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Original Research Article

Effectiveness and safety evaluation of graded levels of N-carbamylglutamate in growing-finishing pigs

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ABSTRACT

The aim of this study was as follows: 1) to investigate the effects of graded levels of N-carbamylglutamate (NCG) on performance, blood biochemical indexes, carcass traits and related indicators in growing-finishing pigs, and 2) to determine the optimal supplemental level. The toxicity of high-dose (much higher than recommended levels) NCG was assessed by routine blood tests and blood biochemical and histopathologic examinations of the heart, liver, spleen, lung, kidney and stomach. One hundred and forty-four growing-finishing pigs (Duroc × Large White × Landrace, 32.24 ± 1.03 kg) were used in a 74-d experiment and each treatment was replicated 6 times with 4 pigs (2 barrows and 2 gilts) per replicate. The dietary treatments were a corn-soybean meal basal diet supplemented with 0% (control), 0.05%, 0.1%, 0.15%, 0.2% or 1% NCG. The first 5 groups were used to explore the optimal supplemental level of NCG, while the control, 0.1% and 1% NCG groups were used to explore the safety of high-dose NCG. Compared with the normal control group, the final body weight and average daily gain tended to be higher in the 0.1% group ($P = 0.08$), the lean percentage tended to be higher in the 0.05% group ($P = 0.07$), the levels of free amino acids in the blood significantly increased in the 0.1% group ($P < 0.05$), both 0.1% and 0.15% NCG supplementation increased the levels of nitric oxide (NO) in serum ($P = 0.07$) and muscle growth- and lipid metabolism-related gene expression ($P < 0.05$) and NCG supplementation improved C18:1N9C monounsaturated fatty acids (MUFA) in a dose-dependent manner ($P = 0.08$). In addition, routine blood tests, blood biochemical indexes and histopathological examination revealed no abnormalities. Overall, increasing the levels of NCG did not linearly improve the above indicators; the 0.1% dose showed the best effect, and a high dose (1%) did not pose a toxicity risk.

1. Introduction

Pigs are a major source of animal protein for human consumption. With the development of society, people have higher requirements for meat quality, and effective and simpler strategies for improving the quality of meat are desirable. Animal nutrition is an important factor affecting meat quality (Wood et al., 2008). Arginine, as the most abundant nitrogen carrier in tissue protein, has significant and diverse metabolic and regulatory effects (Flynn et al., 2002; Southern and Baker, 1983; Wu and Morris, 1998). Previous studies have indicated that arginine could reduce body fat mass in a non-insulin-dependent diabetes mellitus mouse model and in growing-finishing pigs (Fu et al., 2005; Tan et al., 2009; Wu et al., 2007), while enhancing growth performance in suckling piglets and growing-finishing pigs (Kim and Wu, 2004; Tan et al., 2009). However, considering the high cost of adding arginine

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directly, and because effective dosage supplementation could interfere with the absorption and metabolism of other amino acids, alternatives to arginine must be explored.

In recent years, N-carbamylglutamate (NCG), an analog of N-acetyl-L-glutamate (NAG), has been found to initiate the initial enzyme of the urea cycle, namely, carbamoyl phosphate synthase, which in turn promotes the synthesis of endogenous arginine and the production of arginine metabolites, such as nitric oxide (NO) and polyamines (Frank et al., 2007; Wu et al., 2004, 2010). NCG has the advantages of a long half-life and stable metabolism (Wu et al., 2004; Wu and Morris, 1998), and many studies have shown that it has the potential to be used as a substitute for arginine in poultry and ruminant production (Chacher et al., 2014; Ma et al., 2020; Zhang et al., 2019a,b, 2020). In pig production, previous studies have revealed the role of NCG in improving reproductive performance (Liu et al., 2012; Wu et al., 2012) and growth performance (Wu et al., 2010). The regulatory effect on meat quality has been proven in finishing pigs, and dietary NCG supplementation in a protein-reduced diet could increase the longissimus dorsi muscle area, decrease back fat accretion, and produce functional pork with a high content of leucine (Ye et al., 2017). However, to date, little research has investigated the use of NCG in growing-finishing pigs, and no data reported concerning safety evaluation and optimal supplementation dose. Therefore, the objective of this study was to investigate the safety, effectiveness and appropriate dietary supplemental dose of NCG in growing-finishing pigs.

2. Materials and methods

2.1. Animal ethics statement

This study complied with Chinese guidelines on experimental protocols and animal welfare and was approved by the Animal Protection Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences.

2.2. Animals and experimental design

A total of 144 Duroc × Yorkshire × Landrace growing-finishing pigs, with an average initial body weight of 32.24 ± 1.03 kg, were used in a 74-d performance trial. The pigs were allotted to 1 of 6 treatments based on initial body weight in a randomized complete block design with 6 replicates per treatment and 4 pigs per pen. The control diet was formulated based on corn and soybean meal without any supplementation (Table 1). The dietary treatments were either a control diet (corn-soybean meal basal diet) or an NCG diet (corn-soybean meal basal diet) supplemented with 0.05%, 0.1%, 0.15%, 0.2% or 1% NCG. NCG (purity ≥ 97.8%) was purchased from the National Feed Engineering Technology Research Center. The first 5 groups were used to explore the optimal supplemental level of NCG, while the control, 0.1% and 1% NCG groups were used to explore the safety of high-dose NCG. Pigs had free access to water. Feed allowance was equivalent to 4% of BW and was divided into 3 equal meals fed 3 times per day. Body weight was recorded at the beginning and at the end of the experiment.

Table 1
Ingredients and nutrient levels of the basal diet (as-fed basis, %).

Item	Pigs	
	30–60 kg	60–90 kg
Ingredients		
Corn	58.00	67.00
Soybean meal	29.38	23.76
Wheat bran	8.00	6.00
Soybean oil	1.60	0.88
L-Lysine·HCl	0.17	0.01
L-Threonine	0.01	
Monocalcium phosphate	0.65	0.50
Limestone	0.89	0.55
NaCl	0.30	0.30
Premix[1]	1.00	1.00
Nutrient levels[2]		
DE, MJ/kg	14.20	14.20
CP	18.27	16.30
SID Lys	0.98	0.72
SID Met + Cys	0.56	0.50
SID Thr	0.59	0.56
SID Trp	0.20	0.17
Calcium	0.60	0.51
Total P	0.51	0.45
Effective P	0.23	0.19

DE = digestible energy; CP = crude protein.
[1] The premix provided following per kilogram of the basal diets: vitamin A 925.8 μg, vitamin D_3 9.65 μg, vitamin E 15.4 mg, vitamin K_3 2.3 mg, vitamin B_2 3.9 mg, vitamin B_{12} 0.016 mg, niacin 23 mg, Cu (as copper sulfate) 10 mg, Fe (as ferrous sulfate) 100 mg, Mn (as manganese oxide) 10 mg, Zn (as zinc oxide) 100 mg, Se (as sodium selenite) 0.3 mg.
[2] Measured values. All data are the results of a chemical analysis conducted in duplicate.

2.3. Muscle quality measurements

On the final experiment day, pigs with a weight close to the average weight from each replicate were selected and transported to the slaughter site and allowed to rest for 12 h, after which they were electroshocked and slaughtered. The head, hoof, and tail were removed, and the left and right carcasses were cut in half.

The back fat thickness of the 6th–7th ribs, eye muscle area and pH of the left half of the carcass were measured on site. Muscle pH was measured at 3 locations on the 10th rib interface using a hand-held pH meter (model 2000, VWR Scientific Products Co., South Plainfield, NJ, U.S.A.).

After chilling at 2 °C for 24 h, the CIELAB L* (lightness), a* (redness), and b* (yellowness) color of the 10th rib was determined from 3 orientations (middle, medial, and lateral) with a colorimeter with an 8-mm aperture and 0° viewing angle (CR410 chromameter, Minolta, Tokyo, Japan). The illuminant condition was D65. The colorimeter was calibrated according to the manufacturer's guidelines.

The shear force of longissimus dorsi muscle sample was measured by C-LM3 (Harbin, China) muscle tenderness determination device.

The right half of the carcass was taken for tissue segmentation. Skin, bone, lean meat and fat were separated and weighed. Lean meat rate (%) = lean meat weight/carcass weight; fat rate (%) = fat weight/carcass weight.

2.4. Blood sampling and analysis

Blood samples were taken from each of 6 pigs/treatment by venipuncture from the anterior vena cava on d 35 and 74. After collection, the blood samples were placed at room temperature for 2–3 h prior to centrifugation (3,000 × *g* for 10 min), and the serum was obtained and stored at −80 °C for subsequent biochemical analysis. Whole blood samples of the control, 0.1% and 1% groups were collected in EDTA tubes and stored at room temperature for hematology analysis within 6 h of sampling.

Serum amino acid concentrations were determined by an amino acid analyzer (S-433D amino acid analyzer, Sykam GmbH, Eresing, Germany). Serum was deproteinized with 120 mg of salicylic acid per milliliter. The samples were placed in an ice bath for 20 min. Thereafter, the reaction system was adjusted for pH by adding lithium hydroxide solution (2 mol/L), followed by centrifugation at 12,000 × *g*

for 30 min at 4 °C. The supernatant was collected and filtered through a 0.1-μm filter before loading on the amino acid analyzer.

Serum urea nitrogen (SUN), triglycerides (TG), total nitric oxide synthase (TNOS) and NO were determined using commercial test kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The absorbance was read using a multimode microplate reader (iMark, Bio-Rad, USA).

Routine blood indexes were determined by a ProCyte Dx Hematology analyzer (IDEXX Laboratories, America), and blood biochemical indexes were determined by a Hitachi 7020 automatic biochemical analyzer (HITACHI, Japan).

2.5. Total fatty acid detection in longissimus dorsi muscle

The composition of total fatty acids in the longissimus dorsi muscle was determined by an Agilent 6890 N gas chromatographer. The longissimus dorsi muscles (0.5 g) were ground in liquid nitrogen, followed by the addition of 4 mL of chloroacetic methanol, 1 mL of n-hexane, and 1 mL of internal standard fatty acid solution (1 mg/mL 11 carbon fatty acid methyl ester). The samples were then vortexed for 1 min and kept in a water bath at 75 °C for 2 h. After cooling, 5 mL of potassium carbonate solution (70 g/L) was added to the samples and vortexed for 1 min, followed by centrifugation at 200 × *g* for 10 min. The supernatant was then loaded on the gas chromatographer. The concentration of individual fatty acids was quantified according to the following equation: $Ci = m0 \times Ai \times Fi \times Ri/(A0 \times m)$, where Ci is the concentration of individual fatty acids (mg/g), m0 is the weight of internal standard fatty acid (mg), m is the weight of the samples (g), Ai is the peak area of individual fatty acid in the samples, A0 is the peak area of the internal standard fatty acid, Fi is the correction coefficient of fatty acid methyl ester to fatty acid, and Ri is the correction coefficient of the peak area.

2.6. RNA isolation and quantitative RT-PCR determination

Total RNA extraction from longissimus dorsi muscle tissues and cDNA synthesis were conducted using the HiPure Total RNA Mini Kit (Magen, Guangzhou, China) and PrimeScript RT reagent Kit (Takara Biotechnology Co., Ltd., Otsu, Shiga, Japan), respectively. RNA (1 μg) was used to generate cDNA in a volume of 20 μL. The mRNA levels of individual genes were measured by real-time PCR using the TB Green Premix Ex Taq II (Takara Biotechnology Co., Ltd., Otsu, Shiga, Japan) and the LightCycler Real-Time PCR System (Roche, Germany). The primers for the real-time PCR are listed in Table 2, and β-actin was used as a housekeeping gene in this study. The relative mRNA expression of the target genes was determined using the $2^{-\Delta\Delta Ct}$ method.

2.7. Organ histology

The heart, liver, spleen, lung and kidney of the control, 0.1% and 1% groups were removed, trimmed of any superficial fat or blood, blotted dry and weighed ($n = 6$/treatment). Heart, liver, spleen, lung, kidney and stomach samples from the control, 0.1% and 1% groups were also taken and fixed in 4% paraformaldehyde solution and kept in 75% ethanol until processing. The fixe2d tissues were embedded in paraffin, and sections were stained with hematoxylin and eosin and then examined under a light microscope (Nikon Eclipse Ci, Japan). Photomicrographs were captured using a digital camera attached to the microscope (Nikon digital sight DS-FI2, Japan).

2.8. Statistical analysis

Data were subjected to analysis of variance (ANOVA) suited for a randomized complete block design using the general linear model (GLM) procedure (version 9.2, SAS Institute, Inc., Cary, NC, U.S.A.). The results are expressed as the mean ± standard error of the mean (SEM). Statistical differences among groups were analyzed by the Bonferroni multiple comparisons test. *P*-values < 0.05 were considered statistically significant.

Table 2
Primers used for real-time PCR.

Genes	Primer sequences (5′–3′)	Product size, bp	GenBank ID
MyoD	F:CAACAGCGGACGACTTCTATG R:GCGCAAGATTTCCACCTT	383	100136773
MyoG	F: GCAGGGTGCTCCTCTTCA R: AGGCTACGAGCGGACTGA	230	497618
HSL	F:GCAGCATCTTCTTCCGCACA R:AGCCCTTGCGTAGAGTGACA	192	397583
LPL	F:CTCGTGCTCAGATGCCCTAC R: GGCAGGGTGAAAGGGATGTT	147	397537
ACC	F:ATCCCTCCTTGCCTCTCCTA R:ACTTCCCGTTCAGATTTCCG	129	104371
PPARγ	F:GATTTCTCCAGCATTTCCA R:GCTCTTCGTGAGGTTTGTT	184	102097513
PGC-1α	F:GCCCAGTCTGCGGCTATTT R:GTTCAGCTCGGCTCGGATTT	92	397013
GAPDH	F:GTGGCTGATGAGAAGTTCTG R:CAGATCTCTACCGTGTCCAC	233	396823

MyoD = myogenic differentiation antigen; *MyoG* = myoglobin; *HSL* = hormone-sensitive lipase; *LPL* = lipoprotein lipase; *ACC* = acetyl-CoA carboxylase; *PPARγ* = peroxisome proliferator-activated receptor γ; *PGC-1α* = peroxisome proliferator-activated receptor-γ coactivator-1α; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase.

3. Results

3.1. Growth performance

The effects of graded levels of NCG supplementation on growth performance are shown in Table 3. Supplementation with 0.1% and 0.2% NCG significantly improved the final weight and ADG ($P < 0.05$), but had no effects on the ADFI or feed-to-gain ratio (F/G) in the current study.

3.2. Carcass traits and meat quality

The effects of graded levels of NCG supplementation on carcass traits and meat quality are shown in Table 4. NCG (0.05%) significantly increased the cooking holding percentage ($P < 0.05$), but no difference was detected in the other indicators ($P > 0.05$).

3.3. Concentrations of serum amino acids

The serum concentrations of amino acids are presented in Table 5. Graded levels NCG supplementation increased the level of free amino acids in blood to different degrees. Compared with the control group, the 0.1% NCG diet supplemented group had significantly increased serum contents of leucine, isoleucine, methionine, valine, arginine, glutamic acid and citrulline.

3.4. Biochemical indexes

The effects of graded levels of NCG supplementation on the blood biochemical indexes are shown in Table 6. Compared with the control group, dietary supplementation of 0.15% significantly decreased the TG content in serum ($P < 0.05$), and dietary supplementation of 0.1% and 0.15% NCG significantly increased the NO content in serum ($P < 0.05$).

Table 3
Effects of dietary N-carbamylglutamate (NCG) supplementation on growth performance.

Item	NCG, %					SEM	*P*-value
	0	0.05	0.1	0.15	0.2		
Initial weight, kg	32.38	32.49	31.83	32.39	32.13	1.07	0.98
Final weight, kg	86.78^{b}	89.28^{ab}	92.02^{a}	88.65^{ab}	91.38^{a}	1.86	0.08
ADFI, kg	2.09	2.13	2.19	2.10	2.18	0.06	0.31
ADG, g	725.80^{b}	769.93^{ab}	801.23^{a}	768.67^{ab}	801.35^{a}	28.08	0.08
F/G	2.77	2.78	2.73	2.79	2.73	0.07	0.85

ADFI = average daily feed intake; ADG = average daily gain; F/G = the ratio of ADFI to ADG.
$^{a, b}$ Different letters within a row indicate a significant difference among different treatments ($P < 0.05$), $n = 6$.

Table 4
Effect of dietary N-carbamylglutamate (NCG) supplementation on carcass traits and meat quality in growing-finishing pigs.

Item	NCG, %					SEM	*P*-value
	0	0.05	0.1	0.15	0.2		
Back fat depth, mm	16.67	15.76	15.34	16.65	16.53	1.17	0.90
10th rib loin eye area, cm^2	24.33	25.10	23.36	24.49	26.07	1.44	0.75
Lean meat rate, %	59.23	61.04	59.79	59.29	57.69	0.02	0.64
Fat rate, %	14.93	14.90	13.52	12.68	13.86	0.01	0.55
pH $_{45\ min}$	5.73	5.77	5.56	5.77	5.75	0.10	0.50
pH $_{24\ h}$	5.54	5.53	5.53	5.53	5.56	0.02	0.65
Meat color a*	14.25	14.47	14.60	14.66	15.11	0.51	0.82
Meat color b*	4.63	4.94	5.57	4.55	4.94	0.40	0.49
Meat color L*	54.14	53.08	57.36	51.26	52.73	1.79	0.32
Water holding capacity, %	0.22	0.20	0.22	0.19	0.20	0.01	0.26
Cooking holding percentage, %	52.05^{b}	55.96^{a}	50.97^{b}	53.12^{ab}	53.17^{ab}	0.01	0.07
Shear force, N	48.12	50.52	53.68	52.89	50.40	4.38	0.91
Intramuscular fat, %	2.06	2.16	2.13	2.23	2.25	0.11	0.79

$^{a, b}$ Different letters within a row indicate a significant difference among different treatments ($P < 0.05$), $n = 6$.

Table 5
Concentrations of serum amino acids in growing-finishing pigs with N-carbamylglutamate (NCG) supplementation diet (nmol/dL).

Item	NCG, %					SEM	*P*-value
	0	0.05	0.1	0.15	0.2		
Histidine	0.50	0.54	0.55	0.52	0.56	0.06	0.96
Isoleucine	16.10^{b}	17.36^{b}	20.50^{a}	20.50^{a}	20.43^{a}	0.95	<0.01
Leucine	18.18^{c}	20.06^{bc}	22.00^{ab}	20.45^{abc}	22.75^{a}	0.81	0.01
Lysine	11.16	12.82	12.09	13.01	13.96	0.92	0.30
Methionine	17.78^{ab}	20.28^{ab}	21.96^{a}	15.81^{b}	18.93^{ab}	1.40	0.05
Phenylalanine	0.17	0.24	0.15	0.16	0.25	0.41	0.05
Threonine	41.91	34.21	36.98	36.67	46.26	3.40	0.37
Valine	6.79^{ab}	5.61^{b}	6.48^{ab}	7.02^{ab}	7.66^{a}	0.55	0.15
Alanine	1.69^{ab}	1.93^{ab}	2.13^{a}	1.48^{b}	1.88^{ab}	0.17	0.17
Arginine	22.06^{c}	23.43^{bc}	28.56^{ab}	28.57^{ab}	30.07^{a}	2.89	0.03
Aspartic acid	11.95	13.46	12.89	11.96	13.59	0.85	0.50
Glutamate	13.14^{b}	15.60^{ab}	18.65^{a}	11.68^{b}	15.18^{ab}	1.31	0.02
Glycine	10.98	13.25	11.09	10.08	11.72	1.05	0.34
Serine	0.62	0.72	0.60	0.77	0.82	0.09	0.41
Tyrosine	2.08	2.19	2.17	2.57	2.36	0.27	0.73
Proline	40.85	39.33	36.35	42.02	38.68	3.59	0.83
Cysteine	0.96	1.02	1.14	0.94	0.95	0.09	0.62
Citrulline	37.07^{c}	45.85^{ab}	49.32^{a}	38.58^{bc}	45.48^{ab}	2.47	0.01
Ornithine	1.72	1.94	1.93	2.06	2.00	0.13	0.60

$^{a, b, c}$ Different letters within a row indicate a significant difference among different treatments ($P < 0.05$), $n = 6$.

3.5. Total fatty acids composition in the longissimus dorsi muscle

The effects of graded levels of NCG supplementation on the composition of fatty acids in the longissimus dorsi muscle are shown in Table 7. With the increase in the NCG supplemental proportion (0.1%, 0.15% and 0.2%), the monounsaturated fatty acids (MUFA) content showed an increasing trend ($P = 0.08$). In comparison to the control group, the group supplemented with 0.05% NCG had significantly increased contents of C20:1 ($P < 0.05$).

Table 6
Effects of dietary graded levels N-carbamylglutamate (NCG) supplementation on blood biochemical index of the growing-finishing pigs.

Item	NCG, %					SEM	*P*-value
	0	0.05	0.1	0.15	0.20		
SUN, mmol/L	4.54	4.99	5.02	5.09	4.97	0.50	0.94
TG, mmol/L	0.60^{a}	0.61^{a}	0.60^{a}	0.50^{b}	0.53^{ab}	0.05	0.03
TNOS, U/L	16.07	16.72	14.60	14.16	12.91	1.55	0.45
NO, mmol/L	17.72^{b}	23.79^{ab}	25.52^{a}	27.18^{a}	23.96^{ab}	2.19	0.07

SUN = serum urea nitrogen; TG = triglycerides; TNOS = total nitric oxide synthase; NO = nitric oxide.
$^{a, b}$ Different letters within a row indicate a significant difference among different treatments ($P < 0.05$), $n = 6$.

3.6. Effects of NCG on the expression of lipid metabolism related genes

Compared with the control group, 0.15% NCG significantly increased the expression of myogenic differentiation antigen (*MyoD*) ($P < 0.05$), 0.05% and 0.1% NCG significantly increased the expression of hormone-sensitive lipase (*HSL*) and peroxisome proliferator-activated receptor-γ coactivator-1α (*PGC-1α*) ($P < 0.05$), and 0.15% and 0.2% NCG significantly increased the expression of acetyl-CoA carboxylase (*ACC*) ($P < 0.05$) (Table 8).

3.7. Safety evaluation based on growth performance

The effect of high-dose NCG supplementation on growth performance is shown in Table 9. Compared with the control and 0.1% groups, the 1% NCG supplementation group showed no significant difference in growth performance indexes.

Table 7
Effects of dietary N-carbamylglutamate (NCG) supplementation of muscular total fatty acid profile in growing-finishing pigs.

Item, mg/g of muscle	NCG, %					SEM	*P*-value
	0	0.05	0.1	0.15	0.20		
C14:0	1.37	1.42	1.39	1.36	1.31	0.12	0.98
C16:0	25.61	25.49	25.72	25.50	25.10	0.66	0.97
C16:1	3.24	2.79	2.99	3.16	3.31	0.29	0.70
C17:0	0.42	0.49	0.38	0.38	0.37	0.04	0.15
C18:0	13.04	13.69	13.50	13.21	13.16	0.45	0.84
C18:1N9C	33.83^{ab}	30.72^{b}	35.44^{a}	36.01^{a}	38.61^{a}	1.54	0.02
C18:2N6C	14.76^{ab}	17.69^{a}	14.96^{ab}	14.96^{ab}	13.19^{a}	1.34	0.24
C20:0	0.18^{ab}	0.17^{b}	0.21^{ab}	0.19^{ab}	0.22^{a}	0.01	0.08
C20:1	0.74^{b}	0.89^{a}	0.78^{ab}	0.77^{ab}	0.81^{ab}	0.04	0.16
C18:3N3	0.64	0.67	0.70	0.70	0.67	0.05	0.94
C20:3N6	0.44	0.43	0.46	0.47	0.42	0.06	0.97
C20:4N6	3.87^{ab}	5.07^{a}	3.05^{ab}	2.85^{ab}	2.42^{b}	0.79	0.17
C22:6N3	0.15	0.15	0.15	0.18	0.16	0.02	0.69
SFA	40.62	41.25	41.19	40.64	40.16	0.99	0.93
MUFA	37.98^{ab}	34.63^{b}	39.35^{ab}	40.08^{ab}	41.85^{a}	1.69	0.08
PUFA	17.44^{ab}	24.12^{a}	19.46^{ab}	19.28^{ab}	16.97^{b}	2.13	0.17
MUFA/SFA	2.43	1.62	2.17	2.17	2.04	0.35	0.58
PUFA/SFA	0.43	0.59	0.48	0.48	0.42	0.06	0.29

SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acids.
[a, b] Different letters within a row indicate the significant difference among different treatments ($P < 0.05$), $n = 6$.

Table 8
Relative mRNA levels of lipid metabolism related genes.

Item	NCG, %					SEM	*P*-value
	0	0.05	0.1	0.15	0.2		
MyoD	1.14^{b}	1.22^{b}	1.52^{b}	2.87^{a}	1.54^{b}	0.40	0.04
MyoG	1.08	1.09	2.00	1.51	1.92	0.41	0.40
HSL	0.82^{b}	0.58^{b}	2.41^{a}	1.53^{a}	1.40^{ab}	0.38	0.04
ACC	1.04^{c}	1.68^{bc}	1.37^{bc}	2.14^{ab}	2.90^{a}	0.31	0.01
LPL	1.19	1.27	1.27	1.88	1.90	0.31	0.34
PPARγ	1.12	2.53	1.96	2.55	1.96	0.31	0.25
PGC-1α	1.12^{b}	2.53^{a}	2.55^{a}	1.96^{ab}	1.96^{ab}	0.29	0.03

NCG = N-carbamylglutamate; *MyoD* = myogenic differentiation antigen; *MyoG* = myoglobin; *HSL* = hormone-sensitive lipase; *LPL* = lipoprotein lipase; *ACC* = acetyl-CoA carboxylase; *PPARγ* = peroxisome proliferator-activated receptor γ; *PGC-1α* = peroxisome proliferator-activated receptor-γ coactivator-1α.
[a, b, c] Different letters within a row indicate a significant difference among different treatments ($P < 0.05$), $n = 6$.

Table 9
Safety evaluation of growth performance.

Item	NCG, %			SEM	*P*-value
	0	0.1	1		
Initial weight, kg	32.38	31.83	32.21	0.95	0.87
Final weight, kg	86.78^{b}	92.02^{a}	89.30^{ab}	2.10	0.08
ADFI, kg	2.09	2.19	2.09	0.07	0.32
ADG, g	725.80	801.23	752.12	34.06	0.12
F/G	2.77	2.73	2.70	0.06	0.54

NCG = N-carbamylglutamate.
[a, b] Different letters within a row indicate a significant difference among different treatments ($P < 0.05$), $n = 6$.

3.8. Safety evaluation based on organ weights and histology

The effect of NCG supplementation on organ weights is shown in Table 10. NCG supplementation did not affect heart, liver, spleen, lung or kidney weight. Histopathological examination of the heart, liver, spleen, lung, kidney and stomach did not reveal any abnormalities in pigs receiving any of the treatments (Fig. 1).

Table 10
Effect of N-carbamylglutamate (NCG) supplementation on organ weights (%).

Item	NCG, %			SEM	*P*-value
	0	0.1	1		
Heart	0.34	0.37	0.34	<0.01	0.63
Liver	1.77	1.73	1.72	<0.01	0.79
Spleen	0.02	0.02	0.01	<0.01	0.81
Lung	1.94	1.88	1.77	<0.01	0.75
Kidney	0.16	0.16	0.17	<0.01	0.63

Values are the means of 6 replicates.

3.9. Safety evaluation based on hematological parameters

The effect of NCG on hematological parameters is presented in Tables 11 and 12. In the middle of the experiment (d 35), there were no significant differences between the control group and the 1% NCG group. All data were within the normal range.

Table 11 shows the effect of NCG on the serum biochemistry of growing-finishing pigs. There was no significant difference in the serum biochemical indexes among all treatments.

4. Discussion

With the improvement in living standards, the nutritional value and sensory quality of pork have received increasing attention. Producing high-quality pork is conducive to promoting social meat consumption. To pursue a higher lean meat rate, commercial pigs have been strictly selected and bred, which has allowed breakthroughs in lean meat production. But, this has also brought some disadvantages, such as low intramuscular fat, poor flavor and reduced water power of pork (Khan et al., 2015). Therefore, achieving higher meat quality while pursuing a high yield has become an important focus.

Meat quality is related to a series of complex factors (Taheri-Garavand et al., 2019), including breed (Domingo et al., 2015), rearing conditions (Gagaoua et al., 2017; Uhlířová et al., 2018), animal feeding (Qin et al., 2020), animal characteristics at the moment of slaughter (Maggiolino et al., 2019), preslaughter conditions (Acevedo-Giraldo et al., 2020), and meat processing conditions (Bogdanowicz et al., 2018).

Previous studies have shown that NCG can act as a metabolic activator to promote the production of arginine, which in turn produces NO and polyamines through metabolism. Polyamines can promote cell proliferation and protein synthesis and can increase the retention of nitrogen in the body (Wu et al., 2004; Wu and Morris, 1998). Ye et al. (2017) also reported that NCG supplementation was effective in increasing the longissimus dorsi muscle area, decreasing back fat accretion, and producing functional pork with a high content of leucine without any negative impacts on the muscle fatty acid profile in finishing pigs. Consequently, NCG has attracted attention, because it functions as a feed additive to promote growth performance and meat quality. In the present study, a trend to improve growth performance and meat quality was observed. In addition, NCG supplementation improved the expression of muscle growth- and lipid metabolism-related genes. MyoD is a critical myogenic regulatory factor in muscle development, differentiation and regeneration (Johnson et al., 2021), while HSL, ACC and PGC-1α play key roles in lipid metabolism (Arany, 2008; Ribet et al., 2010). Similarly, a previous study showed that arginine differentially regulates the expression of fat metabolic genes, thus favoring lipogenesis in muscle but lipolysis in adipose tissue. Therefore, it is reasonable to speculate that NCG supplementation may regulate genes related to lipid metabolism in adipose tissue and skeletal

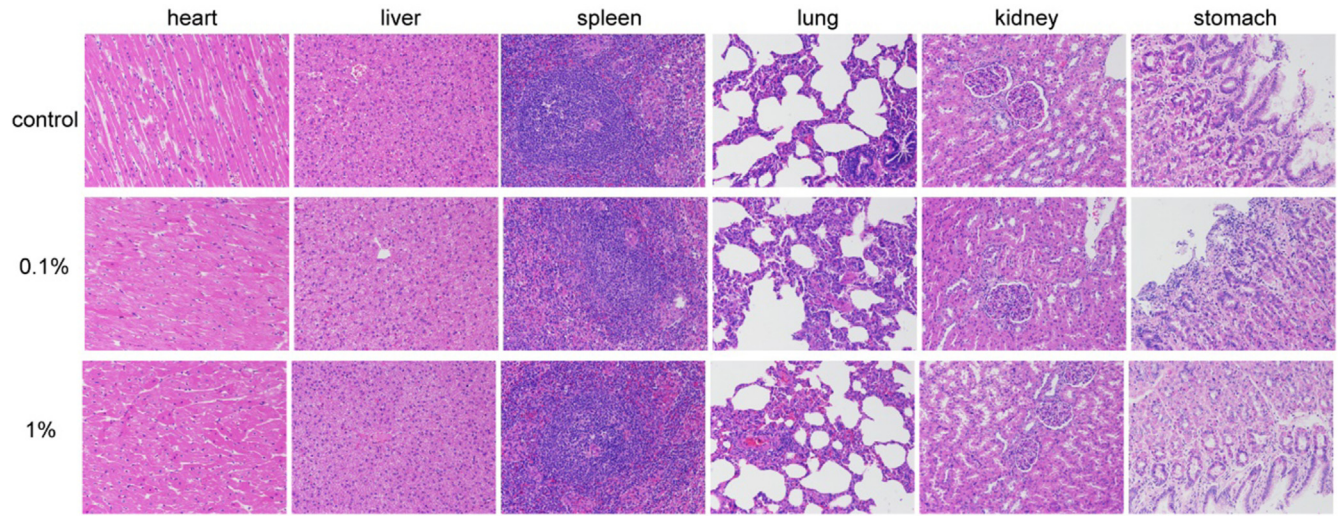

Fig. 1. Photomicrograph of cross section of heart, liver, spleen, lung, kidney and stomach. H&E staining (magnification 200×).

Table 11
Effect of N-carbamylglutamate (NCG) supplementation on serum biochemistry.

Item	NCG, %			SEM	*P*-value
	0	0.1	1		
Day 35					
WBC, 10^9/L	13.53	16.22	11.38	1.62	0.16
RBC, 10^{12}/L	5.90	6.21	5.88	0.31	0.72
Hemoglobin, g/L	102.50	107.33	101.60	5.46	0.74
MCV, fL	58.58	58.70	58.10	0.94	0.91
MCH, pg	17.32	17.25	17.24	0.26	0.98
MCHC, g/L	296.67	295.00	297.40	2.01	0.71
RDW-CV, %	19.98	20.03	20.26	<0.01	0.75
RDW-SD, fL	38.45	38.57	38.82	0.98	0.97
Platelet, 10^9/L	84.80	81.60	78.80	7.78	0.32
Plateletcrit, %	8.67	12.48	6.28	1.74	0.08
PDW, %	14.63	14.95	14.96	0.17	0.33
Day 74					
WBC, 10^9/L	22.57	19.77	21.43	3.22	0.83
RBC, 10^{12}/L	7.38	7.08	6.78	0.17	0.07
Hemoglobin, g/L	136.33[a]	130.33[a]	122.83[b]	2.44	0.01
MCV, fL	61.40	62.07	58.87	1.41	0.27
MCH, pg	18.52	18.40	18.05	0.40	0.70
MCHC, g/L	302.33[ab]	296.67[b]	307.33[a]	2.50	0.03
RDW-CV, %	19.72	20.20	20.13	0.37	0.62
RDW-SD, fL	39.80	41.33	38.72	0.74	0.07
Platelet, 10^9/L	370.17	305.83	355.00	26.82	0.24
Plateletcrit, %	0.32	0.27	0.32	<0.01	0.24
PDW, %	15.17	15.35	15.07	0.22	0.67

WBC = white blood cells; RBC = red blood cells; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RDW = red blood cell distribution width; CV = coefficient of variation; SD = standard deviation; PDW = platelet distribution width.
[a, b] Different letters within a row indicate the significant difference among different treatments ($P < 0.05$), $n = 6$.

Table 12
Effect of N-carbamylglutamate (NCG) supplementation on serum biochemical index.

Item	NCG, %			SEM	*P*-value
	0	0.1	1		
Day 35					
Serum creatinine, μmol/L	111.33	110.67	107.67	4.14	0.80
Blood glucose, mmol/L	4.09	4.23	4.49	0.33	0.69
AST/ALT	0.90	0.96	0.93	0.12	0.95
Alkaline phosphatase, U/L	110.17	128.00	110.83	13.47	0.58
AST, U/L	56.83	47.83	58.50	4.67	0.25
ALT, U/L	60.17[ab]	51.00[b]	66.50[a]	3.69	0.03
Total protein, g/L	66.65	63.02	66.63	1.57	0.20
Albumin, g/L	35.93	36.00	34.90	0.74	0.52
Globulin, g/L	30.72	27.02	31.73	1.81	0.19
A/G	1.19	1.39	1.11	0.10	0.14
Total bilirubin, μmol/L	1.32	1.95	1.05	0.83	0.74
Urea nitrogen, mmol/L	4.70	6.84	5.71	1.05	0.38
C-reactive protein, mg/L	9.10	9.86	12.17	0.97	0.10
Day 74					
Serum creatinine, μmol/L	149.83	156.33	152.00	5.46	0.70
Blood glucose, mmol/L	5.17	5.07	5.22	0.29	0.94
AST/ALT	0.70	0.93	0.80	0.09	0.22
Alkaline phosphatase, U/L	113.33	126.17	91.00	12.89	0.18
AST, U/L	39.33	40.83	34.33	3.33	0.38
ALT, U/L	56.67	45.33	46.83	4.84	0.23
Total protein, g/L	71.58	68.63	71.12	2.11	0.58
Albumin, g/L	37.35	36.40	34.82	0.89	0.16
Globulin, g/L	34.23	32.23	36.30	2.36	0.49
A/G	1.12	1.18	0.99	0.10	0.39
Total bilirubin, μmol/L	2.77	3.97	4.77	1.22	0.52
Urea nitrogen, mmol/L	4.54	5.02	4.54	0.31	0.47
C-reactive protein, mg/L	10.83	14.00	12.80	1.55	0.37

AST = aspartate aminotransferase; ALT = alanine transaminase; A/G = albumin-to-globulin ratio.
[a, b] Different letters within a row indicate a significant difference among different treatments ($P < 0.05$), $n = 6$.

muscle by increasing endogenous arginine, thus increasing muscle weight gain and decreasing body fat content in growing and finishing pigs (Tan et al., 2009, 2011). However, the specific mechanism of action needs further study.

As a structural analog of NAG, NCG functions in a similar manner as NAG. Resistance to cytoplasmic degradation enzymes activates carbamyl phosphate synthase-I (CPS-I) and promotes the synthesis of endogenous arginine (Wu et al., 2004; Ye et al., 2017). In the present study, 0.1% NCG supplementation significantly increased the contents of leucine, isoleucine, methionine, valine, arginine, glutamic acid and citrulline in serum, which indicated the beneficial effects of NCG on amino acid metabolism.

At present, there are few studies on the effect of adding high doses of NCG to the diet of finishing pigs. Previous studies have found that a 10-fold maximum recommended level (3,600 mg/kg) overdose of NCG in Japanese seabass had markedly negative effects with significantly reduced feed efficiency (Hyh et al., 2019). However, in the present study, dietary supplementation with high-dose (approximately 10-fold higher than the recommended dose) NCG had no negative effect on the growth performance of growing-finishing pigs. In addition, the organ index and histology observation results indicate that NCG did not cause damage or adverse

effects on visceral organs. Blood physiological and biochemical indexes can reflect the metabolism and health status of the body (Blood et al., 1983). The present results showed that NCG had no adverse effects on the physiological status of piglets. However, the blood biochemical indicators alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST) and other metabolism-related enzymes could reflect the growth and development of animals to a certain extent (Blood et al., 1983). The results showed that high dose of NCG had no adverse effects on the physiological status of piglets. Therefore, high-dose NCG has no side effects on growing and finishing pigs and has a good safety profile.

5. Conclusion

In conclusion, dietary NCG supplementation could partly improve performance and meat quality, increase specific amino acids metabolism and the MUFA level in muscle, and upregulate the expression level of genes related to muscle growth and lipid metabolism in growing-finishing pigs; thereby improving meat quality and promoting body health. The optimum level of dietary NCG was 0.1% during the overall experimental period to maximize beneficial effects. There were no side effects of high-dose NCG (1%) on the performance, metabolism, tissues and organs of growing-finishing pigs, indicating that NCG is a safe feed additive for growing-finishing pigs.

Author contributions

Chunping Wang: Data curation, Formal analysis, Investigation, Modification; **Lijun Shang**: Writing-Reviewing and Editing, Modification; **Qiuping Guo**: Investigation; **Yehui Duan**: Investigation; **Mengmeng Han**: Investigation; **Fengna Li**: Modification, Supervision; **Yulong Yin**: Modification, Supervision; **Shiyan Qiao**: Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

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

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