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Original Research Article

Alterations on vitamin C synthesis and transportation and egg deposition induced by dietary vitamin C supplementation in Hy-Line Brown layer model

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ABSTRACT

In ovo feeding of vitamin C (VC) has positive effects on the growth performance, immune and antioxidant function in poultry, which indicates that increasing VC content in eggs may be of benefit. This study was to investigate the effects of dietary VC supplementation on VC synthesis and transportation and egg deposition. In Exp. 1, in order to select a suitable animal model, VC content was detected in different eggs from different layer species. Vitamin C content was lower in ISA Brown breeder eggs and Hy-Line Brown layer eggs ($P < 0.05$) then in Arbor Acres breeder eggs. In Exp. 2, a total of 24 Hy-Line Brown layers (42-week-old) were randomly divided into 3 treatments with 8 replicates and fed a basal diet with VC at 0, 200 and 400 mg/kg. Sodium-dependent VC transporter 1 and 2 (*SVCT1* and *SVCT2*) expressions were higher in ileum than in duodenum and jejunum ($P < 0.05$). *SVCT1* expression was higher but *SVCT2* expression was lower in the magnum than in the ovary ($P < 0.05$). L-Gulonolactone oxidase (*GLO*) and *SVCT1* expressions were higher but *SVCT2* was lower in the kidney than in the liver ($P < 0.05$). Dietary VC supplementation at 400 mg/kg increased *SVCT1* expression in duodenum, ovary and magnum, but decreased *GLO* and *SVCT1* expression in liver ($P < 0.05$). Dietary VC supplementation at 200 and 400 mg/kg increased *SVCT2* expression in duodenum, but decreased *GLO* and *SVCT1* expression in kidney and *SVCT2* expression in liver ($P < 0.05$). Dietary VC supplementation promoted VC absorption in duodenum and jejunum, but reduced endogenous VC synthesis in liver and kidney. Although dietary VC supplementation enhanced VC transportation in ovary and magnum, it did not increase VC deposition in produced eggs.

1. Introduction

Embryonic nutrient supplementation is expected to become a great potential way to improve the performance in poultry (Peebles, 2018; Retes et al., 2018). Some researchers have indicated that in ovo feeding of vitamin C (VC) had positive effects on growth performance, immune and antioxidant function in poultry (El-Senousey et al., 2018; Zhang et al., 2019; Zhu et al., 2019, 2020). In humans, more and more studies have proved that the epigenetics diet (including VC), offered for pregnant mothers, leaded to beneficial health outcomes in newborn babies by nutri-epigenetics (Li et al., 2019). Unlike viviparous animals, birds lack a placental structure and embryonic development is completely separated from mothers, thus embryonic VC requirements theoretically rely

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on egg deposition from mother and self-synthesis by embryo. Vitamin C can be synthesized via glucuronate–xylulose cycle in the presence of L-gulonolactone oxidase (*GLO*) by developing embryo (Gan et al., 2018), but its synthesis amount may be insufficient (Nowaczewski et al., 2012), which is why new-hatched chicks usually need to be supplemented with VC in farm or hatchery. And increasing egg VC deposition is a way to improve embryonic VC supplementation.

In eggs, fat-soluble vitamins are merely deposited in yolk, while water-soluble vitamins are deposited in both yolk and albumen (Rehault-Godbert et al., 2019). It is possible for some vitamins to achieve higher enrichment in produced eggs by dietary supplementation in hens, especially all fat-soluble vitamins, but also some water-soluble vitamins (vitamin B_{12}, folic acid and pantothenic acid) (Schiavone and Barroeta, 2011). As for other water-soluble vitamins (including VC), the literatures are scare. According to nutrient requirements of poultry in NRC, VC is not a vitamin that must be added in vitamin premix of poultry, and whether dietary VC supplementation in hens could increase VC content in produced eggs still has not been reported.

Egg yolk and albumen are formed at the hen's ovary and magnum, respectively (Estienne et al., 2020; Nys and Guyot, 2011). Consequently, exploring VC deposition in eggs would be inseparable from detecting the transportation function of ovary and magnum. The source of the hen's VC pool is mainly from exogenous absorption by sodium-dependent VC transporter 1 and 2 (*SVCT1* and *SVCT2*) in the intestine and endogenous synthesis by *GLO* in the liver and kidney (Hooper et al., 2002; Lindblad et al., 2013; Lykkesfeldt and Tveden-Nyborg, 2019; Mandl et al., 2009; Subramanian et al., 2017). As the oxidized form of VC, dehydroascorbic acid can be transported by glucose transporters, but plasma dehydroascorbic acid content was only from about <1% to 2% of plasma VC (Padayatty and Levine, 2016). Therefore, the transportation and distribution of VC in various tissues (including ovary and magnum) mainly depends on blood circulation and sodium-dependent VC transporters (*SVCT*).

In this study, we first selected a better animal model by comparing the egg parameters and VC contents of Arbor Acres breeder hens, ISA Brown breeder hens and Hy-Line Brown layers. Then, we measured the effects of dietary VC supplementation on VC synthesis and transportation and egg deposition. This study will prove whether dietary VC supplementation can achieve egg VC deposition and provide new insights for the approaches of embryonic VC supplementation in poultry.

2. Materials and methods

The use of animals and all experimental protocols were authorized by the Institutional Animal Care and Use Committee of Northwest A&F University (Yangling, Shaanxi, China).

2.1. Experiment 1

2.1.1. Experimental procedures

Experiment 1 was conducted to compare the egg parameters and VC contents among the eggs of Arbor Acres breeder hens, ISA Brown breeder hens and Hy-Line Brown layers. All 3 hen diets were corn-soybean meal without VC supplementation, but with different nutrient levels. On the same day, 15 fertilized eggs were selected from 52-week-old Arbor Acres breeder hens, 19 fertilized eggs were selected from 39-week-old ISA Brown breeder hens and 15 unfertilized eggs were selected from 47-wk-old Hy-Line Brown layers. Every egg was weighed. Fresh albumen and yolk were separated, weighed, and packed into 50-mL cryotube, respectively. After being placed at −80 °C for 24 h, the albumen and yolk samples were immediately put into a pre-cooled vacuum freeze dryer (Siemens, Berlin, Germany) for the preparation of lyophilized powders. Lyophilized powders of albumen and yolk were weighed and stored at −20 °C for further analysis.

2.1.2. Egg parameters

Egg parameters included egg weight, the ratio of yolk fresh weight to albumen fresh weight (YFW:AFW ratio), the ratio of yolk dry weight to albumen dry weight (YDW:ADW ratio), yolk moisture content (YMC) and albumen moisture content (AMC). The weight of lyophilized powders of albumen and yolk represented albumen and yolk dry weights, respectively. The formulas employed were as follows.

$$\text{YFW/AFW (\%)} = \text{YFW/AFW} \times 100$$

$$\text{YDW/ADW (\%)} = \text{YDW/ADW} \times 100$$

$$\text{YMC (\%)} = [(\text{YFW - YDW})/\text{YFW}] \times 100$$

$$\text{AMC (\%)} = [(\text{AFW - ADW})/\text{AFW}] \times 100$$

2.1.3. Measurement of vitamin C content in the eggs

Five milliliters of pre-cooled physiological saline and 1 g of albumen or yolk lyophilized powder were added into a 10-mL centrifuge tube in sequence. The mixture was shaken violently and centrifuged at 664 × *g* for 15 min. Then, the VC content in the supernatant was measured by commercially available kits (A009, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on kit instructions. According to the kit's specification, the sample quality control was strictly implemented. The VC content in the eggs was calculated as follows.

Yolk VC content (YVCC, μg/g of yolk DM) = [the VC content in the supernatant (μg/mL) × 5 mL)/1 g of yolk DM

Albumen VC content (AVCC, μg/g of albumen DM) = [the VC content in the supernatant (μg/mL) × 5 mL]/1 g of albumen DM

2.2. Experiment 2

2.2.1. Experimental procedures

A total of 24 Hy-Line Brown layers (42-week-old) were randomly divided into 3 treatments with 8 replicates and fed a basal diet (Table 1) with 0, 200 and 400 mg/kg VC supplementation, respectively. One hundred and twenty grams of feed per layer was supplied daily to maintain the consistency of VC intake in the same treatment. Drinking water was adequate and the feeding time was at 09:00 and 16:00. The feeding trial lasted for 28 d and the trial time was named sequentially from d 1 to 28. The eggs produced on d 23 and 28 were collected for egg quality analysis and the preparation of lyophilized powder. In the end, all layers were fasted for 12 h. Then, blood samples were taken from the jugular vein, and all layers were euthanized by cervical dislocation. The tissue samples (liver, kidney, ovary and magnum) and the intestinal mucosa samples (duodenum, jejunum and ileum) were collected, snap-frozen in liquid N_2 and then stored at −80 °C for further analysis.

2.2.2. Laying performance and egg quality

The daily produced eggs were weighed and counted. Egg production rate, egg weight and feed-to-egg ratio were calculated. The

Table 1
Composition and nutrient levels of basal diet in Exp. 2 (%, as-fed basis).

Item	Content
Ingredients	
Corn	59.76
Soybean meal	23.00
Distillers dried grains with solubles	4.00
Calcium carbonate	8.65
Dicalcium phosphate	1.05
Soybean oil	1.25
Sodium chloride	0.25
L-Lysine-H_2SO_4	0.13
L-Threonine	0.31
DL-Methionine	0.25
Choline chloride	0.10
Sand	0.25
Vitamin and mineral premix[1]	1.00
Total	100.00
Nutrient levels (calculated)	
Metabolizable energy, kcal/kg	2,700
Crude protein	16.5
Total phosphorus	0.51
Non-phytate phosphorus	0.32
Calcium	3.50

[1] Provided the following per kilogram of diet: manganese 60 mg, copper 8 mg, zinc 80 mg, iodine 0.35 mg, selenium 0.3 mg, vitamin A 8,000 IU, vitamin D_3 1,600 IU, vitamin E 30 mg, vitamin K_3 1.5 mg, thiamine 4 mg, riboflavin 13 mg, pantothenic acid 15 mg, nicotinamide 20 mg, pyridoxine 6 mg, biotin 0.15 mg, folic acid 1.5 mg, cobalamin 0.02 mg. No vitamin C added.

eggs produced on d 23 were analyzed for egg quality, including yolk color, Haugh unit, albumen height, shell strength, and shell thickness.

2.2.3. Measurement of egg parameters and vitamin C content in serum and produced eggs

Egg parameters measurement and lyophilized powders preparation were the same as in Exp. 1. The blood samples were centrifuged at 664 × *g* for 10 min to obtain the serum samples and stored at −20 °C for further analysis. The VC content in lyophilized powders and serum was measured by commercially available kits, which was the same as in Exp. 1.

2.2.4. Quantitative real-time PCR

Total RNA was extracted from the liver, kidney, intestinal mucosa (duodenum, jejunum and ileum) and reproductive tract (ovary and magnum), following TRIzol Reagent protocol (AG21102, AG, Changsha, China). The concentration, purity and integrity of RNA samples were verified and cDNA was synthesized with an *Evo M-MLV* RT Kit for qPCR (AG11707, AG, Changsha, China). *SVCT1* and *SVCT2* expression in the liver, kidney, intestinal mucosa and reproductive tract and *GLO* expression in the liver and kidney were analyzed with a SYBR Green Premix Pro Taq HS qPCR Kit (AG11701, AG, Changsha, China) on the iCycler IQ5 (Bio-Rad, Hercules, CA, USA). Detailed reaction system was referred to our previous description (Zhu et al., 2019). The primers are listed in Table 2. All samples were run in triplicate and the average cycle threshold (Ct) values were normalized to β-actin and quantified by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.3. Statistical analysis

Expressions of *SVCT1* and *SVCT2* between different tissues (ovary vs. magnum, and liver vs. kidney) and *GLO* expression (liver vs. kidney) were compared, and data were analyzed by independent sample t test. All other data were analyzed by one-way ANOVA using the SPSS 21.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was considered at $P < 0.05$ and trends at $P < 0.1$.

Table 2
Primer sequence of target genes in Exp. 2.

Gene	Accession number	Primer sequences	Product Size, bp
β-actin	NM_205518.1	F: ATTGTCCACCGCAAATGCTTC R: AAATAAAGCCATGCCAATCTCGTC	113
SVCT1	XM_004944768.3	F: GCTGTACCAGATCGAGGACG R: AGGTGAAGATGGTGCCGATG	173
SVCT2	XM_025142777.1	F: AGGCAAACACTGGGGTATCG R: GCGAGCATAGAAGCCGTACT	247
GLO	XM_015285218.2	F: GCCAAGGAGGATTCAAGTT R: GATGTCAGAGGGCGAGTG	167

SVCT1 = sodium-dependent vitamin C transporter 1; *SVCT2* = sodium-dependent vitamin C transporter 2; *GLO* = L-gulonolactone oxidase.

3. Results

3.1. Experiment 1

3.1.1. Comparison of egg parameters and vitamin C content among different layer species

As shown in Table 3, egg weight was the heaviest in Arbor Acres breeder hens and was the lightest in Hy-Line Brown layers ($P < 0.05$). Compared with ISA Brown breeder hens and Hy-Line Brown layers, Arbor Acres breeder hens had significantly higher YFW:AFW ratio, YDW:ADW ratio, AMC, YVCC and AVCC ($P < 0.05$). Compared with Arbor Acres breeder hens and ISA Brown breeder hens, Hy-Line Brown layers had significantly higher YMC ($P < 0.05$).

3.2. Experiment 2

3.2.1. Laying performance, egg quality, egg parameters, and vitamin C content in serum and produced eggs

As shown in Table 4, the limited feed supply was executed as 120 g of daily among 3 groups and dietary VC supplementation had no significant effects on egg production rate, egg weight and feed-to-egg ratio ($P > 0.05$). As shown in Table 4, dietary VC supplementation had no significant effects on yolk color, Haugh unit, albumen height, shell strength and shell thickness on d 23 ($P > 0.05$). As shown in Table 5, dietary VC supplementation had no significant effects on egg parameters (Egg weight, YFW:AFW ratio, YDW:ADW ratio, YMC and AMC), serum VC content (SVCC) and VC content in produced eggs (YVCC, AVCC) on d 28 ($P > 0.05$).

3.2.2. Tissue mRNA expression profile of SVCT1, SVCT2 and GLO

As shown in Table 6, compared with duodenum and jejunum, *SVCT1* and *SVCT2* expressions were both significantly higher in ileum ($P < 0.05$). Compared with ovary, *SVCT1* expression was significantly higher in magnum, while *SVCT2* expression was significantly lower ($P < 0.05$). Compared with liver, *GLO* and *SVCT1* expressions were both significantly higher in kidney, while *SVCT2* expression was significantly lower ($P < 0.05$).

3.2.3. Vitamin C absorption: mRNA expressions of SVCT1 and SVCT2 in the intestine

Dietary supplementation of VC at 400 mg/kg increased *SVCT1* expression in the duodenum (Fig. 1A, $P < 0.05$) and had an increasing trend in the jejunum (Fig. 1B, $P = 0.067$). Dietary 200 and 400 mg/kg VC supplementation increased *SVCT2* expression in the

Table 3
Comparison of egg parameters and vitamin C content among Arbor Acres breeder hens, ISA Brown breeder hens and Hy-Line Brown layers in Exp. 1.

Item	Egg varieties			SEM	*P*-value
	Arbor Acres	ISA Brown	Hy-Line Brown		
Egg parameters					
Egg weight, g	72.08[a]	60.94[b]	58.79[c]	0.888	<0.001
YFW:AFW ratio	0.62[a]	0.47[b]	0.47[b]	0.014	<0.001
YDW:ADW ratio	2.57[a]	1.80[b]	1.65[b]	0.067	<0.001
YMC, %	50.75[b]	50.05[b]	54.09[a]	0.427	<0.001
AMC, %	88.11[a]	86.81[b]	86.89[b]	0.119	<0.001
Egg vitamin C content					
YVCC, μg/g of yolk DM	42.07[a]	15.29[b]	19.91[b]	2.455	<0.001
AVCC, μg/g of albumen DM	185.25[a]	126.03[b]	114.73[b]	5.139	<0.001

YFW:AFW ratio = the ratio of yolk fresh weight to albumen fresh weight; YDW:ADW ratio = the ratio of yolk dry weight to albumen dry weight; YMC = yolk moisture content; AMC = albumen moisture content; YVCC = yolk vitamin C content; AVCC = albumen vitamin C content.
[a, b, c] Means within a row with different superscript letters are different at $P < 0.05$.

Table 4
Effects of dietary vitamin C supplementation on laying performance and egg quality of Hy-Line Brown layers in Exp. 2[1].

Item	Vitamin C treatments, mg/kg			SEM	*P*-value
	0	200	400		
Laying performance					
Egg weight, g	60.86	60.61	60.76	0.100	0.605
Egg production rate, %	95.71	92.46	95.36	0.876	0.253
Feed-to-egg ratio	2.13	2.21	2.09	0.024	0.123
Egg quality					
Yolk color	6.87	6.47	6.58	0.119	0.389
Haugh unit	99.48	99.68	100.26	0.925	0.938
Albumen height, mm	10.08	10.07	10.32	0.203	0.851
Shell strength, kg/cm^2	43.92	40.86	48.50	1.457	0.119
Shell thickness, mm	0.36	0.35	0.37	0.006	0.297

[1] Shell strength was measured with the big end of the egg facing up. Shell thickness was the mean shell thickness of the small, middle and big ends of the egg.

Table 5
Effects of dietary vitamin C supplementation on egg parameters and vitamin C content of produced eggs and serum in Hy-Line Brown layers in Exp. 2.

Item	Vitamin C treatments, mg/kg			SEM	*P*-value
	0	200	400		
Egg parameters					
Egg weight, g	59.34	57.80	61.12	0.928	0.369
YFW:AFW ratio	0.418	0.426	0.415	0.008	0.872
YDW:ADW ratio	1.73	1.74	1.68	0.043	0.864
YMC, %	49.10	49.37	49.63	0.144	0.321
AMC, %	87.58	87.53	87.51	0.146	0.981
Egg vitamin C content					
YVCC, μg/g of YDM	13.36	12.37	15.54	1.175	0.558
AVCC, μg/g of ADM	114.57	98.22	107.97	3.958	0.259
Serum vitamin C content					
SVCC, μg/mL	21.42	22.06	22.73	0.688	0.760

YFW:AFW ratio = the ratio of yolk fresh weight to albumen fresh weight; YDW:ADW ratio = the ratio of yolk dry weight to albumen dry weight; YMC = yolk moisture content; AMC = albumen moisture content; YVCC = yolk vitamin C content; AVCC = albumen vitamin C content; SVCC = serum vitamin C content.

duodenum (Fig. 1B), but decreased *SVCT2* expression in the ileum (Fig. 1C, $P < 0.05$). However, VC supplementation had no significant effect on *SVCT1* expression in the ileum (Fig. 1C) and *SVCT2* expression in the jejunum (Fig. 1B, $P > 0.05$).

3.2.4. Vitamin C transport: mRNA expressions of SVCT1 and SVCT2 in the ovary and magnum

Dietary supplementation of VC at 400 mg/kg increased *SVCT1* expression in the ovary (Fig. 2A) and magnum (Fig. 2B, $P < 0.05$). However, dietary VC supplementation had no significant effect on *SVCT2* expression in the ovary (Fig. 2A) and magnum (Fig. 2B, $P > 0.05$).

Table 6
The relative mRNA expression of genes in the intestine (duodenum, jejunum and ileum), reproductive tract (ovary and magnum), liver and kidney of Hy-Line Brown layers in Exp. 2.

Item	Sites			SEM	*P*-value
	Duodenum	Jejunum	Ileum		
SVCT1	1.03[b]	8.03[b]	18.94[a]	2.268	<0.001
SVCT2	1.03[b]	1.16[b]	2.78[a]	0.223	<0.001
	Ovary	Magnum			
SVCT1	1.11	40.65		1.928	<0.001
SVCT2	1.20	0.58		0.267	0.047
	Liver	Kidney			
GLO	1.05	881.18		84.656	<0.001
SVCT1	1.03	6.18		0.451	<0.001
SVCT2	1.04	0.66		0.374	0.010

SVCT1 = sodium-dependent vitamin C transporter 1; *SVCT2* = sodium-dependent vitamin C transporter 2; *GLO* = L-gulonolactone oxidase.
[a, b] Means within a row with different superscript letters are different at $P < 0.05$.

3.2.5. Vitamin C synthesis: mRNA expressions of GLO, SVCT1 and SVCT2 in the liver and kidney

Dietary supplementation of VC at 400 mg/kg decreased *GLO* and *SVCT1* expression in the liver (Fig. 3A, $P < 0.05$). Dietary supplementation of VC at 200 and 400 mg/kg decreased *GLO* and *SVCT1* expression in the kidney (Fig. 3B) and *SVCT2* expression in the liver (Fig. 3A, $P < 0.05$). However, dietary VC supplementation had no significant effect on *SVCT2* expression in the kidney (Fig. 3B, $P > 0.05$).

4. Discussion

Arbor Acres breeder hens, ISA Brown breeder hens and Hy-Line Brown layers all belong to layer species and have similar reproductive tract structure for yolk and albumen synthesis. Nevertheless, different egg parameters among different layer species were indeed observed, which might be related to nutrition (different diets) and genetics (different layer species). Studies have shown that the animal with lower plasma or tissue VC content at baseline are more conducive to use as an animal model for demonstrating benefit or harm from dietary VC supplementation (Lykkesfeldt and Poulsen, 2010; Padayatty and Levine, 2009, 2014), and egg VC content at baseline should be regarded as an important indicator for the selection of the animal model. Therefore, ISA Brown breeder hens and Hy-line Brown layers are both suitable as the animal model. In addition, due to the high price of breeder hens or breeder

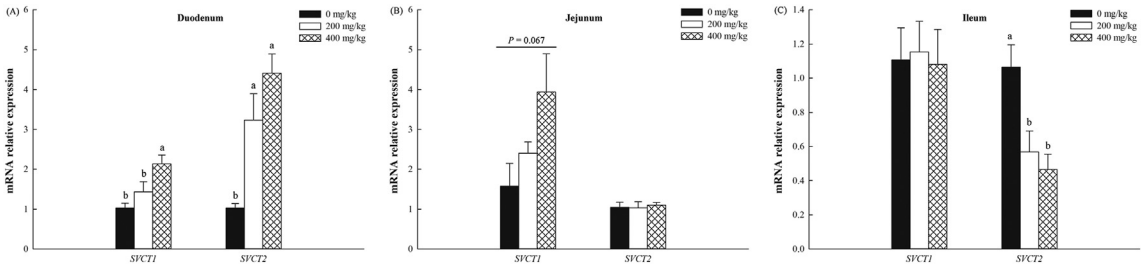

Fig. 1. Effects of dietary vitamin C supplementation on the mRNA expression of *SVCT1* and *SVCT2* in the duodenum (A), jejunum (B) and ileum (C) of Hy-Line Brown layers in Exp. 2. Basal diets were supplemented with vitamin C at 0, 200 and 400 mg/kg. Values are means ± SEM. [a, b] Means with different superscript letters are different ($P < 0.05$, $n = 8$). *SVCT1* = sodium-dependent vitamin C transporter 1; *SVCT2* = sodium-dependent vitamin C transporter 2.

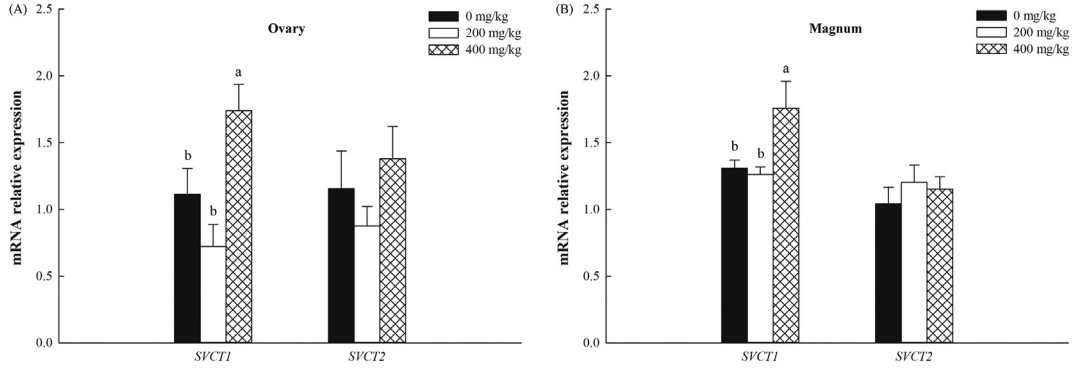

Fig. 2. Effects of dietary vitamin C supplementation on the mRNA expression of *SVCT1* and *SVCT2* in the ovary (A) and magnum (B) of Hy-Line Brown layers in Exp. 2. Basal diets were supplemented with vitamin C at 0, 200 and 400 mg/kg. Values are means ± SEM. [a, b] Means with different superscript letters are different ($P < 0.05$, $n = 8$). *SVCT1* = sodium-dependent vitamin C transporter 1; *SVCT2* = sodium-dependent vitamin C transporter 2.

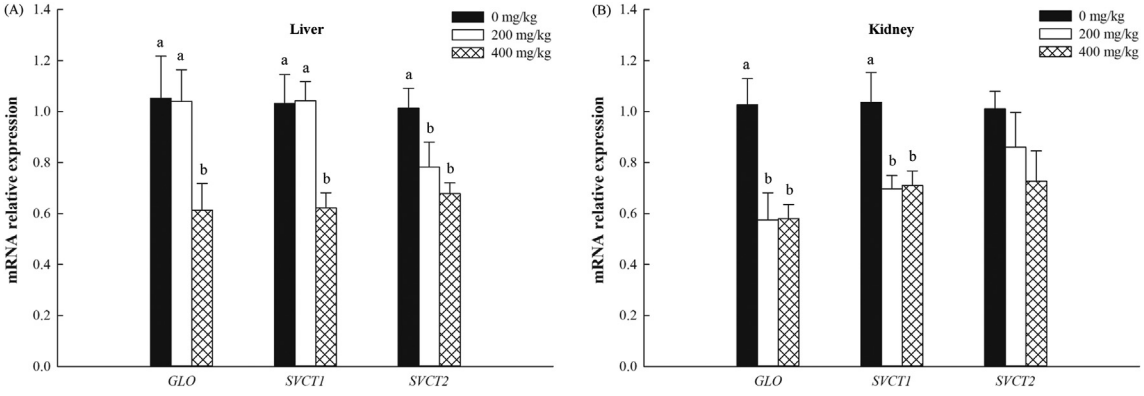

Fig. 3. Effects of dietary vitamin C supplementation on the mRNA expression of *GLO*, *SVCT1* and *SVCT2* in the liver (A) and kidney (B) of Hy-Line Brown layers in Exp. 2. Basal diets were supplemented with vitamin C at 0, 200 and 400 mg/kg. Values are means ± SEM. [a, b] Means with different superscript letters are different ($P < 0.05$, $n = 8$). *GLO* = L-gulonolactone oxidase; *SVCT1* = sodium-dependent vitamin C transporter 1; *SVCT2* = sodium-dependent vitamin C transporter 2.

eggs caused by African swine fever, the farms are reluctant to sell breeder hens, and it is difficult for us to purchase breeder hens. Finally, Hy-Line Brown layers were selected as the animal model in current study for studying the effects of dietary VC supplementation on VC synthesis and transportation and egg deposition.

Some previous studies indicated that the decline of production performance in old layers was inseparable with oxidative stress status (Molnar et al., 2016; Wang et al., 2011). Under the stress conditions, VC supplementation improved the production performance in old layers (72-week-old) (Ahmed et al., 2008) but had no effects on that in young layers (31-week-old) (Wang et al., 2016). Therefore, we speculated that the effects of VC supplementation were related to oxidative stress status in layers, and the anti-stress ability of young layers is better than that of old layers. Obviously, it is more likely to be effective to supplement VC for laying hens with poor anti-stress ability and/or stress ability. In this study, dietary VC supplementation had no significant impact on laying performance and egg quality in 42-wk-old layers (young layers), which supported the above speculation.

In order to explore egg VC deposition induced by dietary VC supplementation, VC absorption in the intestine, VC synthesis in the liver and kidney, and VC transport in the ovary and magnum were detected. *GLO* is the key enzyme for VC synthesis and gene mutations in *GLO* leads to VC synthesis ability loss in some species (like humans) (Linster and Van Schaftingen, 2007). However, Hy-line Brown layers have the ability of VC synthesis, which is much

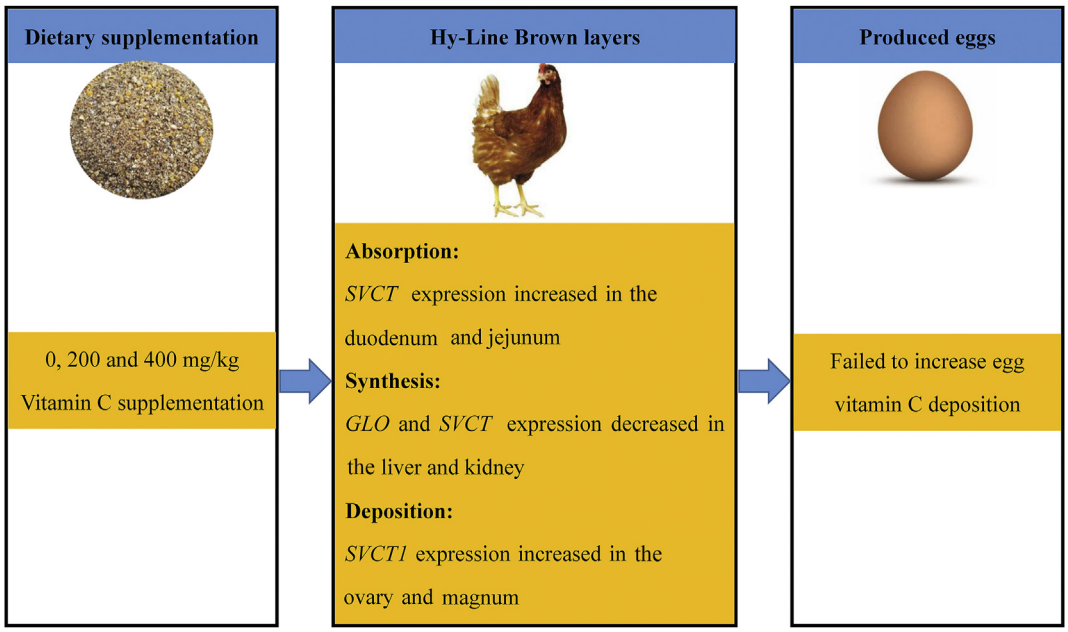

Fig. 4. Graphical illustration of the core findings in this study. *SVCT* = sodium-dependent vitamin C transporters (including *SVCT1* and *SVCT2*); *GLO* = L-gulonolactone oxidase; *SVCT1* = sodium-dependent vitamin C transporter 1.

higher in the kidney than in liver (Gan et al., 2018; Maurice et al., 2002). VC absorption, transportation and distribution are primarily regulated by *SVCT* (Lykkesfeldt and Tveden-Nyborg, 2019).

Tissue mRNA expression profile showed that ileum was the main site for VC absorption with higher *SVCT* expression. Higher *SVCT1* expression and lower *SVCT2* expression in the magnum than those in the ovary indicated that *SVCT1* may be the main VC transporter and may be related to higher VC content in albumen. *GLO* expression was extremely higher in the kidney than that in the liver, indicating that kidney was main tissue for VC synthesis in layers, which was also supported by the results of *GLO* enzyme activity in the liver accounting for only 0.5% to 0.7% of that in the kidney (Gan et al., 2018) and higher *SVCT1* expression in the kidney than that in the liver.

SVCT1 is the absorptive VC transporter with low affinity and high capacity, which is responsible for whole-body homeostasis of VC and is widely expressed in the tissues of endothelial system tissues (including intestine and kidney) and some other tissues (lung, liver and skin) (Savini et al., 2008). And *SVCT2* is the tissue transporter with high affinity and low capacity, which is widely expressed in various tissues and is responsible for tissue transporter for VC (Savini et al., 2008). *SVCT1* is the major transporter for VC in the intestine and higher *SVCT1* expression showed that dietary VC supplementation promoted VC absorption in the duodenum and jejunum. Although dietary VC supplementation decreased *SVCT2* expression in the ileum, *SVCT2* was not a major transporter with low capacity in the intestine. In addition, lower *GLO* expression in the liver and kidney indicated that dietary VC supplementation enhanced the overall VC absorption of the intestine. Compared with VC in plant-derived feed ingredients, VC in the form of additives could contact the intestinal wall faster and earlier in intestinal lumen without digesting plant cells to release VC (Fenech et al., 2019), which may be why dietary VC supplementation promoted VC absorption in the duodenum and jejunum rather than that in the ileum.

Alterations on *SVCT* expression indicated that dietary VC supplementation enhanced VC absorption in the duodenum and jejunum, but surprisingly, no difference in serum VC content was observed in VC supplementation groups. Some research showed that the peak of plasma VC content reached a plateau even if the oral dose continued to increase, and the intravenous VC injection with the maximum tolerable oral dose produced extremely higher (30- to 70-fold) plasma VC content than that of oral (Padayatty et al., 2004). Thus, dietary VC supplementation is not feasible to achieve high plasma VC contents. Vitamin C was rapidly metabolized and excreted within 3 h after oral high-dose VC (the time was shorter after intravenous injection) for the stability of plasma VC content (Furuita et al., 2009). In this study, serum samples were collected from Hy-Line Brown layer after 12 h fasting and this is why no difference in serum VC content was observed.

Dietary VC supplementation significantly reduced *GLO* expression in the liver and kidney. Although dietary VC supplementation increased *GLO* enzyme activity in the liver based on Gan's research, its activity was reduced in the kidney and VC synthesis was primarily in the kidney, as evidenced by *GLO* enzyme activity in the liver accounting for only 0.5% to 0.7% of that in that kidney (Gan et al., 2018; Hooper et al., 2000). Alterations on *SVCT* expression were consistent with *GLO* expression in the liver and kidney, proving that dietary VC supplementation caused the reduction of endogenous VC synthesis.

Yolk and albumen are formed in the ovary and magnum, respectively. In this study, VC content was higher in albumen than in yolk, which may be related with 40-fold higher *SVCT1* expression in the magnum than that in the ovary. But its mechanism of VC secretion into albumen and yolk was unknown (Padayatty and Levine, 2016). The moisture content (53%) is lower and the fat content (28%) is higher in yolk, compared with moisture content (82%) and no fat in albumen (Uni et al., 2012). And in this study, the moisture content was 49% in yolk and 88% in albumen, respectively. Higher *SVCT1* expression, higher moisture content and no fat may benefit VC deposition in albumen rather than in yolk. However, dietary VC supplementation had no effect on VC deposition in yolk and albumen, which was contrary with higher *SVCT1* expression in

the ovary and magnum. Whether VC was taken orally or intravenously, the plasma VC content could be quickly returned stability by metabolism and excretion (Padayatty et al., 2004). Thus, it was difficult to keep a high dose of plasma VC content through VC supplementation (Lykkesfeldt and Tveden-Nyborg, 2019; Padayatty et al., 2004). The follicle maturation process is accompanied by the deposition of lipids (Schneider, 2016), which may not benefit VC deposition. And, it only takes 1.5 h to complete albumen formation in the magnum (Estienne et al., 2020), which gives a very short window time for VC deposition. In addition, limited increase in *SVCT1* expression may make it difficult to activate the secretion of the ovary and magnum, so dietary VC supplementation failed to increase VC deposition in produced eggs.

Obviously, there are some limitations in this study. According to industry routine dosage (dietary supplementation of VC at 200 mg/kg), VC supplementations at 0, 200 and 400 mg/kg were selected. However, the higher-dose vitamin C group should be contained in the current study, which may be more convincing to demonstrate the effects of dietary VC supplementation on VC deposition in produced eggs. In addition, egg VC deposition is affected by VC transport and secretion in the ovary and magnum. However, there is no research basis for the mechanism of VC secretion, and VC secretion was not detected in this study.

5. Conclusion

In summary, based on egg VC content at baseline and the availability of animal materials, Hy-Line Brown layer was selected as an animal model. Dietary VC supplementation had no significant impacts on laying performance and egg quality in 42-week-old Hy-line Brown layers. *GLO* expression indicated that the ability of VC synthesis was extremely stronger in the kidney than in the liver. And, a higher VC content in albumen than in yolk may be related to the higher *SVCT1* expression in the magnum than in ovary. Lower *GLO* expression in the liver and kidney indicated that dietary VC supplementation enhanced the overall VC absorption of the intestine. Although *SVCT* expression indicated that the ileum was the main site for VC absorption, dietary VC supplementation enhanced intestinal VC absorption by promoting VC absorption in the duodenum and jejunum. And, dietary VC supplementation increased *SVCT1* expression in the ovary and magnum, but had no significant effects on VC deposition in produced eggs (Fig. 4).

Author contributions

Yufei Zhu, **Wei Guo**, **Jianfei Zhao** and **Xiaojun Yang** conceived and designed the experiments; **Yufei Zhu**, **Wei Guo**, **Jianfei Zhao**, **Kailong Qin** and **Jiakun Yan** mainly performed the experiments; **Yufei Zhu** and **Yanli Liu** analyzed the data and wrote the manuscript; **Wei Guo**, **Jianfei Zhao**, **Zhouzheng Ren**, **Xin Yang, Yanli Liu** and **Xiaojun Yang** participated in the revision of the manuscript.

Conflict of 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.

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References

Ahmed W, Ahmad S, Ahsan U, Kamran Z. Response of laying hens to vitamin C supplementation through drinking water under sub-tropical conditions. Avian Biol Res 2008;1:59–63.
El-Senousey HK, Chen B, Wang JY, Atta AM, Mohamed FR, Nie QH. In ovo injection of ascorbic acid modulates antioxidant defense system and immune gene expression in newly hatched local Chinese yellow broiler chicks. Poultry Sci 2018;97:425–9.
Estienne A, Brossaud A, Reverchon M, Rame C, Froment P, Dupont J. Adipokines expression and effects in oocyte maturation, fertilization and early embryo development: lessons from mammals and birds. Int J Mol Sci 2020. https://doi.org/10.3390/ijms21103581.
Fenech M, Amaya I, Valpuesta V, Botella MA. Vitamin C content in fruits: biosynthesis and regulation. Front Plant Sci 2019. https://doi.org/10.3389/fpls.2018.02006.
Furuita H, Ishida T, Suzuki T, Unuma T, Kurokawa T, Sugita T, Yamamoto T. Vitamin content and quality of eggs produced by broodstock injected with vitamins C and E during artificial maturation in Japanese eel Anguilla japonica. Aquaculture 2009;289:334–9.
Gan L, Fan H, Nie W, Guo Y. Ascorbic acid synthesis and transportation capacity in old laying hens and the effects of dietary supplementation with ascorbic acid. J Anim Sci Biotechnol 2018. https://doi.org/10.1186/s40104-018-0284-7.
Hooper CL, Maurice DV, Lightsey SF, Toler JE. Factors affecting ascorbic acid biosynthesis in chickens. I. Adaptation of an assay and the effect of age, sex and food deprivation. J Anim Physiol Anim Nutr 2000;84:48–56.
Hooper CL, Maurice DV, Lightsey SF, Toler JE. Factors affecting ascorbic acid (AsA) biosynthesis in chickens. II. Effect of dietary AsA and strain of chicken. J Anim Physiol Anim Nutr 2002;86:326–32.
Li S, Chen M, Li Y, Tollefsbol TO. Prenatal epigenetics diets play protective roles against environmental pollution. Clin Epigenet 2019. https://doi.org/10.1186/s13148-019-0659-4.
Lindblad M, Tveden-Nyborg P, Lykkesfeldt J. Regulation of vitamin C homeostasis during deficiency. Nutrients 2013;5:2860–79.
Linster CL, Van Schaftingen E. Vitamin C - biosynthesis, recycling and degradation in mammals. FEBS J 2007;274:1–22.
Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 2001;25:402–8.
Lykkesfeldt J, Poulsen HE. Is vitamin C supplementation beneficial? Lessons learned from randomised controlled trials. Br J Nutr 2010;103:1251–9.
Lykkesfeldt J, Tveden-Nyborg P. The pharmacokinetics of vitamin C. Nutrients 2019. https://doi.org/10.3390/nu11102412.
Mandl J, Szarka A, Banhegyi G. Vitamin C: update on physiology and pharmacology. Br J Pharmacol 2009;157:1097–110.
Maurice DV, Lightsey SF, Abudabos A, Toler JE. Factors affecting ascorbic acid biosynthesis in chickens: III. Effect of dietary fluoride on L-gulonolactone oxidase activity and tissue ascorbic acid (AsA) concentration. J Anim Physiol Anim Nutr 2002;86:383–8.
Molnár A, Maertens L, Ampe B, Buyse J, Kempen I, Zoons J, Delezie E. Changes in egg quality traits during the last phase of production: is there potential for an extended laying cycle? Br Poultry Sci 2016;57:842–7.
Nowaczewski S, Kontecka H, Krystianiak S. Effect of in ovo injection of vitamin C during incubation on hatchability of chickens and ducks. Folia Biol-Krakow 2012;60:93–7.
Nys Y, Guyot N. Egg chemistry, production and consumption. In: Nys Y, Bain M, VanImmerseel F, editors. Improving the safety and quality of eggs and egg products. London: Woodhead Publishing; 2011. p. 83–132.
Padayatty SJ, Levine M. Antioxidant supplements and cardiovascular disease in men. Jama-J Am Med Assoc 2009;301:1336.
Padayatty SJ, Levine M. Vitamin and mineral supplements in the primary prevention of cardiovascular disease and cancer. Ann Intern Med 2014;160:654.
Padayatty SJ, Levine M. Vitamin C: the known and the unknown and goldilocks. Oral Dis 2016;22:463–93.
Padayatty SJ, Sun H, Wang YH, Riordan HD, Hewitt SM, Katz A, Wesley RA, Levine M. Vitamin C pharmacokinetics: implications for oral and intravenous use. Ann Intern Med 2004;140:533–7.
Peebles ED. In ovo applications in poultry: a review. Poultry Sci 2018;97:2322–38.
Rehault-Godbert S, Guyot N, Nys Y. The golden egg: nutritional value, bioactivities, and emerging benefits for human health. Nutrients 2019. https://doi.org/0.3390/nu11030684.

Retes PL, Clemente AHS, Neves DG, Esposito M, Makiyama L, Alvarenga RR, Pereira LJ, Zangeronimo MG. In ovo feeding of carbohydrates for broilersa systematic review. J Anim Physiol Anim Nutr 2018;102:361–9.
Savini I, Rossi A, Pierro C, Avigliano L, Catani MV. *SVCT1* and *SVCT2*: key proteins for vitamin C uptake. Amino Acids 2008;34:347–55.
Schiavone A, Barroeta AC. Egg safety and nutritional quality. In: VanImmerseel F, Nys Y, Bain M, editors. Improving the safety and quality of eggs and egg products. London: Woodhead Publishing; 2011. p. 289–320.
Schneider WJ. Lipid transport to avian oocytes and to the developing embryo. J Biomed Res 2016;30:174–80.
Subramanian VS, Srinivasan P, Wildman AJ, Marchant JS, Said HM. Molecular mechanism(s) involved in differential expression of vitamin C transporters along the intestinal tract. Am J Physiol-Gastrl 2017;312:340–7.
Uni Z, Yadgary L, Yair R. Nutritional limitations during poultry embryonic development. J Appl Poultry Res 2012;21:175–84.
Wang A, Xie F, Wang YH, Wu JL. Effects of vitamin C supplementation on growth performance and antioxidant status of layer ducklings. J Anim Physiol Anim Nutr 2011;95:533–9.
Wang JP, He KR, Ding XM, Luo YH, Bai SP, Zeng QF, Su ZW, Xuan Y, Zhang KY. Effect of dietary vanadium and vitamin C on egg quality and antioxidant status in laying hens. J Anim Physiol Anim Nutr 2016;100:440–7.
Zhang H, Elliott KEC, Durojaye OA, Fatemi SA, Schilling MW, Peebles ED. Effects of in ovo injection of L-ascorbic acid on growth performance, carcass composition, plasma antioxidant capacity, and meat quality in broiler chickens. Poultry Sci 2019;98:3617–25.
Zhu YF, Bodinga MB, Zhou JH, Zhu LQ, Cao YL, Ren ZZ, Yang XJ. Effects of in ovo injection of vitamin C on heat shock protein and metabolic genes expression. Animal 2020;14:360–7.
Zhu YF, Li SZ, Sun QZ, Yang XJ. Effect of in ovo feeding of vitamin C on antioxidation and immune function of broiler chickens. Animal 2019;13:1927–33.