PHYSIOLOGY AND REPRODUCTION

Improvement of eggshell quality by dietary N-carbamylglutamate supplementation in laying chickens

Yanfen Ma, Jinwei Yao, Shuo Zhou, Yuling Mi, Jian Li, and Caiqiao Zhang[1]

Department of Veterinary Medicine, College of Animal Sciences, Zhejiang University, Hangzhou 310058, P.R. China

ABSTRACT Egg quality defects seriously reduce the quality grade and increase egg breakage in egg marketing activities. In this study, the effect of N-carbamylglutamate (**NCG**) on eggshell quality was investigated by evaluating calcium absorption and calcification in laying hens. A total of 30 newly hatched female Hy-Line chicks were randomly assigned to the control group (basal diet) and treatment group (basal diet supplemented with 1% NCG). At 25 wk, eggs from each group were obtained to assess egg quality parameters. Blood samples were collected for analysis of mineral, hormone, and amino acids levels at 16 h after laying egg. Uterine tissues were removed and fixed in 4% neutral paraformaldehyde solution or kept in liquid nitrogen for mineral determination, quantitative PCR, and Western blot. Results showed that the egg quality (eggshell thickness, strength and percentage, egg specific gravity, and eggshell effective thickness) was significantly increased while effective thickness of mastoid layer, width of mastoid gap, and mammillary knobs were significantly decreased by dietary NCG supplementation ($P < 0.05$). The levels of minerals (Ca, P, Fe, Mg, Na, K) in eggshell, plasma, and uterus were remarkably elevated ($P < 0.05$). Meanwhile, the concentrations of calcium metabolism–related hormones (17β-estradiol, parathyroid hormone, and calcitonin) were increased in the NCG group ($P < 0.05$). Moreover, expression of calbindin 1, carbonic anhydrase 2, ovalbumin, ovotransferrin, ovocleidin-17, ovocleidin-116, and clusterin mRNAs, as well as calbindin 1 and ATP2A1 proteins in uterus, duodenum, and kidney, was all upregulated in hens fed with NCG ($P < 0.05$). In addition, the number of blood vessels in the uterus, height of uterine mucosal folds, villus length in endometrium, and areas of uterine mucosal folds were significantly increased in the NCG group ($P < 0.05$). In conclusion, dietary 1% NCG supplementation during 0 to 25 wk can improve eggshell quality through changes in endometrial morphology, expression of calcium metabolism–related genes, and secretion of related hormones to promote eggshell formation in the laying hens.

Key words: N-carbamylglutamate, laying chicken, eggshell quality, eggshell calcification

INTRODUCTION

Poultry eggs represent one of the largest human protein sources required all over the world. In 2017, the production of poultry eggs in China and the U.S. was about 529 and 106.7 billion, respectively, ranking the top 2 worldwide (U.S. Department of Agriculture, 2019). However, owing to the fragility of the eggshell, broken and cracked eggshells in egg marketing activities such as egg collecting, packaging, transporting, storing, and selling bring to huge economic loss for the egg industry (Mertens et al., 2006). A previous study reported that approximately 6 to 8% of total eggs produced was downgraded because of eggshell damage. Therefore, one of the most effective strategies to decrease the loss of eggs is to reduce the rate of eggshell breakage.

Eggshell is formed mainly in the uterus (shell gland). It is a highly ordered calcareous structure which is composed of 95% minerals and 3.5% organic ingredients (Nys et al., 1999). It can be divided into 6 layers: cuticle layer, vertical crystal layer, palisade layer, mastoid layer, inner membrane, and outer membrane. Eggshell will be likely cracked when the strength of the shell is less than the strength of the insults to which they are exposed. The shape, size, and specific gravity of eggs; the thickness of the shell; and the distribution of material in the shell determine the strength of the eggshell. From the ultrastructure, palisade and mammillae layers of the eggshell are the thickest part (Chen et al., 2015). The effective

thickness of the mastoid layer is measured from the basal caps of the mammillary knobs to the point at which the palisade columns begin to fuse, and the width of the mastoid gap is the distance between the 2 basal caps of the mammillary knobs. It was reported that the strength of eggshell was positively correlated with the total thickness of the eggshell and the density of the mammillary knobs but was negatively correlated with the effective thickness of the mastoid layer and the width of mastoid gaps (Hincke et al., 2011).

The most mineral in the eggshell is calcium (in the form of calcium carbonate) together with much less magnesium to form the radiating crystals. The duodenum and jejunum are the main sites for calcium absorption. The process of intestinal Ca^{2+} absorption consists of 3 major steps: entry of Ca^{2+} through the brush border, facilitated diffusion or movement to the basal membrane, and extrusion through the basal membrane (Bar, 2009). Transfer of calcium from the gut lumen to the uterus lumen via blood is the predominant route for eggshell calcification, with supplementary routes from bone resorption and reabsorption from the kidney.

In poultry production, feed additives are often used to improve production performance. For example, dietary supplementation with a mixture of daidzein and Chinese herbs increased egg production and eggshell strength as well as blood plasma Ca, P levels in post-peak, brown laying hens (Xiao et al., 2019). Dietary bamboo vinegar supplementation decreased the damaged egg rate by reducing pathogenic bacteria, stimulating intestinal functions, and improving eggshell quality of laying hens (Rattanawut et al., 2018). Amino acids (**AA**) can also improve the quality of the eggshells. Khattak and Helmbrecht (2018) reported that tryptophan (**Trp**) supplementation improved eggshell weight, shell density, and shell thickness. N-Carbamylglutamate (**NCG**), a structural analogue of N-acetyl-L-glutamate, has been shown to increase endogenous synthesis of arginine (**Arg**) (Wu et al., 2004). Oral supply with NCG improved the expressions of AA transporters Slc6a19, Slc7a9, and Slc1a1 and increased intestinal absorptive function in the weaned piglets (Yang et al., 2013). Furthermore, dietary NCG supplementation improved daily weight gain and growth performance in the yellow-feather broilers through modifying homeostasis of Arg metabolism (Hu et al., 2019). In addition, our previous study manifested dietary NCG enhanced ovarian angiogenesis by elevating plasma Arg and nitric oxide (**NO**) concentrations and ultimately improved follicular development in the chicken (Ma et al., 2019). In view of the importance of eggshell quality in egg production and the function of NCG, we assume that NCG may promote eggshell quality in laying hens through changes in calcium metabolism and calcification. Therefore, the present experiment was conducted to explore the effect of dietary NCG supplementation on eggshell quality by evaluating changes in calcium absorption and deposition in the laying hens.

MATERIALS AND METHODS

Experimental Design and Animal Raising

Fertilized Hy-Line chicken eggs were incubated at 38.5°C and 60% humidity. After hatching, 30 female chicks were randomly divided into 2 groups (15/group): control group (basal diet) and treatment group (basal diet supplemented with 1% NCG) for 25 wk. The composition, nutrient level, and metabolic energy of the basal diets are listed in Table 1, and chickens had *ad libitum* access to feed and water. Conventional vaccination procedure was adopted in the management. Temperature, humidity, and photoperiod are listed in Supplementary Table 1.

This study was carried out in accordance with the Guiding Principles for the Care and Use of Laboratory Animals of Zhejiang University. The experimental protocols were approved by the Committee on the Ethics of Animal Experiments of Zhejiang University (No. ZJU2015-156-12).

Sample Collection

At 23 to 24 experimental weeks, the time of laying egg was recorded for each hen. At the 25th wk, 60 eggs from each treatment group were randomly collected to assess egg quality parameters. For each hen, based on the records of laying time and oviposition cycle, at 16 h after laying eggs, blood samples were collected into centrifugal tubes from the wing veins of hens for plasma preparation. After that, the hens were sacrificed after anesthesia, and the uterine tissue, duodenum, and kidney were collected and washed in cold PBS (pH 7.4) 3 times to remove contaminated blood. In 5 hens of each group, the uterine tissues from the middle part were obtained for fixation in 4% neutral paraformaldehyde solution. The rest of the uterine tissues were kept in liquid nitrogen for subsequent analyses.

Table 1. Composition, nutrient level, and metabolic energy of the basal diets.

Ingredients	Content (%)		
	0–60 D	61–120 D	>120 D
Corn	65.5	69	62
Bran	2.5	8	0
Soybean meal	29	20	25
Limestone	0	0	8
Premix[1]	3	3	5
Nutrient concentration			
Crude protein	18.9	16.1	16.8
Calcium	0.86	0.83	3.47
Available phosphorus	0.39	0.37	0.52
Metabolic energy (MJ/kg)	11.95	12.18	11.81

[1]Supplied the following per kilogram of premix: dl-a-tocopherol acetate ≥ 280 mg; menadione sodium bisulfite, 30–96 mg; thiamine nitrate ≥ 38 mg; vitamin B_2 ≥ 60 mg; pyridoxine hydrochloride ≥ 60 mg; vitamin B12 ≥ 0.2 mg; nicotinamide ≥ 420 mg; D-calcium pantothenate ≥ 420 mg; folic acid ≥ 12.0 mg; D-biotin ≥ 3.0 mg; choline chloride ≥ 5.6 g; vitamin A acetate, 160,000–200,000 IU; vitamin D3, 4.4–100 mg; Cu, 140–420 mg; Fe, 1.6×10^3–1.3×10^4; Mn, 1.2×10^3–3.0×10^3; Zn, 1.2×10^3–2.4×10^3; Se, 2.0–6.0 mg; I, 6.0–18 mg; methionine, 2.6–5.2%; P, 3.6–10.0%; Ca, 5.0–20%; NaCl, 4.0–9.0%; H_2O ≤ 1%.

Eggshell Quality

The egg weight, eggshell thickness, and eggshell strength were measured using a digital egg tester (n = 30) (DET6000, NABEL Co., Ltd., Kyoto, Japan). Eggshells were washed under tap water, dried at room temperature, and weighed. Eggshell percentage was calculated as eggshell weight/egg weight × 100%. Another 30 eggs were used to examine specific gravity by saline solution floating method. In brief, gradient concentration sodium chloride solution (1.068–1.208 g/cm^3, increased by 0.04 g/cm^3 every adjacent concentration) was prepared. The eggs were put in sodium chloride solution from lower concentration to higher concentration. The concentration of sodium chloride solution is the specific gravity of the egg when the egg was suspended.

Scanning Electron Microscopy

To examine ultrastructure of eggshell, 5 eggs were randomly collected in each group and the interior contents of eggs were removed. Eggshells were washed with tap water as clean as possible. Two pieces of eggshell samples (about 1 cm^2/piece) were cut from the equatorial region of each egg. And the membranes of shells were carefully peeled from the edge of the shell inward using tweezers (this step is not needed when analyzing membrane fibers and cross section of shells). Then, eggshell samples were boiled in 2% NaOH solution for 10 min to dissolve all of the protein materials incorporated in the eggshell (Liao et al., 2013). Finally, shell samples were kept in room temperature and dried. All shell samples were mounted inner side uppermost on aluminum stubs and coated with gold powder using an ion coater. These samples were examined by scanning electron microscopy at 2.0 or 3.0 kV (JEOL JSM-T330 A, HITACHI Ltd., Tokyo, Japan).

The effective thickness of shells (including the palisade layer, vertical crystal layer, and cuticle layer, μm) and the mastoid layer (μm) and width of the mastoid gap (μm) and mammillary knobs (μm) were measured on Image J Software after scanning the cross section of eggshells. The effective thickness of the mastoid layer was measured from the basal caps of the mammillary knobs to the point at which the palisade columns first fused. The width of the mastoid gap was the distance between the 2 basal caps of the mammillary knobs. The width of the mammillary knobs was analyzed as mammillary knobs length/mammillary knobs number (DeHoff and Rhines, 1968). The smaller the width of mammillary knobs, the more the mammillary knobs per unit length, which means the higher the density. Each group had 5 replicates, and 2 samples were examined for each egg, with 3 images taken for each sample.

Determination of Mineral Levels in the Eggshell and Uterus

For assay of elements in eggshells, interior contents of eggs were removed and shells were washed with tap water as clean as possible. The membranes of shells were carefully peeled from the edge of the shell inward using tweezers. Finally, shell samples were dried at room temperature. For each egg, 100 mg of shell was cut from the equatorial region and then put into the digestion tube, with 5.0 mL of nitric acid and 1.0 mL of 30% hydrogen peroxide for microwave digestion. After acid draining, the samples were transferred into flask, then diluted with ion-free water up to 50 mL. In each group, 5 samples were used to analyze elements levels. For assay of minerals in the uterus, approximately 300 mg of the uterine tissue was weighted after homogenization. The detection procedures were the same as those for eggshell.

Determination of Plasma Minerals and Hormones

Five blood samples were collected in centrifugal tubes from the wing veins of hens for plasma preparation. Determination of plasma estradiol (**E$_2$**), parathyroid hormone (**PTH**), and calcitonin (**CT**) levels was carried out with ARCHITECT Estradiol Reagent Kit (01191UI00, Abbott, Lisnamuck, Ireland), Roche PTH Reagent Kit (34169303, Roche, Mannheim, Germany), and Roche CT Reagent Kit (37352501, Roche, Mannheim, Germany) in accordance with the manufacturer's protocols. Concentrations of minerals (Ca, P, Mg, Fe, Na, Cl, and K) in plasma were determined with reagents from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Analysis of Plasma AA

Five plasma samples were weighted (about 0.5 g) and acid hydrolyzed with 4.0 mL of 6 mol/L HCl in vacuum-sealed hydrolysis vials at 110°C for 22 h. The contents of AA were analyzed using an AA analyzer (L-8900 Hitachi-Hitech, Japan).

Histological Observation of the Uterine Tissue

Five uterine tissues were randomly collected from each group and fixed in 4% neutral paraformaldehyde solution over 24 h at 4°C. Uterine tissues were dehydrated in a grade ethanol (70, 80, 90, 95, 100% I and 100% II, respectively), cleared in xylene, and embedded with paraffin. The embedded samples were sectioned at 5-μm thickness for hematoxylin and eosin (HE) staining that was performed following a conventional protocol (Liu et al., 2018). The width between the 2 adjacent

uterine mucosal folds and the width of uterine mucosal folds were measured using Eclipse 80i microscope (Nikon, Tokyo, Japan). Each group had 5 replicates, and 3 samples were examined for each uterine tissue, with 3 images taken for each sample.

Quantitative PCR Analysis

Total RNA was extracted from the uterine tissues, duodenum, and kidney using Trizol reagent (Vazyme, Nanjing, China). RNA concentrations were measured using a NanoDrop 2000c (Thermo Scientific, Waltham). In accordance with the manufacturer's protocol, the cDNA was generated from 2 µg of total RNA using a HiScript II first Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). Then, quantitative PCR (qPCR) was performed in triplicate with SYBR Premix Ex TaqTM (Vazyme, Nanjing) in ABI 7500 HT Real-Time PCR machine (Applied Biosystems, Foster City, CA). The qPCR conditions were as follows: 95°C for 10 min and then 40 cycles of 95°C for 30 s, 64°C for 34 s, and 72°C for 30 s. The $2^{-\Delta\Delta Ct}$ formula method was used to analyze relative mRNA expression calibrated with β-actin as the reference gene. Primers are listed in Table 2. Each group had 3 replicates, and 3 samples were examined for each uterine tissue.

Western Blot

The uterine tissues, duodenum, and kidney were homogenized in 500 µL of ice-cold radioimmunoprecipitation lysis buffer supplemented with 1 mmol/L phenylmethanesulfonyl fluoride (Beyotime, Shanghai, China) and proteinase inhibitors (Beijing Solarbio Science & Technology Co., Beijing, China). Bicinchonininc acid protein assay kit (Nanjing Jiancheng Bioeng Ins, Nanjing, China) was used to determine total protein concentration. A total of 24 µg of protein was separated with SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (0.22 µm, Millipore, Bedford). After blocking with 5% skim milk, the PVDF membrane was incubated with corresponding primary antibodies including rabbit anti-CALB1 (1:1,000, ET1702-54) and rabbit anti-ATP2A1 (1:500, ER1803-34, Hangzhou HuaAn Biotechnology Co., Hangzhou, China) at 4°C overnight. Then, the PVDF membrane was incubated with biotinylated goat anti-rabbit IgG (1:100, Santa Cruz Biotechnology, Dallas, TX) at room temperature for 1 h. Immunological signals were detected by an enhanced chemiluminescence kit (Bio-Rad, Hercules, CA) using a ChemiScope 3400 Mini machine (Clinx, Shanghai, China). The band intensities were quantified using Quantity One Software, and the results were normalized to β-actin.

Statistical Analysis

All results were expressed as the mean ± standard error of the means (**SEM**). Data were analyzed by one-way analysis of variance (**ANOVA**) with the post hoc Dunnett's test and the independent samples t-test using SPSS, version 16.0, software (SPSS Inc., Chicago, IL). The level was considered up to statistically significant when $P < 0.05$.

RESULTS

Eggshell Quality

At 18 to 19 experimental weeks, hens started laying eggs. At the 24th wk, the laying rate reached 90%. In each group, 10 eggs/D for 6 consecutive days were randomly collected to assess egg quality parameters. After supplementation with 1% NCG in the diet, eggshell thickness, eggshell strength, and eggshell percentage were significantly increased (Figure 1A–C). Egg specific gravity was also increased remarkably. The percentage of eggs (egg specific gravity $\geq$ 1.128 g/cm^3) was 86.7% in the NCG group, while it was 52.4% in the control group. However, the percentage of eggs (egg specific gravity $<$ 1.128 g/cm^3) was decreased from 47.6 to 13.3% compared with the control group (Figure 1D).

Table 2. Sequences of the primers for PCR.

Gene name	Accession no.	Primer sequence (5′-3′)	Product size (bp)
CALB1	NM_205513.1	F: TGTTATGGAGTGCAGGATGG	131
		R: TAGAGCGAACAAGCAGGTGA	
OC-116	AM076827.1	F: AATGCCCCACTAACTGCTCC	81
		R: AATGGGGTGTTCAATGGGTAA	
OVA	AH002466.2	F: GAGTGGCATCAATGGCTTCTG	123
		R: TCTAGGGCCATACCTGCTCAA	
OVO	NM_205304.1	F: TGACTTCCACCTCTTTGGGC	108
		R: GAATCCATCAGCGAGGGGAC	
CA2	NM_205317.1	F: CTCCTCCGACAAGTCAGTGC	214
		R: TACGACGGCCAAACCATCAG	
OPN	U01844.1	F: GTCACAGACTTCCCCACAGAG	193
		R: TCCTCACCACCCTTCCCAATA	
OC-17	KF835610.1	F: GCAATGCCTTCGTCTGCAAAG	132
		R: CTGCTGTGGGTCCGTTTATTG	
CLU	NM_204900.1	F: TCCACGCTTCATGGAGATCG	115
		R: GCGTTTGACCCACTGATGTG	
β-actin	NM_205518	F: ACACCCACACCCCTGTGATGAA	136
		R: TGCTGCTGACACCTTCACCATTC	

Ultrastructure of Eggshell

To examine the effects of NCG on the ultrastructure of eggshells, 5 eggshell samples in each group were examined by scanning electron microscopy (Figure 2A, 2C). After supplementation with 1% NCG, effective thickness of shells (including the palisade layer, vertical crystal layer, and cuticle layer) was 268.4 μm, while it was 224.2 μm in the control group (Figure 2B, a). The effective thickness of the mastoid layer and width of the mastoid gap and mammillary knobs in the NCG group decreased significantly by 43.0, 27.3, and 23.3%, respectively, when compared with the control group (Figure 2B, b–d). Furthermore, the vertical crystal layer and palisade layer in the NCG group were smoother, the structures were more compact, the number of stomata was fewer, and the number of cracks was less (Figure 2C, a–d) than in the control group. In addition, the width of mammillary knobs in the NCG group was smaller than in the control group, which means there were more mammillary knobs per unit length. Therefore, the density of the closely packed mastoid was increased in the NCG group than in the control group (Figure 2C, e and f). However, fibers in the shell membranes layer displayed no obvious change (Figure 2C, g and h).

Effects of NCG on Mineral Contents in the Eggshells, Uterus, and Plasma

Results of mineral analysis showed that dietary NCG supplement remarkably promoted mineral content in the eggshells, uterus, and plasma. In eggshells, levels of Ca, P, Fe, Mg, Na, and K were increased by 3.5, 33.1, 62.8, 16.0, 13.0, and 28.8%, respectively, after feeding with 1% NCG (Figure 3A). In the uterus, relative to the control group, concentrations of Ca, P, Fe, Mg, Na, and K were significantly increased by 19.0, 11.5, 13.3, 17.0, 8.1, and 13.9%, respectively (Figure 3B). The levels of Ca, P, Fe, Mg, Na, and K in the plasma were higher by 346.0, 86.3, 65.4, 77.4, 2.2, and 13.5%, respectively (Figure 3C).

Effect of NCG on Plasma Hormone Levels

N-Carbamylglutamate administration remarkably raised plasma E_2, PTH, and CT concentrations. As shown in Figure 4, E_2 level was 1307.7 pmol/L in the NCG group, while it was 877.7 pmol/L in the control group with an increment of 49.0% (Figure 4A). Meanwhile, the level of PTH and CT was increased by 46.2 and 24.7%, respectively, as compared with the control group (Figure 4B, 4C).

Effects of NCG on Plasma AA Levels

After supplementation with 1% NCG for 24 wk, plasma AA were measured. As shown in Table 3, dietary supplementation with 1% NCG significantly increased the content of AA, including aspartic acid, threonine, serine, glycine, alanine, cysteine, valine, isoleucine, leucine, phenylalanine, histidine, arginine, and proline, as compared with the control group.

Morphological Changes of the Uterus

Hematoxylin and eosin staining showed that the number of blood vessels in the uterus was significantly increased after supplementation with 1% NCG
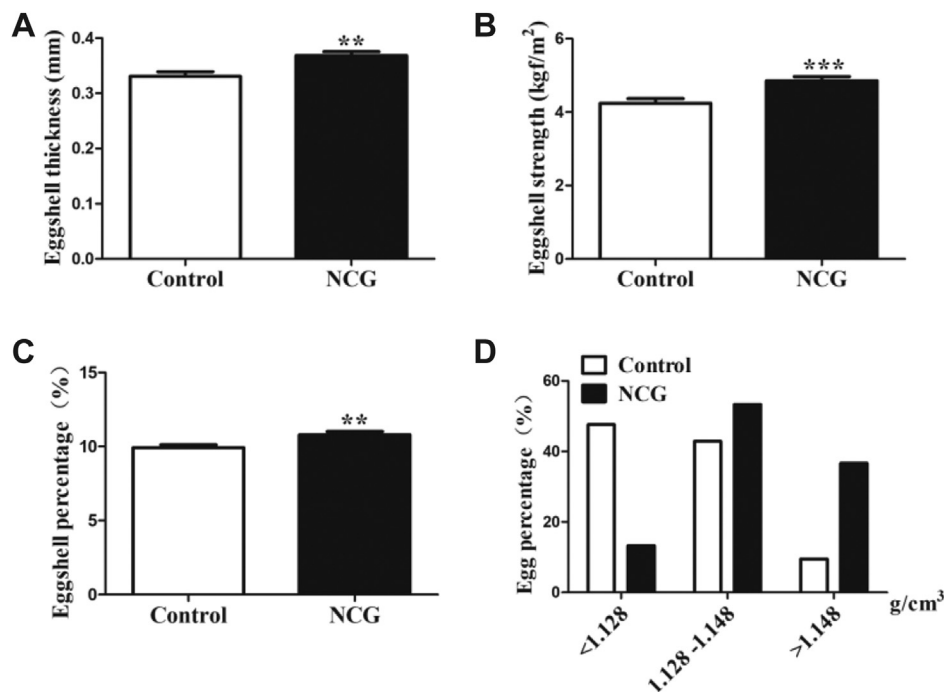

Figure 1. Effect of N-carbamylglutamate (NCG) on eggshell quality. Eggs were collected in the age of 23 to 24 wk of hens. (A) Eggshell thickness (mm). (B) Eggshell strength (kgf/m²). (C) Eggshell percentage (%). (D) The percentage of egg specific gravity (%). Asterisks indicate significant differences (n = 30, values are means ± SEM; **$P < 0.01$ and ***$P < 0.001$).
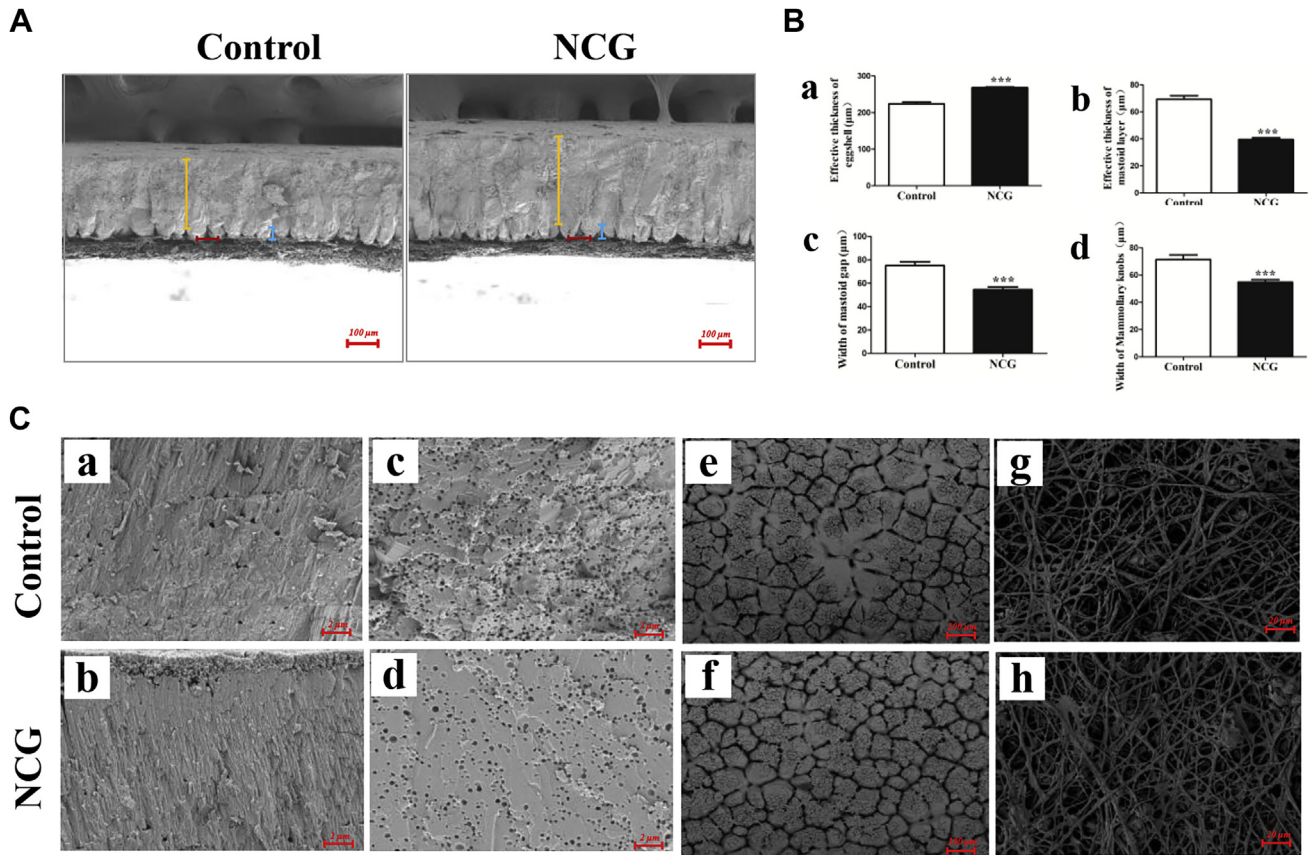
Figure 2. Ultrastructure of eggshell. (A) Effect of NCG on the ultrastructure of eggshell. Yellow line: effective thickness of eggshells; blue line: effective thickness of the mastoid layer; red line: width of the mastoid gap. Scale bar: 100 μm. (B, a) Effective thickness of shells (μm). (B, b) Effective thickness of the mastoid layer (μm). (B, c) Width of the mastoid gap (μm). (B, d) Width of mammillary knobs (μm). (C, a and b) Vertical crystal layer. Scale bar: 2 μm. (C, c and d) Palisade layer. Scale bar: 2 μm. (C, e and f) Mastoid layer. Scale bar: 100 μm. (C, g and h) Eggshell membranes. Scale bar: 20 μm. Asterisks indicate significant differences (n = 5, values are means ± SEM; ***$P < 0.001$). Abbreviation: NCG, N-carbamylglutamate.

(Figure 5). Moreover, based on Figure 6A, the height of uterine mucosal fold was increased significantly in the NCG group (Figure 6B, a). In addition, supplementation with NCG obviously increased the length of villus in uterine mucosal and the areas of uterine mucosal folds (Figure 6B, b and c). The width between 2 adjacent uterine mucosal folds manifested no obvious changes (Figure 6B, d). And the width of uterine mucosal folds in the 2 groups displayed no significant increase (Figure 6B, e).

Effects of NCG on Expression of Calcification-Related Genes and Proteins

After NCG administration, a dramatical elevation in the levels of eggshell calcification–related genes calbindin

D28k (***CALB1***), carbonic anhydrase II (***CA2***), osteopontin (***OPN***), ovalbumin (***OVA***), ovotransferrin (***OVO***), ovocleidin-17 (***OC-17***), ovocleidin-116 (***OC-116***), and clusterin (***CLU***) mRNA was detected from the uterus, duodenum, and kidney by using real-time qPCR (Figures 7A, 7B; 8A, 8B; 9A, 9B). The *CALB1* and *CA2* were calcium transport–related genes. The levels of *CALB1* mRNA in the uterus, duodenum, and kidney were increased by 37.7, 273.2, and 202.5%, respectively, compared with the control group. Meanwhile, the levels of *CA2* mRNA in the uterus, duodenum, and kidney were increased by 83.2, 97.7, and 134.0%, respectively, compared with the control group (Figures 7A, 8A, 9A). Simultaneously, in the uterus, duodenum, and kidney, transcription of eggshell calcification–related
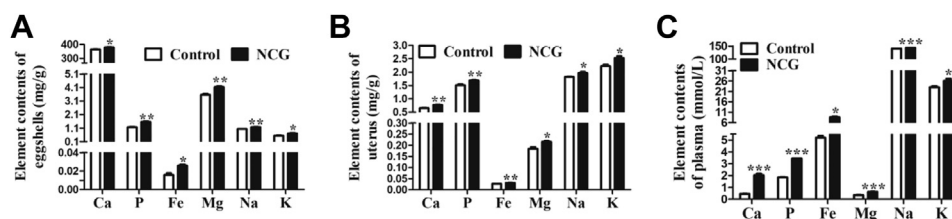

Figure 3. Effects of NCG on mineral contents. (A) Effects of NCG on mineral contents of eggshells. (B) Effects of NCG on mineral contents of uterus. (C) Effects of NCG on mineral contents of plasma. Asterisks indicate significant differences (n = 5, values are means ± SEM; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$). Abbreviation: NCG, N-carbamylglutamate.
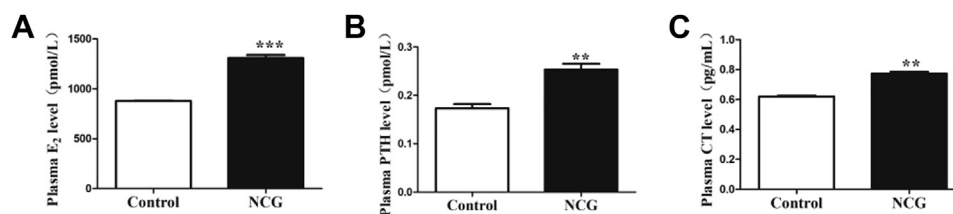
Figure 4. Effects of NCG on plasma hormone levels. (A) Plasma E_2 level (pmol/L). (B) Plasma PTH level (pmol/L). (C) Plasma CT level (pg/mL). Asterisks indicate significant differences (n = 5, values are means ± SEM; **$P < 0.01$ and ***$P < 0.001$). Abbreviations: NCG, N-carbamylglutamate; CT, calcitonin; PTH, parathyroid hormone.

genes (*OPN*, *OVA*, *OVO*, *OC-17*, *OC-116*, and *CLU*) was upregulated in different degrees (Figures 7B, 8B, 9B). In addition, the proteins of CALB1 and ATP2A1 were detected by Western blot, and results showed that expression of these proteins were all upregulated significantly in the NCG group (Figures 7C, 8C, 9C).

DISCUSSION

Eggshell is a unique natural protective barrier that defends egg contents and the embryo from microorganism invasion and physical damage while allowing the exchange of water and gases through stomas during embryo development. Furthermore, $CaCO_3$ in eggshell can provide calcium for embryo development when the yolk reserve is insufficient (Nys et al., 2004). In addition, hatchability correlates positively with eggshell thickness (Liao et al., 2013). Eggshell structural and textural features that are important for shell strength, such as the density of mammillary knobs, the effective thickness of the mastoid layer, and the width of the mastoid gap, are closely related to eggshell strength (Radwan, 2015). Improved eggshell strength can effectively reduce the loss of eggs in egg marketing activities. In this study, the effect of dietary NCG supplementation on eggshell quality was investigated. After dietary supplementation with 1% NCG, eggshell quality was improved significantly. Eggshell thickness, strength, and percentage were increased remarkably by 9.5, 14.5, and 8.7%, respectively. Meanwhile, scanning electron microscopy showed that dietary supplementation with NCG, effective thickness of shells, and the density of mammillary knobs were increased markedly, while the effective thickness of the mastoid layer and the width of the mastoid gap were significantly decreased.

Eggshell is formed in the poultry oviduct, mainly in the uterus and isthmus. Eggshell mineralization includes 3 phases: initiation, growth, and arrest phases (Gautron et al., 1997). Mineral and organic matrix required for shells mineralization in uterine fluid is secreted daily by the uterus. Ca^{2+} and bicarbonate (HCO^{3-}) fluid are sufficiently supplied from the blood via transepithelial transport in the uterus through ion channels, ion pumps, and ion exchangers (Brionne et al., 2014). This process involves additional ion (Na^+, K^+, Mg^{2+}, and Cl^-) transfer to maintain cell homeostasis. The present study revealed that levels of Ca, P, Fe, Mg, Na, and K were significantly increased in the plasma, uterus, and eggshells after supplementation with dietary NCG. Matrix proteins play essential roles during eggshell formation. In this study, 1% NCG supplementation induced upregulated expression of *CALB1*, *CA2*, *OC-17*, *OC-116*, *OVO*, *OVA*, *CLU*, *OPN* mRNAs and CALB1, ATP2A1 proteins in the uterus, duodenum, and kidney.

Table 3. Effect of NCG feeding on plasma AA levels.

	Control	1% NCG
AA (g/100 g plasma)	Mean ± SEM	Mean ± SEM
Aspartic acid	0.132 ± 0.0010	0.169 ± 0.0012***
Threonine	0.078 ± 0.0021	0.099 ± 0.0035*
Serine	0.110 ± 0.0028	0.129 ± 0.0044*
Glutamic acid	0.263 ± 0.0055	0.271 ± 0.0017
Glycine	0.050 ± 0.0015	0.067 ± 0.0026**
Alanine	0.078 ± 0.0011	0.096 ± 0.0029**
Cysteine	0.047 ± 0.0004	0.055 ± 0.0007***
Valine	0.069 ± 0.0012	0.087 ± 0.0023***
Methionine	0.037 ± 0.0008	0.033 ± 0.0012
Isoleucine	0.064 ± 0.0024	0.077 ± 0.0005**
Leucine	0.121 ± 0.0042	0.163 ± 0.0026**
Tyrosine	0.069 ± 0.0021	0.077 ± 0.0009
Phenylalanine	0.052 ± 0.0007	0.071 ± 0.0014***
Lysine	0.095 ± 0.0017	0.104 ± 0.0030
Histidine	0.034 ± 0.0007	0.044 ± 0.0005**
Arginine	0.080 ± 0.0019	0.095 ± 0.0003**
Proline	0.052 ± 0.0011	0.059 ± 0.0011*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (n = 5 hens per treatment). Abbreviations: AA, amino acid; NCG, N-carbamylglutamate.
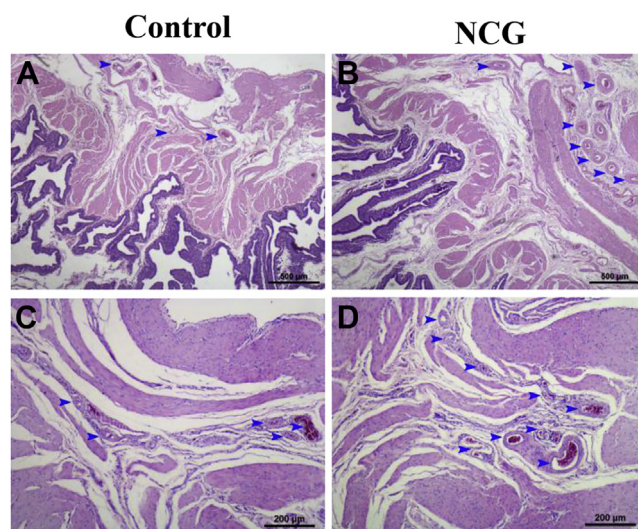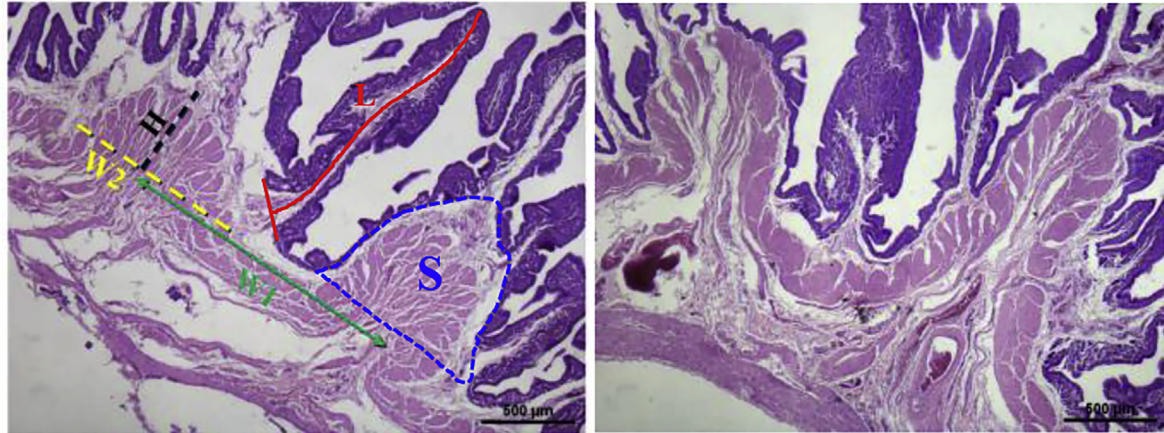

Figure 5. Effects of NCG on the number of uterus blood vessels. (A, B) Scale bar: 500 μm. (C, D) Scale bar: 200 μm. Blue arrowheads: blood vessels. Abbreviation: NCG, N-carbamylglutamate.
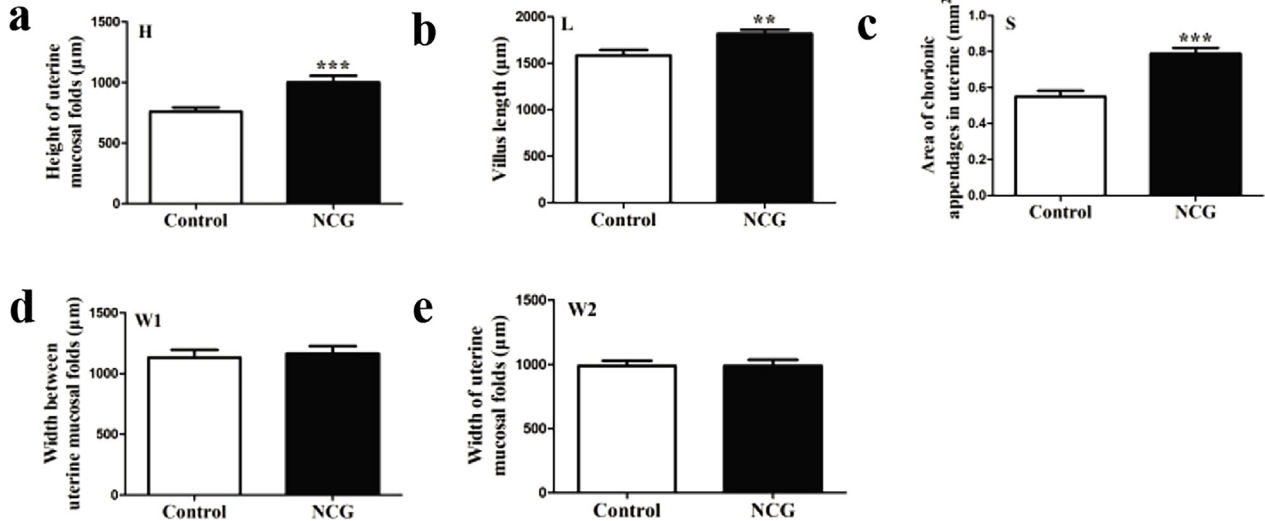
Figure 6. Effect of NCG on morphological changes of the uterus. (A) Morphology of the uterus. Black line (H): Height of the uterine mucosal fold; red line (L): length of villus in the endometrium; green line (W1): width of the 2 adjacent uterine mucosal folds; yellow line (W2): width of the uterine mucosal folds; blue line (S): the areas of the uterine mucosal folds. Scale bar: 500 μm. (B, a) The height of the uterine mucosal fold (H: μm). (B, b) The length of villus in the endometrium (L: μm). (B, c) The areas of the uterine mucosal folds (S, $μm^2$). (B, d) The width of the 2 adjacent uterine mucosal folds (W1: μm). (B, e) The width of the uterine mucosal folds (W2: μm). Asterisks indicate significant differences (n = 5, values are means ± SEM; **$P < 0.01$ and ***$P < 0.001$). Abbreviation: NCG, N-carbamylglutamate.

These changes contributed to ion transport and absorption that facilitate eggshell mineralization.

Ca^{2+} is one of the most efficiently regulated plasma constituents in birds. In the laying hens, approximately 2.4 g Ca^{2+} is required over 18 h to produce a shelled egg of 60 g. In the peak production stage, a hen lays almost one egg every day during a 52-wk period. It was calculated that the t½ for ^{45}Ca uptake by the chick femur is less than 10 min, while a rabbit, dog, and rat took about 30 min (Shaw and Dacke, 1989; Bronner and Stein, 1992). Therefore, the throughput of Ca^{2+} in hens is much more active than in mammals.

In birds, classical Ca^{2+}-regulating hormones include PTH, CT, and 1,25-dihydroxy vitamin D_3 (Dacke, 1979). Parathyroid hormone is secreted by parathyroid glands in response to decreased plasma Ca^{2+} level (Dacke, 2015). Calcitonin blocks osteoclastic resorption of medullary bone when an egg is in the magnum of the oviduct, while PTH stimulates osteoclastic bone resorption for eggshell formation during the period when an egg is in the shell gland. This study manifested that NCG supplement markedly elevated plasma levels of PTH and CT. Presumably, elevated plasma PTH led to an increase of the plasma Ca^{2+} level, while elevated CT promoted the deposition of calcium in the eggshell. These 2 hormones act together to maintain avian Ca^{2+} levels for speedy demand of eggshell formation.

In addition to PTH and CT, E_2 is another pivotal Ca^{2+}-regulating hormone. The onset of osteoporosis is related to estrogenic activity, which stimulates medullary bone formation for eggshell formation (Fleming et al., 1998). It was reported that subcutaneous injection of estradiol 3-benzoate in laying hens increased shell thickness and improved shell deformation (Wistedt et al., 2014). Phytoestrogen
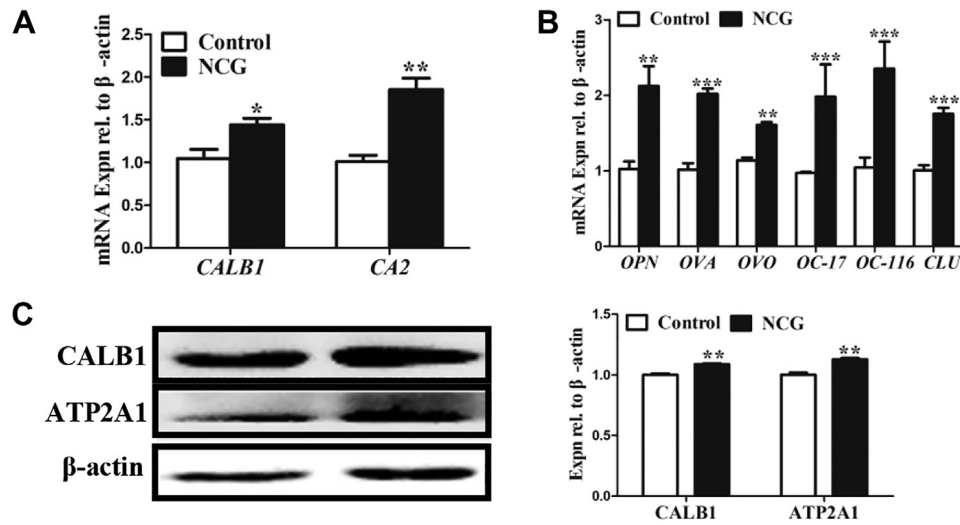
Figure 7. Effects of NCG on expression of eggshell formation–related genes and proteins in the uterus. (A) Expression of Ca^{2+} transport–related genes. (B) Expression of eggshell calcification–related genes (C) Expression of CALB1 and ATP2A1 proteins. Asterisks indicate significant differences (values are means ± SEM; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$). Abbreviation: NCG, N-carbamylglutamate.

source supplementation could improve reproductive performance, egg quality, steroidogenesis, hormonal profile, and the antioxidative status in the aging chicken (Saleh et al., 2019). In this study, the level of plasma E_2 was increased significantly in the NCG group that is in favor of eggshell formation.

N-Carbamylglutamate was able to increase gene expression of nitric oxide synthase 3 and vascular endothelial growth factor A in the pregnant cows, thus increasing litter size (Cai et al., 2018). HE staining in this study showed that the density of blood vessels in the uterus was significantly increased after supplementation with 1% NCG. Plasma AA analysis showed that feeding with 1% NCG significantly increased plasma AA levels (including Arg). A previous study reported that supplementation with glutamate and NCG in

combination displayed better favorable effect on proliferation of the intestinal epithelium cells than glutamate alone (Wu et al., 2012). Furthermore, NCG treatment enhanced the capacity of antioxidants in the rat jejunum under oxidative stress (Xiao et al., 2016). In addition, dietary NCG supplementation improved daily weight gain and growth performance in the yellow-feather broilers through modifying homeostasis of Arg metabolism (Hu et al., 2019). Presumably, NCG may enhance the capacity of intestinal absorption and thereafter elevate the levels of AA, ions, and proteins. Especially, Arg can be converted to NO and citrulline by the catalysis of the NO synthase. Therefore, the increased concentration of NO resulting from elevated Arg promoted angiogenesis and thus increased the transport ability of nutrients.
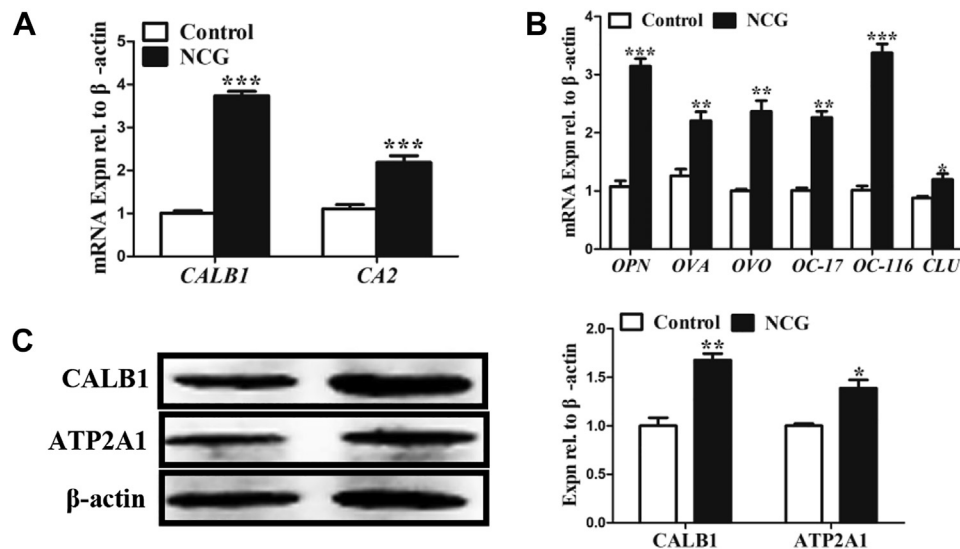

Figure 8. Effects of NCG on expression of eggshell formation–related genes and proteins in the duodenum. (A) Expression of Ca^{2+} transport–related genes. (B) Expression of eggshell calcification–related genes (C) Expression of CALB1 and ATP2A1 proteins. Asterisks indicate significant differences (values are means ± SEM; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$). Abbreviation: NCG, N-carbamylglutamate.
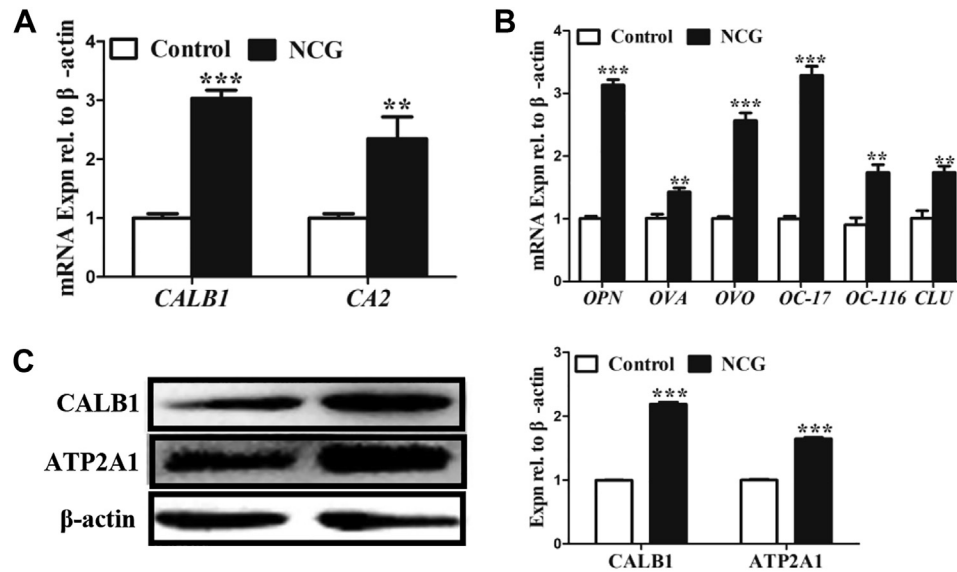
Figure 9. Effects of NCG on expression of eggshell formation–related genes and proteins in the kidney. (A) Expression of Ca^{2+} transport–related genes. (B) Expression of eggshell calcification–related genes. (C) Expression of CALB1 and ATP2A1 proteins. Asterisks indicate significant differences (values are means ± SEM; **$P < 0.01$ and ***$P < 0.001$). Abbreviation: NCG, N-carbamylglutamate.

Eggshell is mainly formed in the isthmus and uterus portions of the oviduct. This study revealed that the height of uterine mucosal folds, the length of villus in the endometrium, and the areas of uterine mucosal folds were increased significantly after NCG supplementation. This result indicated that the secretory ability of the uterine mucosa was enhanced by NCG feeding. Hence, transport of the ions into the uterus fluid was increased, leading to accelerated calcification and improvement of the eggshell quality.

Dietary 1% NCG supplementation upregulated calcium metabolism–related genes and hormones, along with an increased number of blood vessels of the uterus. These changes contributed to accelerated ion transport and absorption that facilitated eggshell mineralization. In summary, dietary NCG administration remarkably improves eggshell quality through changes in endometrial morphology, calcium metabolism–related genes expression, and hormone secretion to promote calcification and improvement of the eggshell quality.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.04.004.

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