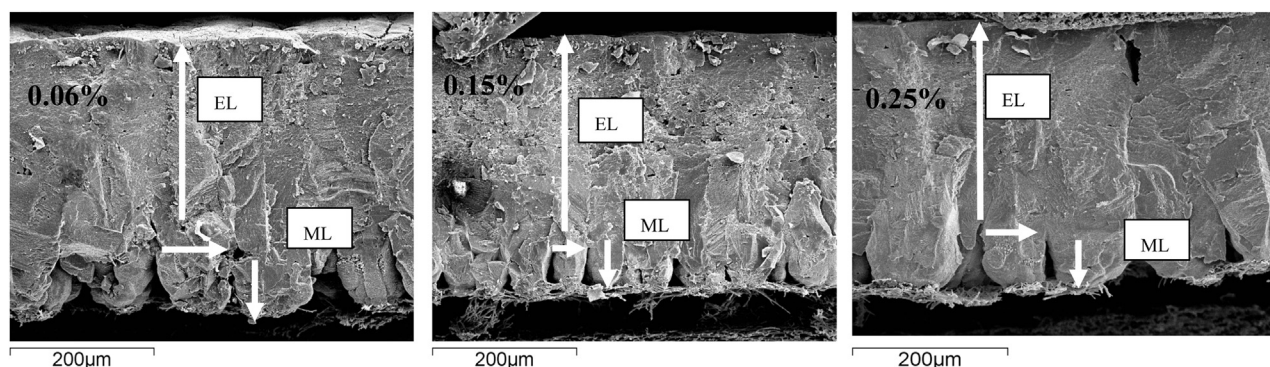


Figure 2. Effect of dietary Cl levels by substitution of Na_2SO_4 for dietary NaCl on the eggshell ultrastructure of layers fed the experimental diets (scanning electron microscope images, magnification, 200 \times). The 0.06, 0.15, and 0.25% were fed a corn–soybean meal diets with 0.06, 0.15, and 0.25% Cl, respectively. ML, mammillary layer; EL, effective layer.

Table 8. Effect of dietary Cl levels by substitution of Na₂SO₄ for dietary NaCl on eggshell ultrastructure of laying hens (54 wk of age)¹.

Item	Cl level (%)			SEM	ANOVA ²
	0.06	0.15	0.25		
Mammillary knob width (μm)	93.04 ^a	75.18 ^b	76.60 ^b	1.74	<0.001
Mammillary thickness (μm)	90.90 ^a	65.67 ^c	84.20 ^b	2.41	<0.001
Effective thickness (μm)	263.27 ^c	318.89 ^a	280.19 ^b	5.48	<0.001
Total thickness (μm)	354.17 ^c	384.56 ^a	364.39 ^b	3.72	<0.001
Mammillary layer (%)	25.70 ^a	17.07 ^c	23.13 ^b	0.80	<0.001
Effective layer (%)	74.30 ^c	82.93 ^a	76.87 ^b	0.80	<0.001

^{a,b,c} Means within a row with no common superscripts differ significantly ($P < 0.05$).

¹Data are the mean of 8 replicates (4 eggs of each replicate).

²Comparisons among groups of all variables were evaluated from one-way ANOVA and Duncan test.

eggshell weight, could be obtained when NaCl was partly replaced by Na₂SO₄ in laying hen diets maintaining Cl level at 0.10 and 0.15%. Dietary Cl levels of 0.13 and 0.14% had been recommended previously in consideration of eggshell quality (Faria et al., 2000; Murakami et al., 2003). The effects of Cl levels were evaluated by quadratic regression analysis in this study, and a reliable equation was obtained for breaking strength of eggshell (51–54 wk): $y = -419.04x^2 + 128.41x + 28.88$ ($P < 0.05$, $R^2 = 0.44$). The optimal Cl level in laying hen diets was calculated as 0.1532% when dietary NaCl was replaced by Na₂SO₄. Then, we further analyzed the eggshell ultrastructure among the groups of dietary Cl level at 0.06, 0.15, and 0.25%. Consistent with the observation of eggshell thickness, the ultrastructural data showed increased total thickness in the group of dietary 0.15% Cl. The increased total thickness mainly resulted from the effective layer of eggshell, which contributes to two-thirds of shell thickness and is crucial for eggshell breaking strength (Radwan et al., 2010; Radwan, 2016). Besides, decreased thickness of the mammillary layer and width of the mammillary cones, also observed in the group of dietary 0.15% Cl, were reported to improve the bonding strength between mammillary cones and be finally beneficial to eggshell resistance (Dunn et al., 2012). Thus, apart from the eggshell component, improved eggshell ultrastructure may be the other reason for the increased eggshell strength in the group of dietary 0.15% Cl.

In conclusion, complete substitution of dietary NaCl by Na₂SO₄ in diets may induce Cl deficiency and depress laying performance and eggshell quality. Na₂SO₄ could partly replace NaCl in diets for laying hens (43–54 wk of age) without adverse effects on performance at the dietary Cl level from 0.10 to 0.25%. And better eggshell quality could be obtained when NaCl was partly replaced by Na₂SO₄ in laying hen diets maintaining Cl level at ~0.15%.

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