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Dietary N-Carbamylglutamate (NCG) alleviates liver metabolic disease and hepatocyte apoptosis by suppressing ERK1/2-mTOR-S6K1 signal pathway via promoting endogenous arginine synthesis in Japanese seabass (*Lateolabrax japonicus*)

H.Y. Huang[a], P. Chen[a], X.F. Liang[a], X.F. Wu[a], C.P.Wang[b], X. Gu[a,**], M. Xue[a,c,d,*]

[a] National Aquafeed Safety Assessment Center, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing, 100081, China
[b] Beijing Animore Sci. & Tech. Co., Ltd, Beijing, 100193, China
[c] Key Laboratory of Feed Biotechnology of Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing, 100081, China
[d] National Engineering Research Center of Biological Feed, 100081, China

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ABSTRACT

N-Carbamylglutamate (NCG), an analogue of N-acetylglutamate (NAG), can promote the synthesis of endogenous Arginine (Arg) in mammals, but not well studied in fish. This study was conducted to investigate the capacity of Arg endogenous synthesis by NCG, and the effects of various dietary NCG doses on growth performance, hepatic health and underlying nutrient regulation metabolism on ERK1/2-mTOR-S6K1 signaling pathway in Japanese seabass (*Lateolabrax japonicus*). Four experimental diets were prepared with NCG supplement levels of 0 (N0), 360 (N360), 720 (N720) and 3600 (N3600) mg/kg, in which N360 was at the maximum recommended level authorized by MOA, China in fish feed, and the N720 and N3600 levels were 2 and 10-fold of N360, respectively. Each diet was fed to 6 replicates with 30 Japanese seabass (initial body weight, IBW = 11.67 ± 0.02 g) in each tank. The results showed that the dietary NCG supplementation had no significant effects on the SGR and morphometric parameters of Japanese seabass, but 360–720 mg/kg NCG inclusion promoted PPV, while the 10-fold (3600 mg/kg) overdose of NCG had remarkably negative effects with significantly reduced feed efficiency, PPV and LPV. We found that Japanese seabass can utilize 360–720 mg/kg NCG to synthesis Arg to improve the amino acid metabolism by increasing plasma Arg and up-regulating intestinal ASL gene expression. Increased plasma GST and decreased MDA indicated the improved antioxidant response. Dietary NCG inclusion decreased plasma IgM and down-regulated the mRNA levels of inflammation (TNF-α and IL8), apoptosis (caspase family) and fibrosis (TGF-β1) related genes in the liver. The immunofluorescence examination revealed significantly decreased hepatic apoptosis and necrosis signals in the NCG groups. The ameliorated liver function and histological structure were closely related to the improved lipid metabolism parameters with decreased plasma VLDL and hepatic TG and NEFA accumulation, down-regulated fatty acid and cholesterol synthesis and simultaneously increased lipolysis gene mRNA levels, which regulated by inhibiting phosphorylation of ERK1/2-mTOR-S6K1 signaling pathway. Consuming 3600 mg/kg of dietary NCG is not safe for Japanese seabass culturing with the significantly increased FCR and decreased protein and lipid retention, and reduced plasma ALB. Accordingly, the observed efficacy and safety level of dietary NCG in the diet of Japanese seabass is 720 mg/kg.

1. Introduction

Arg is an intermediate of the ornithine cycle and can be synthesized from citrulline, and is an essential precursor for the synthesis of biologically important metabolites, including nitric oxide (NO), polyamines, creatine and ornithine [1]. Although the Arg is classified as a semi-essential or conditional essential amino acid (CEAA) in mammals [2], it is an essential amino acid (EAA) in fish and plays a vital role in nutrient metabolism and immunological response on animals [3,4]. The Arg acts the functions depending on the developmental stage, the

* Corresponding author. National Aquafeed Safety Assessment Center, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing, 100081, China.
** Corresponding author.
E-mail addresses: guxu@caas.cn (X. Gu), xuemin@caas.cn (M. Xue).

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health status of the individual and also in terms of feeding habits [5]. Arg is considered an EAA in carnivores mammals, such as cat [6] and ferrets [7], which requires much higher Arg than that of the omnivorous rat, dog and piglet [8,9] and are unable to produce Arg endogenously as they lack the key synthase enzymes, and this is also believed to be the reason for a lack of Arg synthesis in fish [10]. Similarly, carnivorous fish have higher Arg requirements than omnivorous and herbivorous fish [11]. Thus, endogenous synthesis of Arg must play a crucial role in maintaining Arg homeostasis in fish, as reported for human [12], piglets [13] and mice [14]. Furthermore, growing evidence indicates that Arg supplementation may provide novel and effective therapies for obesity and metabolic syndromes, such as the fatty liver and hepatic steatosis [4,15,16]. Additionally, the benefits of optimal dietary Arg had been reported on antioxidant status, immune response improvement, inflammation alleviation and intestinal morphology refining on several fish species, including yellow catfish (*Pelteobagrus fulvidraco*) [17], hybrid striped bass (*Morone saxatilis*) [18], and blunt snout bream (*Megalobrama amblycephala*) [19]. Besides, the Arg deficiency caused growth reduction and poor protein retention, which had been shown in coho salmon (*Oncorhynchus kisutch*), European seabass (*Dicentrarchus labrax*) and Indian major carp (*Cirrhinus mrigala*) [20–22]. However, at present, Arg is too expensive to be used in commercial fish feed, and in some fish, there is a problem of unsynchronized absorption, which interferes with other amino acid metabolism [23]. Consequently, it would be an economical and effective strategy to increase the endogenous Arg biosynthesis in animals.

N-Carbamylglutamate (NCG) as an analogue of N-acetylglutamate (NAG) by activating intestinal pyrroline-5-carboxylic acid synthase and carbamyl phosphate synthase I (the rate-limiting enzymes responsible for both the ornithine cycle and the Arg synthetic pathway) promotes the synthesis of endogenous Arg [24], and this had been confirmed in mammals, including sows, piglets, rats, and ruminants, with enhanced plasma Arg concentration, improved growth performance and antioxidant response, and inhibited inflammation [25–30]. Moreover, compared with the NAG, NCG has the advantages of low cost, high absorption rate, and stable metabolism *in vivo* [31]. However, Only two studies researched the function of NCG in fish and found that mirror carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus*) may have the capability to use NCG to synthesis Arg [32,33]. Mirror carp and Nile tilapia as omnivorous fish are much less fishmeal-rely on species than Japanese seabass. Besides, almost all fish are ammonia excretion animals, and they might own quite different ornithine cycle and Arg synthetic pathway from mammals and birds [34]. Although the full complement of enzymes of the ornithine cycle exists in a number of teleost species, including tench (*Tincatinca*), catfish (*Silurus glanis*), perch (*Perca fluviatils*), rainbow trout (*Oncorhynchus mykiss*) and common sole (*Solea solea*), the activities are expressed during embryogenesis and quickly down-regulated during later life stages [35]. Until now, whether Japanese seabass (*Lateolabrax japonicus*) could utilize NCG to biosynthesis Arg *in vivo* is not confirmed.

The Arg requirement on fish is with very large variation (1.8–3.1% in diet or accounting for 3.8–8.1% of dietary crude protein levels) depending on different developmental stages and feeding habits [36]. Japanese seabass, a eurythermal and euryhaline carnivorous species, is one of the most important species for marine culture in China and other countries in the southeast Asia [37]. Carnivorous fish were fishmeal-rely species, and it is more difficult to use plant proteins than omnivorous and herbivorous fish, and the Arg requirement is higher [38]. At present, there is no report on Arg requirement of Japanese seabass. However, fish muscle amino acid composition can be used as an ideal protein model for the essential amino acid requirements of feed [39]. Lin et al. (2010) and Wang et al. (2003) analyzed muscle amino acid composition of Japanese seabass and found that its arginine content was accounting for 5.27–5.80% of the protein content [40,41]. In the past few decades, in fish feed, lysine and methionine are considered as the first and the second limiting amino acids and were paid much more attention than the dietary Arg deficiency. Arg deficiency induced liver metabolic disease including fatty liver and liver fibrosis [42,43]. Extracellular signal–regulated kinases (ERKs) are widely expressed protein kinase intracellular signaling molecules that are involved in functions including cell proliferation, differentiation, cell death and survival [44]. Bost et al. reported that ERK is necessary for pre-adipocyte differentiation, and that ERK activation promotes the expression of key adipogenesis regulators C/EBPα, β and δ, and PPARγ [45]. mTOR-S6K1 signaling pathway plays a very important regulatory role in metabolic diseases (fatty liver, obesity, diabetes, etc.) [46–48]. Until now, there were no reports of Arg or NCG functions on nutrient metabolism and related disease by regulating the ERK-mTOR-S6K1 pathway in fish. Hence, the objectives of the present study were to investigate the capacity of Arg endogenous synthesis by NCG, and the effects of various doses of dietary NCG on growth performance, hepatic health and underlying nutrient regulation metabolism on ERK1/2-mTOR-S6K1 signaling pathway in Japanese seabass.

2. Materials and methods

During the feeding period, the experimental fish were maintained in compliance with the Laboratory Animal Welfare Guidelines of China (Decree No. 2 of Ministry of Science and Technology, issued in 1988).

2.1. Experimental diets

The tested NCG (97.5%) was supplied by Animore Sci. & Tech. Co., Ltd, Beijing, China, and the protocols were following the technical guidance of tolerance studies of feed additives in target animals (EFSA, 2008) [49]. Four experimental diets were prepared by adding 0 (N0), 360 (N360), 720 (N720) and 3600 (N3600) mg/kg of NCG to a low-Arg basal diet (Arg content is 1.98%, accounting for 4.5% of crude protein levels), in which 360 mg/kg was the maximum recommended level reported in our previous study (Huang et al., 2019, accepted, in press) and authorized by MOA, China in fish feed, and the 720 mg/kg and 3600 mg/kg dose were designed to be 2 and 10-fold of the 360 mg/kg dose for the tolerance test to obtain the safety margin. Each diet was extruded into 2 mm diameter pellets under the following extrusion condition as: feeding section (90 °C/5 s), compression section (150 °C/5 s) and metering section (120 °C/4 s) using a Twin-screwed extruder (EXT50A, YANGGONG MACHINE, Beijing, China). The diet formulation and analyzed chemical compositions are shown in Table 1. The amino acid profile of the experimental diets is shown in Table 2.

2.2. Experimental fish, feeding and sampling

Juvenile Japanese seabass were obtained from Weihai Yulong Aquafarm, Shandong, China. All fish were acclimated in laboratory conditions and fed the control experimental diet with NCG (N0) for 4 weeks before the commencement of the trial. Fish (initial body weight = 11.67 ± 0.02 g) were randomly selected and distributed into tanks after 24 h of starvation with 30 fish per tank (256 L) and six tanks per treatment. The water temperature was maintained at (26 ± 2) °C, pH = 7.5–8.5, dissolved oxygen (DO) > 7.0 mg/L and ammonia nitrogen levels < 0.5 mg/L. Aeration was supplied to each tank 24 h per day, and the photoperiod was 12D: 12 L. Fish were fed to apparent satiation twice daily at 08:00 and 16:00 for 70 days.

The fish from each tank were batch weighed after starvation for 24 h at the end of the growth trial. Twelve fish for each treatment (2 fish from each tank) were randomly selected and anaesthetized with chlorobutanol (300 mg/mL). The body weight, body length, liver, viscera and adipose tissue weight were recorded individually to calculate condition factor (CF), hepatosomatic index (HSI), viscerosomatic index (VSI), and visceral adipose index (VAI) respectively. Blood samples were drawn from the caudal part of the sedated fish using anticoagulant syringes with 2% NaF and 4% potassium oxalate. Blood samples were

Table 1
Formulation and compositions of experimental diets (g/kg).

Ingredients	N0	N360	N720	N3600
Fishmeal[a]	350	350	350	350
Corn gluten meal	100	100	100	100
Soybean meal	30	30	30	30
Wheat Gluten	50	50	50	50
Krill meal	50	50	50	50
Wheat flour	180	180	180	180
Wheat shorts	59	59	59	59
Cassava starch	50	50	50	50
Fish oil	45	45	45	45
Soy lecithin	20	20	20	20
Soy oil	30	30	30	30
Dihydrogen phosphate	18	18	18	18
Vitamin and mineral premix[b]	14	14	14	14
a-cellulose[c]	4	3.64	3.28	0.4
NCG[c]	0	0.36	0.72	3.60
Total	1000	1000	1000	1000
Analyzed chemical composition (g/kg, dry matter basis)				
Moisture	656	671	698	678
Crude Protein	437	434	427	434
Crude Lipid	145	141	146	146
Crude Ash	89.7	88.1	86.6	86.9
Gross Energy (MJ/kg)	22.1	22.3	21.9	21.9

[a] The fishmeal used in the experiment included 15% low-temperature dried fish meal (999, Denmark) and 20% domestic fishmeal (Shandong Chishan Fishmeal Factory, Rongcheng).

[b] Vitamin and mineral premix (mg/kg diets): VA 20 mg, VB_1 10 mg,VB_2 15 mg, VB_6 15 mg,VB_{12} 8 mg, VE 400 mg, VK_3 20 mg,VD_3 10 mg, Niacinaminde 100 mg, Ascorbyl calcium phosphate 1000 mg, inositol 200 mg, Calcium pantothenate 40 mg, biotin 2 mg, folic acid 10 mg, choline chloride 4000 mg, Corn gluten meal 150 mg; CuSO4·5H2O 10 mg, $FeSO_4·H_2O$ 300 mg, $ZnSO_4·H_2O$ 220 mg, $MnSO_4·H_2O$ 25 mg, 3 mg, Na_2SeO_3 5 mg, $CoCl_2·6H_2O$ 5 mg, $MgSO_4$ 2000 mg, Zeolite 332 mg.

[c] a-cellulose and NCG were premixed with vitamin and mineral premix.

Table 2
Amino acids content of experimental diets (g/kg, dry matter basis).

	N0	N360	N720	N3600
Essential amino acids				
Arginine	19.8	19.3	19.2	19.3
Histidine	9.30	9.00	9.00	9.00
Isoleucine	16.7	16.2	16.1	16.3
Leucine	33.7	32.8	32.8	33.0
Lysine	21.9	21.2	21.2	21.3
Methionine	9.50	9.20	9.20	9.30
Phenylalanine	17.5	17.1	17.0	17.1
Threonine	14.8	14.4	14.4	14.5
Tryptophan	4.00	4.0 = 0	4.00	4.00
Valine	19.4	18.9	18.8	18.9
Non-essential amino acids				
Asparagic acid	31.9	31.0	31.1	31.2
Cysteine	4.70	4.60	4.70	4.70
Glutamic acid	73.1	71.0	72.0	72.6
Glycine	18.5	18.0	18.1	18.1
Proline	24.5	23.8	24.0	23.8
Serine	16.5	16.3	16.2	16.3

centrifuged at 4000 rpm for 10 min at 4 °C to obtain plasma. Two liver samples from each tank were collected for histology (fixed in 4% paraformaldehyde fixative), and the liver and distal intestine tissues from the same fish were sampled and fast frozen in liquid nitrogen for RNA isolation. All samples were stored at −80 °C until analysis.

2.3. Chemical analysis

All chemical analyses of the diets and liver samples were carried out in duplicate according to AOAC (2006). The dry matter was analyzed by drying the samples to a constant weight at 105 °C. Crude protein (CP) was determined using a Kjeltec™ 2300 Unit (Foss, Hillerød, Denmark) by the method of Kjeldahl, and the CP content was estimated by multiplying nitrogen by 6.25. Crude lipid was analyzed by acid hydrolysis with a Soxtex System HT 1047 Hydrolyzing Unit (Foss, Hillerød, Denmark), followed by Soxhlet extraction using a Soxtex System 1043 (Foss, Hillerød, Denmark). Ash was analyzed by combustion in a muffle furnace (CWF1100, Carbolite, Derbyshire, UK) at 550°Cfor 16 h. Gross energy was determined using an IKAC2000 Calorimeter (C2000, IKA, Staufen, Germany). The free amino-acid concentrations in the plasma were analyzed by Automatic amino acid analyzer (S-433D, Sykam, Germany).

2.4. Hematological and liver homogenate parameters

Hematological parameters, including plasma TC (total cholesterol), TG (triglyceride), HDL-C (high density lipoprotein cholesterol), LDL-C (low density lipoprotein cholesterol), VLDL (very low-density lipoproteins), ALT (alanine aminotransferase), AST (aspartate aminotransferase), Ammonia, IgM (Immunoglobulin M), NO (nitric oxide), SOD (Superoxide dismutase), MDA (Malondialdehyde), T-AOC (Total antioxidative capability), CAT (Catalase), GST (glutathione S-transferase), GSH-Px (Glutathione peroxidase) were determined by assay kits (Nanjing jiancheng Co., Nanjing, China) following the protocols given by the supplier. Hepatic non-esterified fatty acid (NEFA) were measured by assay kits of Wako Pure Chemical Industries, Ltd. (Japan).

2.5. Histopathological and immunofluorescence examination of the liver

After 24 h of fixation, all liver samples were dehydrated by the standard procedures, and the samples were embedded in paraffin and cut to 6 μm sections. Liver sections were stained following the protocols of hematoxylin and eosin (H&E) staining and observed by light microscopy (Leica DM2500, Leica, Solms, Germany).

The immunofluorescence test for activated caspase-3 was as follows. Liver sections were deparaffinized, rehydrated and rinsed with PBST (0.1% Tween-20 in phosphate buffered saline, PBS). Antigen retrieval was obtained by maintaining slides in citrate antigen retrieval solution (pH 6.0) in a pressure cooker for 10 min. Sections were blocked 30 min by serum-free blocking buffer (Dako, USA). Sections were incubated with the polyclonal cleaved caspase-3 antibody (Abcam, Shanghai, China; dilution 1:300) overnight at 4 °C. After washing with PBST, sections were incubated with Alexa Flour 555 goat anti-rabbit antibody (Life Technology, USA) for 1 h at room temperature. After 3 washes with PBST, sections were mounted with anti-fade mounting medium that contains DAPI (VECTASHIELD, Vector Laboratories, H-1200) for nuclei staining. The terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was performed following the manufacturer's instructions (One Step TUNEL Apoptosis Assay Kit; Beyotime Biotechnology, China). The fluorescent signal was captured using a confocal microscope (Zeiss LSM700, Germany) in merge format [50].

2.6. RNA isolation, reverse transcription and mRNA level analysis

Total RNA was isolated from liver tissue and distal intestine using miRNA easy Mini kit (QIAGEN Sciences, MD, USA), spectrophotometrically quantified using a NanoDrop 2000 (Thermo, USA) and electrophoresed on a 1% denaturing agarose gel to test the integrity. For each reverse transcription reaction, 1.0 μg of total RNA was first treated with gDNA Eraser to remove genomic DNA contaminants and was then subjected to cDNA synthesis by reverse transcription in a 20 μL volume using aniScript cDNA Synthesis Kit (Bio-Rad, USA).

The core fragment of all the genes was obtained from the database of RNA-seq of Japanese seabass. EF1α (GenBank accession no. JQ995147), a housekeeping gene whose expression was found to be unaffected by the treatment in the present experiment, was used as an

Table 3
Primer sequences for real-time PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Products length (bp)	TM(°C)	E-Value (%)
ACC 1	AATCAACATCCGCCTGACTCCAAC	CCTGCTTGTCTCCGTATGCTTGG	176	59.0	90.2
ASL	TCAGTCCTGACATGTTGGCC	TGTACTGCTCCACACTGCTG	222	57.6	92.2
ASS	CCCAGGAGGCACAATTCTGT	CGCACAAAATCACACTCCGG	153	57.6	93.8
ATGL	CTTCCTCTCCGCAACAAGTC	TGGTGCTGTCTGGAGTGTTC	211	55.8	100.0
Caspase-3	ATCACAGCAACTACGCCTCATTCG	GCCTCTGCAAGCCTGGATGAAG	176	61.6	98.9
Caspase-8	AAGACGCATCTGTTCGCTTCCTG	GCGACAGCTTCAGCCTATCCATC	116	61.6	98.9
Caspase-9	TGCGGAGGAGGTGAACGAGAC	CGGTTCGTCGGACATGCTCAG	138	62.8	90.5
C/EBP α	CACGGACAACGACAGACTGA	GCCACACACCAACTCACGTA	213	60.5	93.4
C/EBP β	TCTGATTCCAGGGTGTCCTC	TCATCTGCTCAGCCACTCTG	181	57.1	98.8
CPS-I	GAGACACCAGTTTGCCCAGT	ATTTGTGATGAGGGGGACAC	152	58.0	95.3
EF1α	AATCGGCGGTATTGGAACTG	TCCACGACGGATTTCCTTGA	205	58.5	102.0
ERK	GACATTATCCGCACGCCAAC	AGATGTGGTCGTTGCTCAGG	118	57.5	104.0
FASN	AGGCATTGTGGAGGGTGTAG	CCAGTCCACCAGTGATGATG	233	56.8	97.1
HMGCR	GGAAGAGGAAGAGGACAACAAGCC	GAACCATGACCAGGCCAAGCG	80	56.6	101.0
HSL	TGATGTTTGCCAAGAAGCTG	CTGATGGACTGGTGCTCTGA	228	57.8	93.8
IL1β	CTGAACATCAAGGGCACAGA	GTTGAAGGGGACAGACCTGA	192	60.8	92.8
IL8	GAGCTGATTCCTGCCAACTC	CCGATCTGTTCAGGGTGTTC	153	55.8	98.0
P5CS	TGAGATTCTGCTGTCCCAGA	GCAAGTGCTGTATTGGGTGA	163	60	91.0
PPAR α	GACAACGATGCCCTCAGCTC	CGGCACAAACTCGACACTCA	193	58.0	92.7
PPAR γ	AGGCCTGCTGAATGTGAAGC	GCTGGATGAAGTGGACGTGG	170	58.0	93.3
TNF-α	GACTCCATAGGCAGCAAAGC	AGAAAGTCTTGCCCTCGTCA	205	60.8	103.2
TGF-β1	GCAGGCAGTGAAAGAAAAGG	CTAATGGCCTCAATGCGTTT	200	63.9	98.5

ACC 1: acetyl-CoA carboxylase 1; ASL: Argininosuccinate Lyase; ASS: argininosuccinate synthetase; ATGL: adipose triglyceride lipase; Caspase: cysteine-aspartic proteas; C/EBP: CCAAT-enhancer-binding protein; CPS-I: Carbamoyl-Phosphate Synthase 1; EF1α: eukaryotic translation elongation factor 1 alpha; ERK: extracellular regulated protein kinases; FASN: fatty acid synthase; HMGCR: 3-Hydroxy-3-Methylglutaryl-CoA Reductase; HSL: hormone-sensitive triglyceride lipase; IL: interleukin; P5CS: pyrroline-5-carboxylate synthetase; PPAR: peroxisome proliferator activated receptor; TNF-α: tumor necrosis factor; TGF-β1: transforming growth factor-β1.

endogenous reference to normalize the template amount. Specific primers of these genes were designed according to the partial cDNA sequences of these genes using the Japanese seabass transcriptome analysis shown in Table 3. RT-qPCR analysis was performed using a CFX96TM Real-Time System (Bio-Rad, USA) in a 20 μL reaction volume containing iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA).

Serial dilutions of cDNAs generated from liver tissues were used to generate a standard curve to determine the amplification efficiency (E-values) of target and reference genes. The E-values ranged from 90% to 110% (Table 3). The RT-PCR temperature profile for all genes was 95 °C for 30 s followed by 40 cycles of 10 s at 95 °C, 30 s at Tm (Table 3) and 40 s at 72 °C. After the final cycle of PCR, the melting curves were systematically monitored (65 °C temperature gradient at 0.05 °C/10 s from 65 to 95 °C). During the detection, each sample was run in triplicate. PCR-grade water in place of the template served as the negative control. The expression values were calculated as $(1 + \text{E-values})^{-\Delta\Delta Ct}$ [51].

2.7. Western blotting

The western blotting experiment method is as follows [52]: liver tissues were homogenized in RIPA buffer (Beyotime, China) with an added protease inhibitor (Roche, Germany) and phosphatase inhibitor (Roche, Germany). The protein concentration was measured using a BCA Protein Quantification Kit (Beyotime, China). Protein extracts were run on gels in SDS-PAGE electrophoresis buffer (Beyotime, China) and blotted onto nitrocellulose membranes (Millipore, USA). Ponceau S staining (Beyotime, China) was used to observe the effect of transfer film. After blocking for 2 h at room temperature, immunoblots were incubated overnight at 4 °C in primary antibodies, namely, anti-ERK1/2 (Cell Signal Technology, USA), anti-P-ERK1/2 (GENE TEX, USA), anti-mTOR (Cell Signal Technology, USA), anti-P-mTOR (Cell Signal Technology, USA), anti-S6K1 (Cell Signal Technology, USA), anti-P-S6K1 (Cell Signal Technology, CST, USA) and anti-GAPDH (Goodhere, China). Blots were then incubated for 1 h in goat anti-rabbit IgG-HRP secondary antibody (CWBIO, China). Proteins were detected using enhanced chemiluminescence (ECL) (Millipore, USA). Quantification was performed using Image J software.

2.8. Statistical analysis

Statistical differences between two groups were analyzed by the unpaired student's *t*-test and differences between multiple groups of data were analyzed by one-way ANOVA. Homogeneity of variance was confirmed by Levene's test before ANOVA and differences among the means were analyzed by Duncan's multiple-range test using SPSS Statistics 17.0 (IBM. Inc. USA). $P < 0.05$ was considered statistically significant. The graphics were drawn using GraphPad Prism 6.0 (GraphPad Software Inc. USA).

3. Results

3.1. Growth performance, morphometric parameters and whole-body composition

The results of growth performance and morphometric parameters are presented in Table 4. All groups showed high survival rate (> 97.5%), but the survival rate of the N3600 group was significantly lower than other groups ($P < 0.05$). With the increased NCG intake, SGR was no difference among groups ($P > 0.05$), 360 and 720 mg/kg NCG inclusion significantly improved PPV, but fish fed 3600 mg/kg NCG increased FR and FCR and reduced PPV and LPV significantly ($P < 0.05$). No significant difference was observed among the four groups in VSI, HSI, CF, VAI and whole-body composition ($P > 0.05$). The lipid content in liver tissue was decreased significantly in the N360 group and N3600 group compared with the N0 group ($P < 0.05$).

3.2. Liver function, plasma immunology and anti-oxidation responses

In Table 5, the liver function biomarkers, including ALT, AST, TP and ALB were stable in N0, N360 and N720 groups, but fish in the N3600 group showed significantly lower plasma ALB ($P < 0.05$). Compared with N0 group, plasma ammonia in N360 and N720 groups were significantly decreased ($P < 0.05$). The immunological

Table 4
Effects of dietary NCG on the growth performance and morphometric parameters in Japanese seabass (means ± SEM, n = 12)[a].

	N0	N360	N720	N3600
Growth performance				
Survival (%)[b]	100 ± 0.00[b]	99.2 ± 0.83[b]	100 ± 0.00[b]	97.5 ± 0.83[a]
FBW (g/fish)[c]	91.0 ± 0.50	89.1 ± 1.82	89.5 ± 0.71	90.6 ± 1.76
SGR (%/d)[d]	3.02 ± 0.01	2.99 ± 0.03	2.99 ± 0.04	3.01 ± 0.02
FR (%bw/d)[e]	2.09 ± 0.01[a]	2.08 ± 0.01[a]	2.10 ± 0.01[a]	2.14 ± 0.01[b]
NCG intake (mg/kgbw/d)[f]	/	7.48 ± 0.05[a]	15.1 ± 0.05[b]	76.8 ± 0.55[c]
FCR[g]	0.92 ± 0.00[a]	0.92 ± 0.00[a]	0.93 ± 0.00[a]	0.95 ± 0.00[b]
PPV (%)[h]	42.5 ± 0.39[b]	44.8 ± 0.59[c]	44.1 ± 0.59[c]	35.7 ± 0.04[a]
LPV(%)[i]	90.8 ± 3.68[b]	96.2 ± 1.76[b]	89.8 ± 2.18[b]	78.2 ± 1.73[a]
Morphometric parameters				
VSI (%)[j]	13.3 ± 0.58	12.4 ± 0.32	12.7 ± 0.37	12.3 ± 0.28
HSI (%)[k]	1.83 ± 0.09	1.75 ± 0.12	1.81 ± 0.13	1.79 ± 0.17
CF (g/cm^3)[l]	1.33 ± 0.02	1.31 ± 0.02	1.26 ± 0.04	1.34 ± 0.03
VAI (%)[m]	7.49 ± 0.35	7.22 ± 0.27	7.56 ± 0.27	7.19 ± 0.22
Whole body composition (g/kg, dry matter basis)				
Crude Protein	156 ± 23.0	161 ± 18.0	161 ± 17.0	158 ± 11.0
Crude Lipid	112 ± 36.0	116 ± 17.0	113 ± 28.0	119 ± 27.0
Moisture	681 ± 41.0	682 ± 21.0	677 ± 35.0	675 ± 28.0
Crude Ash	40.5 ± 10.0	422 ± 12.0	420 ± 10.0	389 ± 10.0
Liver tissue (g/kg, on wet basis)				
Lipid content	42.9 ± 0.40[b]	35.7 ± 3.40[a]	41.0 ± 1.80[ab]	34.1 ± 1.30[a]

"/" means: not intake.
[a] Values (mean ± SEM) in the same row with different superscript letters are significantly different ($P < 0.05$).
[b] Survival, % = 100 × final fish number/initial fish number.
[d] SGR (specific growth rate, %/d) = 100 × [Ln (FBW) - Ln (IBW)]/days. FBW is final body weight, IBW is initial body weight.
[e] FR (feeding rate, %) = 100 × feed intake/[(Wf + Wi + Wd)/2]/days. Wf is the final total weight, Wd is the total weight of dead fish, Wi is the initial total weight. The same below.
[f] NCG intake (mg/kg bw/d) = NCG contents × feed intake/[(Wf + Wi + Wd)/2]/days.
[g] FCR (feed conversion ratio) = feed intake/(Wf + Wd - Wi).
[h] PPV (Protein productive value, %) = 100 × (final total weight × terminal fish protein content - initial total weight × initial fish protein content)/(total food intake × feed protein content).
[i] LPV (Lipid productive value, %) = 100 × (final total weight × terminal fish fat content - initial total weight × initial fish lipid content)/(total food intake × feed lipid content).
[j] VSI (viscera somatic index, %) = 100 × viscera weight/whole body weight.
[k] HSI (hepatosomatic index, %) = 100 × liver weight/whole body weight.
[l] CF (condition factor, g/cm^3) = 100 × (body weight, g)/(body length, cm) 3.
[m] VAI (visceral adipose index, %) = 100 × visceral adipose weight/whole body weight.

indicators, plasma IgM was significantly reduced in NCG inclusion groups and lowest in N360 and N720 groups. Plasma NO level in the N360 group was significantly higher than that in the N720 group but no difference with the N0 group. The antioxidant response was also improved by NCG inclusion. There was no significant difference in plasma T-AOC, GST-PX and SOD among groups ($P > 0.05$). But, 720 mg/kg dietary NCG improves antioxidant responses by decreasing plasma MDA concentration and increasing the GST level.

3.3. Hepatic inflammatory and apoptosis/necrosis responses along with histological analysis

In Fig. 1A, four typical phenotypes are observed on the hepatic samples of the Japanese seabass. Four phenotypes of hepatic histopathological examination with symtom from light to severe by H&E staining for histology examination, apoptosis signals of cleaved caspase-3 (red color signal), and TUNEL (TdT-mediated dUTP Nick-End Labeling) assay (green color signal) for cell apoptosis and tissue necrosis

Table 5
Effects of dietary NCG on Hematological parameters of Japanese seabass (means ± SEM, n = 8).

	N0	N360	N720	N3600
Liver function				
ALT (U/L)	1.16 ± 0.36	1.53 ± 0.55	1.67 ± 0.20	3.18 ± 0.89
AST (U/L)	7.34 ± 1.06[ab]	6.84 ± 1.25[a]	6.42 ± 1.02[a]	10.97 ± 2.63[b]
TP (g/L)	24.9 ± 1.11	24.0 ± 0.63	24.6 ± 1.25	22.5 ± 0.96
ALB (g/L)	18.4 ± 0.26[b]	17.7 ± 0.63[ab]	18.5 ± 0.48[b]	16.6 ± 0.81[a]
Ammonia (μmol/L)	315 ± 32.6[b]	232 ± 12.4[a]	217 ± 26.3[a]	279 ± 26.3[ab]
Immunological biomarkers				
IgM (g/L)	5.11 ± 1.68[b]	1.11 ± 0.38[a]	1.12 ± 0.29[a]	2.34 ± 1.13[ab]
NO (μmol/L)	4.67 ± 0.69[ab]	5.1 ± 0.64[b]	3.74 ± 0.74[a]	4.54 ± 1.74[ab]
Antioxidant responses				
T-AOC (U/mL)	11.9 ± 0.52	12.4 ± 1.13	13.6 ± 0.29	14.7 ± 1.51
GST (U/mL)	52.3 ± 3.69[a]	57.0 ± 1.29[ab]	59.6 ± 0.33[b]	59.8 ± 0.52[b]
GSH-PX (U/mL)	94.7 ± 13.1	113 ± 9.10	118 ± 13.4	98.5 ± 10.8
SOD (U/mL)	9.99 ± 0.12	10.6 ± 0.31	10.3 ± 0.26	10.3 ± 0.27
MDA (nmol/mL)	33.6 ± 2.43[bc]	29.8 ± 1.71[ab]	27.7 ± 1.65[a]	37.7 ± 1.39[c]

Values (mean ± SEM) in the same row with different superscript letters are significantly different ($P < 0.05$).

with DNA damage and DAPI for nucleus, in which, (I): no obvious abnormity phenotype with well shaped hepatocytes and clearly located cell nuclei, and with the negative response to cleaved caspase-3 and TUNEL signals. (II): with fatty liver or steatohepatitis phenotypes, which indicated intense vacuoles in the hepatocytes resembling lipid, mildly activated caspase-3, but negative to TUNEL signal. (III): Hepatocyte nuclear dense phenotype, with unclear liver cord, which is usually a precursor to liver fibrosis in the clinic with highlighted cleaved caspase-3 and clear TUNEL signal. (IV): severe vacuolation along with hepatic fibrosis symptoms, along with intensive apoptosis (cleaved caspase-3) and necrosis signal (TUNEL) around the fibrosis tissues. The hepatic histopathological examination results of each group are shown in Fig. 1B. All NCG inclusion groups showed refined liver health phenotype than that in N0 group. In the N0 group, there were only two samples were generally normal (phenotype I), six with fatty liver (phenotype II), one sample showed a dense cell nucleus and (phenotype III), and three with severe vacuolation cells along with fibrosis symptoms (phenotype IV). All dietary NCG inclusion groups showed improved phenotypes of hepatic health and best in fish fed N720 diet, in which five samples were observed as no obvious abnormity (phenotype I), five samples appeared in fatty liver (phenotype II), no phenotype III sample and only two samples with lightly fibrosis symptom. We also observed positively modified hepatic health phenotypes with the significantly down-regulated mRNA levels of those genes related inflammatory cytokines (TNFα, IL1β, IL8 and TGFβ1) and cell apoptosis (caspase family) (Fig. 1C and D). In general, dietary NCG inclusion significantly improved liver health with lower down-regulated inflammatory and apoptosis responses.

3.4. Endogenous synthesis of Arg

The results of mRNA expression of Arg synthesis related genes in intestinal of Japanese seabass are shown in Fig. 2. The mRNA expression of ASL-the key synthesis enzyme of Arg in N720 group was increased compared with other groups ($P < 0.05$). Moreover, plasma Arg content in N360 and N720 groups increased significantly compared N0 group ($P < 0.05$), and ornithine in plasma of N720 group was also significantly increased (Table 6), which indicated that Japanese seabass could utilize NCG to synthesis Arg *in vivo*. Concentrations of all other measured plasma free amino acids were no difference among the four groups of Japanese seabass.

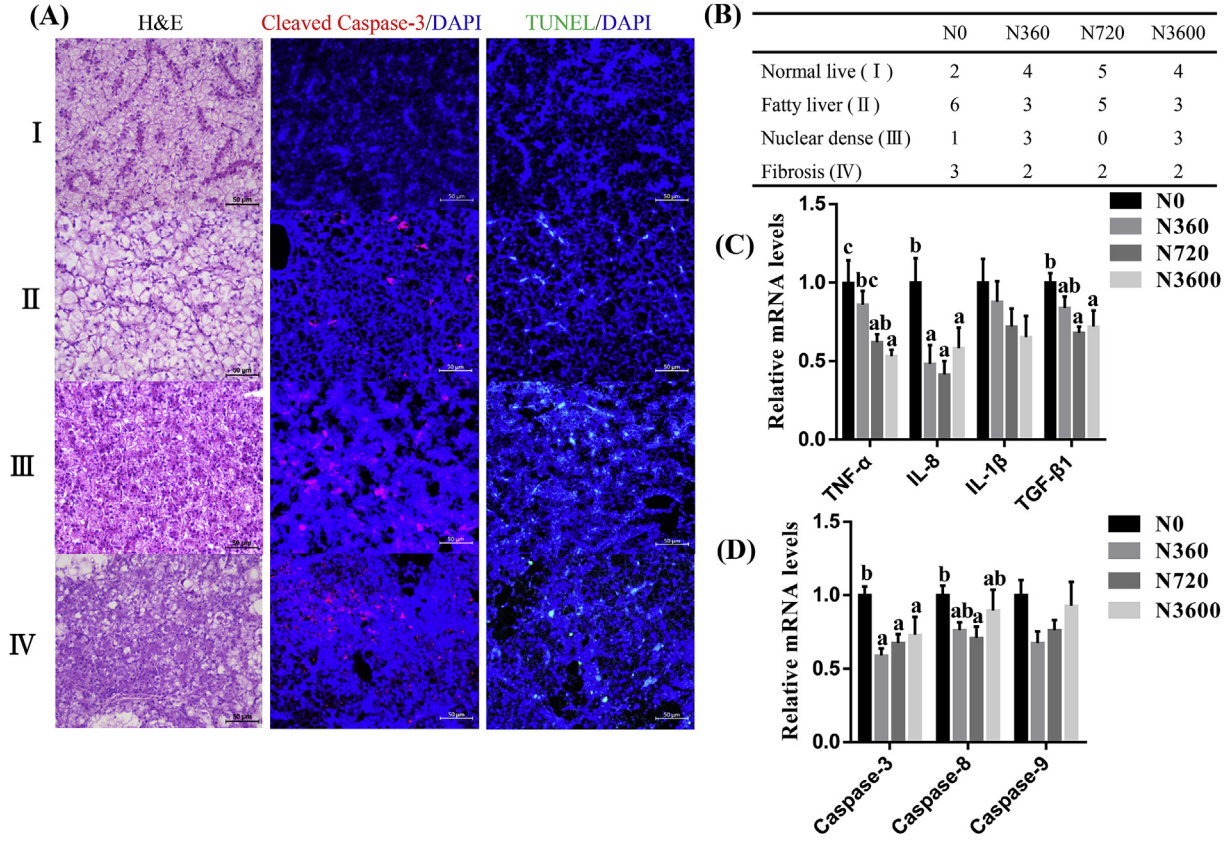

Fig. 1. Dietary NCG improved hepatic health by reducing inflammatory and apoptosis responses. A: Histopathological analysis of Japanese seabass. Four phenotypes of hepatic histopathological examination with symptom from light to heavy by H&E staining for histology examination; apoptosis signals of cleaved caspase-3 in red color; TUNEL (TdT-mediated dUTP Nick-End Labeling) assay for cell apoptosis and tissue necrosis with DNA damage in green color and DAPI for nucleus (bar = 50 μm, n = 12), in which, (I): no obvious abnormity. (II): fatty liver or steatohepatitis. (III): Nuclear dense, unclear liver cord. (IV): severe vacuolation along with hepatic fibrosis symptoms. (B): Phenotypes statistical results (n = 12). (C): The mRNA levels of inflammatory cytokines (TNF-α: tumor necrosis factor α; IL-8: interleukin 8; IL-1β: interleukin 1β; TGF-β1: transforming growth factor β1; n = 8). (D): The mRNA levels of caspase family genes (caspase-3, caspase-8 and caspase-9, n = 8). Values are means with their standard errors. Values marked with different letters are significantly different ($P < 0.05$, mean ± SEM, n = 8). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

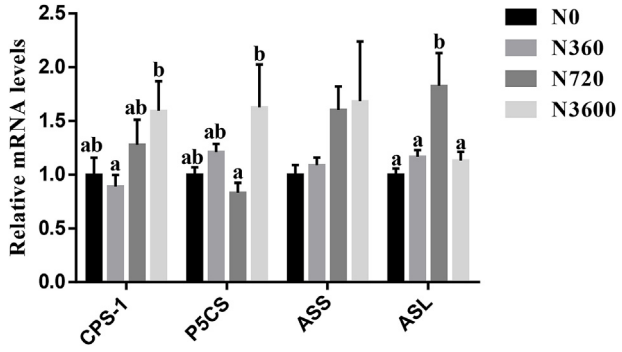

Fig. 2. Endogenous arginine synthesis related genes expression in intestine of Japanese seabass. The key enzyme of arginine synthesis-ASL was up-regulated in N720 group. Values having different letters are significantly different ($P < 0.05$, mean ± SEM, n = 8).

Table 6
Free amino acids content on plasma of Japanese seabass (mg/kg, means ± SEM, n = 3).

	N0	N360	N720	N3600
amino acids in ornithine cycle				
Arginine	97.1 ± 10.4^{a}	185 ± 29.5^{b}	150 ± 2.08^{b}	76.9 ± 3.73^{a}
Ornithine	0.90 ± 0.10^{a}	0.60 ± 0.10^{a}	2.00 ± 0.70^{b}	0.90 ± 0.10^{a}
Citrulline	2.40 ± 0.10	2.90 ± 1.00	1.20 ± 0.50	3.20 ± 0.80
Glutamate	10.6 ± 1.30	22.7 ± 9.60	15.8 ± 6.80	10.6 ± 0.90
Proline	15.2 ± 2.80^{a}	12.6 ± 2.80^{a}	13.6 ± 4.90^{a}	26.8 ± 2.00^{b}
Other essential amino acids				
Histidine	10.2 ± 0.50	9.70 ± 1.60	10.2 ± 0.70	10.8 ± 1.20
Isoleucine	15.3 ± 1.10	14.4 ± 1.60	15.3 ± 3.90	15.2 ± 1.80
Leucine	40.9 ± 4.10	49.5 ± 12.9	41.7 ± 11.8	39.5 ± 4.40
Lysine	119 ± 25.2	250 ± 94.4	161 ± 74.7	98.0 ± 4.90
Methionine	5.50 ± 1.70	7.90 ± 3.90	3.80 ± 1.10	7.50 ± 2.00
Phenylalanine	18.4 ± 0.80	17.4 ± 2.80	17.5 ± 1.70	19.5 ± 2.80
Threonine	1.10 ± 0.40	0.50 ± 0.20	3.50 ± 2.70	0.50 ± 0.10
Tryptophan	3.90 ± 0.10	4.20 ± 1.50	5.50 ± 0.30	3.90 ± 1.10
Valine	27.2 ± 2.30	28.3 ± 5.80	29.3 ± 1.30	27.8 ± 3.40

Values (mean ± SEM) in the same row with different superscript letters are significantly different ($P < 0.05$).

3.5. NCG inclusion in diet alleviated liver metabolic disease and improved lipid metabolism by regulating ERK1/2-mTOR-S6K1 pathway

In Fig. 3A, the significantly reduced hepatic TG and NEFA contents were observed in all NCG inclusion groups ($P < 0.05$). Besides, plasma VLDL in N360 group was significantly decreased compared with N0 group ($P < 0.05$) (Fig. 3B). The mRNA levels of lipid metabolism related genes in liver tissues were shown in Fig. 3C. Fish feed NCG improved lipid metabolism with down-regulated lipogenesis (FASN), cholesterol synthesis (HMGCR), and adipocyte differentiation gene expression (C/EBPα), and simultaneously up-regulated lipolysis (ATGL) gene mRNA level in N720 group ($P < 0.05$). There was no significantly different on ACC1, PPAR γ and C/EBPβ mRNA levels ($P > 0.05$).

Western blot analysis showed that total and phosphorylated ERK1/2

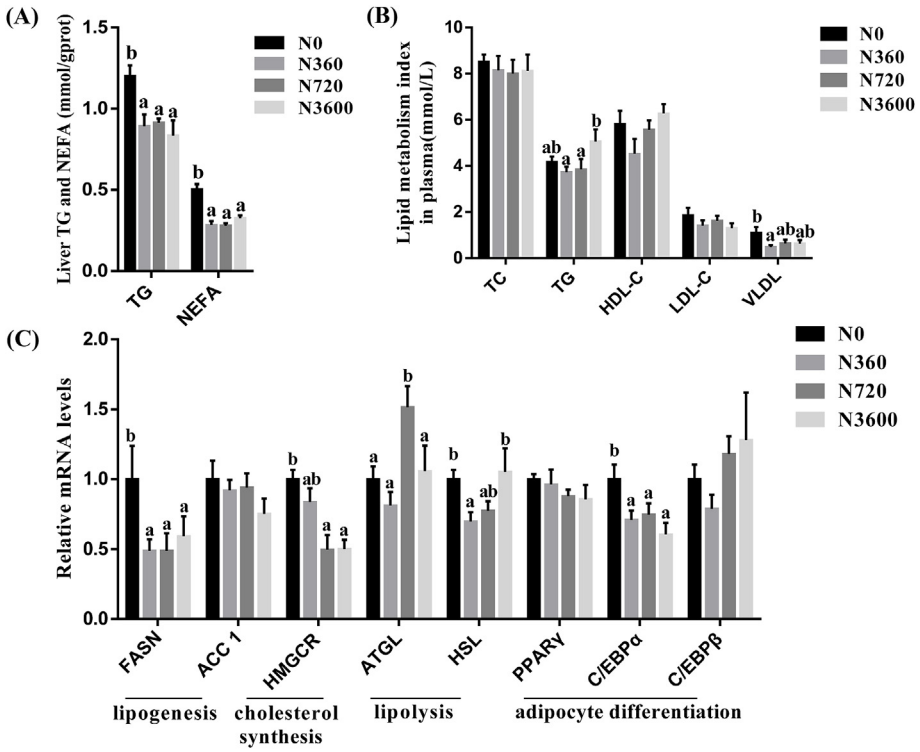

Fig. 3. Lower lipid accumulation in liver with NCG inclusion in Japanese seabass diet, with down-regulated lipogenesis, adipocyte differentiation and cholesterol synthesis genes expression and up-regulated lipolysis gene mRNA levels. (A): Hepatic TG, NEFA concentration (mmol/gprot); (B): Plasma lipid metabolism index (mmol/L); (C): The mRNA levels of lipid metabolism related genes in liver tissues; Values having different letters are significantly different ($P < 0.05$, mean ± SEM, n = 8).

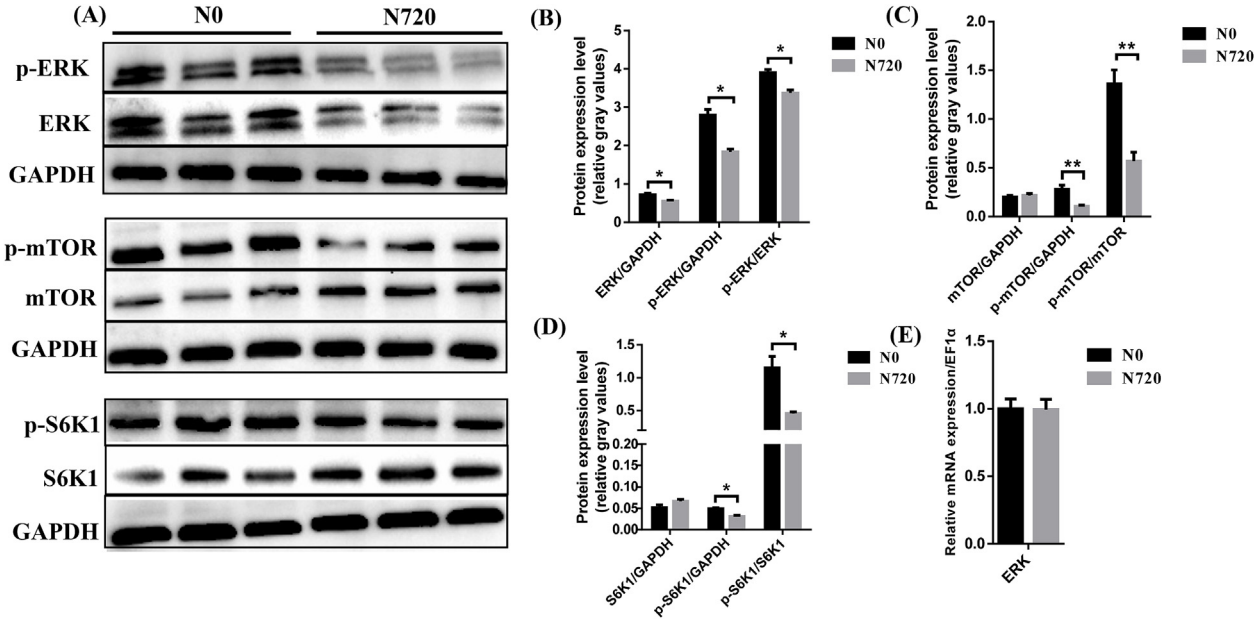

Fig. 4. Dietary NCG inhibited the ERK1/2-mTOR-S6K1 protein phosphorylation in liver tissue. Western blot results of total and phosphorylated (p-) ERK1/2, mTOR and S6K1 protein (A); The grayscale analysis of ERK 1/2 and *p*-ERK (B), mTOR and *p*-mTOR (C), S6K1 and p-S6K1 (D); The mRNA levels of ERK (E); $*P < 0.05$, $**P < 0.01$ indicates a significant difference between two groups.

protein expression in N720 group was significantly inhibited compared with the N0 group (Fig. 4A, B). Simultaneously, phosphorylation of mTOR and down-stream S6K1 protein in the liver were significantly attenuated by NCG treatment in N720 group (Fig. 4A, C, 4D). These data suggest that dietary NCG inhibited the activities of ERK1/2-mTOR-S6K1 signaling pathway to regulate the lipid metabolism. There was no significant difference in the ERK mRNA level ($P > 0.05$) (Fig. 4E), presuming that the ERK translation level was inhibited or promoted protein degradation by NCG supplementation.

4. Discussion

Arg plays an important role in cell proliferation, wound healing, removing ammonia from the body, immune function, and the release of hormones in animals, including teleost [53]. The requirement for Arg varies greatly from 3.8 to 8.1% of crude protein between fish at different developmental stages and different feeding habits [36]. Until now, there is no report on Arg requirement of Japanese seabass. However, Lin et al. (2010) and Wang et al. (2003) analyzed muscle amino acid composition of Japanese seabass and found that its arginine

content was accounting for 5.27–5.80% of the protein content [40,41]. In the present study, although we did not find significant effects of dietary NCG inclusion on the SGR and morphometric parameters of Japanese seabass, 360–720 mg/kg NCG inclusion promoted PPV and decreased FCR, which indicated that the basal Arg level (Arg content is 1.98%, accounting for 4.5% of crude protein levels) didn't meet the requirement in Japanese seabass. The 10-fold (3600 mg/kg) overdose of NCG has remarkably negative effects with increased FCR and decreased PPV and LPV in Japanese seabass. The positive responses of animals with optimal level of dietary Arg had been widely reported, including growth performance and meat quality ameliorating, nutrient retention, antioxidative capacity and immune responses improvement, and so on in fish [54–56], and even on the mammals, such as pigs [57], Lambs [28] and rats [58], which has capability of endogenous synthesis of Arg. Wu et al. (2004) reported that NCG activates *in vivo* synthesis of Arg, and it was widely studied on mammals and poultry [59]. In mammals, supplementation with NCG is an economic and effective way to enhance piglets growth and protein deposition [26,60]. Moreover, Huang et al. (2015) reported that 4000 mg/kg NCG supplementation could promote growth performance and increase the contents of plasma Arg in Yellow Hair Chicken [30].

Different from the mammal and bird, fish is ammonia excretion animal and it's ornithine cycle could be very specific [61]. Arg is not EAA for mammals, but definitely is essential for fish because of the deficiency in endogenous synthesis capability, and the capability of fish utilizing NCG to synthesis Arg is not well certificated yet. The Arg synthesis pathway occurs in various organs, such as the small intestine, kidneys and fewer in liver. It is now generally accepted that endogenous synthesis of Arg in humans, pigs, dogs, sheep and rats involves the intestinal–renal axis in which citrulline released by the small intestine is converted into Arg via argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) [62,63], and the *de novo* synthesis of Arg is transported to the liver for catabolism [34]. Enteric citrulline is funneled into renal tissue or used for enterocyte conversion into Arg [64]. Although the full complement of enzymes of the ornithine cycle have been shown to exist in a number of typical teleost species [35], including Oyster Toadfish (*Opsanus tau*) [65], channel catfish (*Ictalurus punctatus*) [66], and rainbow trout (*Salmo gairdneri*) [67], it was unknown if these fish could synthesis Arg with NCG. Until now, only two pieces of literature reported fresh water teleost might synthesis Arg with NCG. Cheng et al. (2015) reported that the diet containing 4000 mg/kg NCG effectively improved the Arg level in the blood and reduced the liver fat deposition of Nile tilapia [34]. Wang et al. (2018) found that 1600 mg/kg NCG increased the growth performance, feed utilization, plasma Arg, antioxidant status and intestinal immune response of mirror carp [32]. Results from our study showed that NCG promoted the synthesis of endogenous Arg in Japanese seabass by increasing the plasma Arg, and also the up-regulated ASL mRNA expression in intestine. This phenomenon is similar to the report of Wu et al. (2010), in which dietary Arg or NCG enhanced the plasma Arg, ornithine, and proline concentration of weanling piglets [25]. Besides, Frank et al. reported that nursing piglets in NCG-treated group had greater plasma Arg and somatotropin levels [26]. This study is the first time to prove that NCG promoted the synthesis of endogenous Arg in Japanese seabass.

Arg requirement for carnivorous animals, such as cats and ferrets are higher than those omnivores. Similarly, the carnivorous fish, such as-Japanese seabass has higher Arg requirements than omnivorous and herbivorous fish, which is fishmeal-rely species and more difficult to utilize plant proteins. Arg deficiency causes growth retardation, liver and intestinal injury, impaired immune and antioxidant system, hyperammonemia, and even death in animals [6,68–71]. On the contrary, overdose Arg could inhibit other EAA absorption, such as lysine [23], increase blood urea nitrogen [72], acute necrotizing pancreatitis [73], and even induce lethal effect [74]. Gerard et al. (1997) reported a fatal case in human induced by an accidental overdose of Arg hydrochloride [74]. Therefore, both efficacy and tolerance need to be well evaluated before authorized an additive in market. Hong (2015) reported that the NCG had no subchronic toxicity by subchronic toxicity test (45 mg/kg bw/d, 90 days) on Mouse. Additionally, NCG is not toxic to animals on the basis of studies with infants with a total dose of 80–100 mg/kg per day [75] and rats with 1 mmol/L level in the drinking water [76]. However, in the present study, 3600 mg/kg NCG inclusion or 76.8 mg/kg bw/d intake induced significantly negative effects on protein and lipid retention in Japanese seabass, which increased nitrogen emissions and had a negative impact on aquaculture water discharge. Ammonia is toxic to all vertebrates causing convulsions, coma and death. Fish have various strategies in order to ameliorate the problem of ammonia toxicity, including being detoxified to glutamine, excreted by urea, and reduced via amino acid catabolism [77]. In the present study, compared with the Arg-deficient group (N0), fish fed N360 and N720 diets showed significantly lower plasma ammonia and higher plasma Arg, suggesting that NCG inclusion improved nitrogen metabolism and reduced the nitrogenous waste by balanced EAA profile in Japanese seabass.

Moreover, the antioxidant defense system is highly associated with the physical health status of animals [78,79], which can remove excessive reactive oxygen species (ROS) and protect cells from oxidative stress damage [80]. In fish, the MDA contents were widely used as biochemical indicators of the oxidative product of lipids [81,82]. It has been known that GST can reduce lipid hydroperoxides through their Se-independent glutathione peroxidase activity and these enzymes can also detoxify the ROS products [83]. In this study, plasma clinical parameters clearly demonstrated the efficacy of NCG in the N720 group for improving antioxidant responses by decreasing MDA and increasing GST concentrations. However, the abnormal rebounded plasma ammonia, decreased plasma Arg indicated that overfed NCG could be a burden for nitrogen metabolism.

Numbers of evidence suggest that the ROS produced by lipid peroxidation can further cause the production of cytokines (TNF-α, IL-8, and TGF), promoting inflammation apoptosis and even necrosis [84–87]. In the present study, we observed the refined liver functions, and reduced inflammatory and apoptosis responses with NCG intake groups compared with the N0 group. The caspase family is normally present in cells in an inactive zymogen state and is activated primarily by self-activation, transactivation, and non-caspase protease activation [88]. Caspase family members play important regulatory roles in the regulation of apoptosis in biological processes by activating promoter-type caspase: such as caspase-8 and caspase-9 cascades activate the caspase-3 via extrinsic and intrinsic (mitochondrial apoptosis) apoptosis pathway, respectively [89]. Caspase-3 plays a role as executor of apoptosis, and its cleavage reflects the degree of apoptosis activity [90]. Furthermore, TNFα is classified as a pro-inflammatory mediator that induces cell death via playing an initiating role in hepatocyte apoptosis [91,92]. IL-8 is an important pro-inflammatory cytokine that recruits and activates macrophages and neutrophils to remove cell debris and invades microorganisms and facilitates the regeneration of injured tissues [93]. In this study, severe hepatic fibrosis and steatosis were observed in the N0 group, combined with high levels of TNFα, IL-8, and caspase-8 mRNA expression, and strong activation of caspase-3 signal (Fig. 1). The symptom of liver disease was much refined in the N720 group, that the most of the fish showed normal-shaped liver histological structure with alleviated inflammatory and apoptosis responses, including lower plasma IgM and low mRNA expression of pro-inflammatory cytokines and less signal of apoptosis and DNA damage.

Although Wang et al. (2018) reported the positive responses of dietary NCG improved intestinal health by inhibiting the intestinal pro-inflammatory response of common carp, the mechanism of NCG or Arg regulating the nutrient metabolism and related metabolic disease are unclear. Studies pointed out that abnormal lipid accumulation contributes to the production of cytokines and apoptosis and then induce steatohepatitis and fibrosis [86,94,95]. Nanji et al. (2001) found that

Arg reversed inflammatory and fibrotic in alcohol-induced liver injury patients [42]. Similar studies have shown that fatty liver induced by Arg deficiency could be reversed by refeeding an Arg or adenine enriched diet [43]. In the present study, we found that NCG supplement in the diet of Japanese seabass improved liver health effectively and dramatic attenuation of integral features of steatohepatitis, including reduced hepatic TG and NEFA accumulation, decreased plasma VLDL and ammonia, increased plasma antioxidant capacity, and suppression of inflammation and apoptosis. Moreover, in this study, clearly down-regulated mRNA levels of lipogenesis and cholesterol synthesis genes (FASN and HMGCR), adipocyte differentiation and proliferation genes (C/EBPα), and up-regulated lipolysis genes (ATGL) illustrated the lipid metabolism regulation function of NCG in N720 group.

Protein kinases play a predominant regulatory role in nutrient metabolism and immune function [96]. ERK1/2 are members of the mitogen-activated protein kinase (MAPK) super family that can mediate cell proliferation and apoptosis [44]. Studies have shown that ERK would be necessary to initiate the pre-adipocyte into the differentiation process, and the activity of ERK is necessary for the expression of the crucial adipogenic regulators C/EBPα, β and δ and PPARγ [45]. ERK as one of the upstream targets of mammalian target of rapamycin (mTOR), is also a central integrator of various extracellular signals [48], which induce tuberous sclerosis complex (TSC1/2) phosphorylation and lead to mTOR activation. mTOR is proving to be a critical link between nutrient metabolism and immune function [97]. Recent studies have shown that mTOR signaling has various roles in different tissues and acts in different regulating functions in metabolic disease [46]. In muscle, mTOR plays a crucial role in regulating protein synthesis, and in adipose tissue, and mTOR activation promotes adipogenesis by activating PPAR-γ. In liver, mTOR activation also promotes hepatic lipogenesis [46]. S6 kinase 1 (S6K1) is a protein kinase that acts as a downstream effector of mTOR [98], which often performs same functions as mTOR [99]. Chakrabarti et al. (2010) reported that mTOR promotes fat storage in mammalian cells by inhibiting lipolysis and stimulating *de novo* lipogenesis [100] by regulating the expression of many lipogenic genes, including FASN and ACC1 [101]. Furthermore, studies have reported that rapamycin-mediated mTOR inhibition reduces mRNA and protein levels of PPAR-γ and C/EBP-α and the expression of numerous lipogenic genes *in vitro* [102]. In the present study, phosphorylation of ERK, mTOR and S6K1 protein in liver were significantly decreased by NCG supplementation and total level of ERK was also attenuated. This can be demonstrated that dietary NCG alleviates the liver disease and hepatocyte apoptosis by suppressing ERK1/2-mTOR-S6K1 protein phosphorylation to inhibiting lipogenesis and up-regulating lipolysis gene expression via promoting endogenous arginine synthesis in Japanese seabass. Additionally, the reasons for the decrease in total protein level include reduced protein synthesis or increased protein degradation. Among them, protein synthesis is mainly regulated by transcription and translation processes, and protein degradation comprises two major systems: lysosomal degradation and ubiquitin mediated proteolysis [103]. We observed no significant difference in the ERK mRNA level, presuming that NCG supplementation suppressed ERK1/2-mTOR-S6K1 signal pathway via inhibiting the ERK translation level or promoting protein degradation.

In conclusion, Japanese seabass has the ability to utilize NCG to synthesis Arg, and the balanced EAA profile benefits to the protein retention, liver function and histological structure refining. Dietary supplement 360–720 mg/kg NCG attenuates the Arg-deficiency-induced liver metabolic disease and hepatocyte apoptosis by suppresses ERK1/2-mTOR-S6K1 protein phosphorylation to inhibiting lipogenesis and up-regulating lipolysis gene expression via promoting endogenous arginine synthesis in Japanese seabass. Consuming 3600 mg/kg of dietary NCG is not safe for Japanese seabass culturing with the significantly decreased protein and lipid retention, the abnormal rebounded plasma ammonia and MDA, and reduced plasma ALB. Although the 360 mg/kg dose was the maximum recommended dose authorized by MOA, China, which mainly based on the efficacy in increased protein retention and reduced visceral adipose index. In the present study, the further studies of NCG on nutrient metabolism regulation and liver disease alleviation responses indicated that the 2-fold inclusion level (720 mg/kg) would be optimal and safe dose for Japanese seabass.

Acknowledgments

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