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Original Research Article

N-carbamoylglutamate improves lipid metabolism, inflammation, and apoptosis responses in visceral adipocytes of Japanese seabass (*Lateolabrax japonicus*), in vivo and in vitro

Haoyan Huang [a], Xiaoran Zhang [a], Xiaofang Liang [a], Xiufeng Wu [a], Xu Gu [a], Juan Han [b, *], Min Xue [a, c, *]

[a] National Aquafeed Safety Assessment Center, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing, 100081, China
[b] Institute of Food and Nutrition Development, Ministry of Agriculture and Rural Affairs, China
[c] Feed Processing Innovation Team, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing, 100081, China

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ABSTRACT

This study applied in vivo and in vitro methods to investigate the effect of dietary N-carbamoylglutamate (NCG) on lipid metabolism, inflammation and apoptosis related-gene expression in visceral adipose tissue and isolated adipocytes of Japanese seabass (*Lateolabrax japonicus*). A basal diet and a test diet supplemented with 720 mg/kg NCG were fed to the fish for 10 weeks. During the growth trial, no mortality and no significant differences in growth performance were observed in fish between the 2 groups ($P > 0.05$). Plasma Arg content and mRNA level of argininosuccinate synthetase (*ASS*) in adipose tissue were significantly increased, which indicated that NCG inclusion promoted endogenous Arg synthesis. Thereafter, the potential effects of NCG treatment on lipid metabolism-related genes expression were studied through in vivo and in vitro methods. In the present study, we successfully established a primary adipocytes culture system and isolated pre-adipocytes in vitro of Japanese seabass for the first time. Both the results in vivo and in vitro showed that NCG treatment decreased the mRNA levels of genes related to adipogenesis (fatty acid synthase, *FASN*), cholesterol synthesis (3-hydroxy-3-methylglutaryl-CoA reductase, *HMGCR*) and fat deposition (lipoprotein lipase [*LPL*] and leptin), which revealed the underlying mechanism of NCG on reducing fat deposition. The results of this study demonstrated that NCG inclusion reduced the expression of inflammatory and apoptosis cytokines markedly in vivo and in vitro. In conclusion, NCG did exert beneficial effects on ameliorating adipogenesis, inflammation and apoptosis via promoting Arg endogenous synthesis in Japanese seabass.

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1. Introduction

Currently, global aquaculture has developed rapidly with the extension of large-scale and intensive farming models, and most fish are farmed and fed with formulated diets. However, due to non-integrated feed or excess energy intake of commercial diets, excess fat accumulation in fish abdomins could induce metabolic disturbances, imbalanced immune systems and lower quality product (Chatzifotis et al., 2010; Cheng et al., 2006). Therefore, potential regulatory mechanisms to decrease fat deposition in fish have received considerable attention in recent decades.

Growing evidences from animal studies indicates that diets supplemented with gradient dietary Arg are effective in reducing excess fat accretion in fatty rats (Fu et al., 2005; Jobgen et al., 2008), in growing-finishing pigs (Tan et al., 2011), broiler chickens (Fouad et al., 2013) and Nile tilapia (*Oreochromis niloticus*) (Li et al., 2020). Several studies have shown that Arg may affect multiple metabolic pathways involving fatty acid and glucose syntheses, amino acid degradation, metabolism of energy substrates and nutrient partitioning in mammals (Jobgen et al., 2006; Wu et al., 2009).

* Corresponding author.
E-mail addresses: hanjuan@caas.cn (J. Han), xuemin@caas.cn (M. Xue).
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Additionally, the benefits, e.g., antioxidant and immune responses improvement, inflammation alleviation, and apoptosis inhibition of optimal or slightly extra dietary Arg, have been reported on several fish species, including yellow catfish (*Pelteobagrus fulvidraco*) (Zhou et al., 2015), hybrid striped bass (*Morone saxatilis*) (Cheng et al., 2012), blunt snout bream (*Megalobrama amblycephala*) (Liang et al., 2018) and Japanese seabass (Huang et al., 2019). Hence, inclusion of dietary Arg is particularly important in improving animal health.

The relatively high cost and its antagonistic action with other amino acid metabolism (Jones, 1964), has limited the potency of Arg as a commercial feed additive. Consequently, an alternative strategy to increase endogenous Arg biosynthesis is supplementation with N-carbamylglutamate (NCG), a metabolically stable analogue of N-acetylglutamate (NAG), which has been demonstrated to increase plasma Arg concentration and promote the synthesis of endogenous Arg by activating the rate-limiting enzymes responsible for both the ornithine cycle and the Arg synthetic pathway both in mammals and teleost (Frank et al., 2007; Huang et al., 2019, Wang et al., 2019a,b). Compared with Arg inclusion in animal feed, supplementation of NCG has the advantages of lower cost, higher absorption rate, and more stable metabolism (Chacher et al., 2013). Moreover, NCG serves as a safe and metabolically stable feed additive, and is beneficial for ameliorating fatty liver and hepatocyte apoptosis in Japanese seabass (*Lateolabrax japonicus*) (Huang et al., 2019). Further studies have shown that dietary NCG supplementation has stimulated endogenous Arg synthesis, enhanced the antioxidant statuses and improved the immune response in several fish species, including mirror carp (*Cyprinus carpio*), Nile tilapia (*Oreochromis niloticus*) and Japanese seabass (Cheng et al., 2015; Huang et al., 2019, Wang et al., 2019a,b). However, little information is available as to the physiological change of supplementation with NCG on lipid metabolism in fish visceral adipose tissue (VAT).

Therefore, the objective of the current experiment was to investigate the potential regulatory mechanism of Arg endogenous activator-NCG on lipid metabolism and inflammation-related gene expression in VAT and adipocytes of 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 was supplied by Animore Sci. & Tech. Co., Ltd, Beijing, China, and the purity of NCG was not lower than 98%. Our previous study indicated that dietary 720 mg/kg NCG inclusion was the optimal and effective dose for increasing endogenous Arg synthesis in Japanese seabass (Huang et al., 2019). In this experiment, a basal diet, and a test diet with 720 mg/kg NCG were prepared. The 2 experimental diets are referred to hereafter as N0 and NCG diets. The sources of ingredients, diet formulation and analyzed chemical compositions are shown in Table 1. All diets were well mixed with coefficient of variation (CV) $\leq$5%, and then extruded into 2 mm diameter pellets under the following extrusion conditions: 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). Fish oil and soy oil were added by a pilot vacuum coater (FAMSUN, Yangzhou, China). All diets were stored at −20 °C refrigeration before using. The amino acid profile of the experimental diets is shown in Table 2.

Table 1
Formulation and compositions of experimental diets (g/kg, as is basis).

Item	N0	NCG
Ingredients [1]		
Fishmeal	350	350
Corn gluten meal	100	100
Soybean meal	30	30
Wheat Gluten	50	50
Krill meal	50	50
Wheat flour	180	180
Wheat middling	59	59
Cassava starch	50	50
Fish oil	45	45
Soy lecithin	20	20
Soy oil	30	30
Monocalcium phosphate	18	18
Vitamin and mineral premix [2]	14	14
α-Cellulose[3]	4	3.28
NCG[3]	0	0.72
Total	1000	1000
Analyzed chemical composition		
Moisture	65.6	69.8
Crude protein	437	427
Crude lipid	145	146
Crude ash	89.7	86.6
Gross energy, MJ/kg	22.1	21.9

NCG = N-carbamoylglutamate.
[1] The fishmeal used in the experiment included 150 g/kg low-temperature dried fish meal (999, Denmark) and 200 g/kg domestic fishmeal (Shandong Chishan Fishmeal Factory, Rongcheng). Corn gluten meal, soybean meal, wheat gluten meal, soy lecithin and soy oil were supplied by Bohai Oil Co., Ltd (Qingdao, China); wheat flour, wheat middling and cassava starch were produced by NanKou Flour Mill (Beijing, China); fish oil was provided by JinHai grain and oil industry Co., Ltd (Qinhuangdao, China). Krill meal were provided by Liaoning Ocean Fishery Co., Ltd. (Dalian, China).
[2] Vitamin and mineral premix provided the following per kilogram of diets: vitamin A 20 mg; vitamin B_1 10 mg; vitamin B_2 15 mg; vitamin B_6 15 mg; vitamin B_{12} 8 mg; vitamin E 400 mg; vitamin K_3 20 mg; vitamin D_3 10 mg; niacinaminde 100 mg; ascorbyl calcium phosphate 1,000 mg; inositol 200 mg; calcium pantothenate 40 mg; biotin 2 mg; folic acid 10 mg; choline chloride 2,000 mg; corn gluten meal 150 mg; $CuSO_4.5H_2O$ 10 mg; $FeSO_4 \cdot H_2O$ 300 mg; $ZnSO_4 \cdot H_2O$ 220 mg; $MnSO_4 \cdot H_2O$ 25 mg; KIO_3 4 mg; Na_2SeO_3 0.5 mg; $CoCl_2 \cdot 6H_2O$ 0.5 mg; $MgSO_4$ 2,000 mg; zeolite 332 mg.
[3] α-Cellulose and NCG were premixed with vitamin and mineral premix.

2.2. Experimental fish, feeding and sampling

Juvenile Japanese seabass were obtained from Weihai Yulong Aquafarm, Shandong, China. All fish were acclimatised in laboratory

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

Item	N0	NCG
Arginine	19.8	19.2
Histidine	9.30	9.00
Isoleucine	16.7	16.1
Leucine	33.7	32.8
Lysine	21.9	21.2
Methionine	9.50	9.20
Phenylalanine	17.5	17.0
Threonine	14.8	14.4
Tryptophan	4.00	4.00
Valine	19.4	18.8
Asparagic acid	31.9	31.1
Cysteine	4.70	4.70
Glutamic acid	73.1	72.0
Glycine	18.5	18.1
Proline	24.5	24.0
Serine	16.5	16.2

NCG = N-carbamoylglutamate.

conditions and fed the N0 diet 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 6 tanks per treatment. The water temperature was maintained at (26 ± 2) °C, pH = 7.5 to 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 everyday, and the photoperiod was 12 h dark (D):12 lighting (L). Fish were fed to apparent satiation twice daily at 08:00 and 16:00 for 70 d.

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, 6 replicates in each treatment) were randomly selected and anaesthetized with chlorobutanol (300 mg/mL). The body weight, body length and adipose tissue weight were recorded individually to calculate condition factor (CF) 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 centrifuged at 1,800 × *g* for 10 min at 4 °C to obtain plasma. Two VAT samples from each tank were sampled and fast frozen in liquid nitrogen for RNA isolation, and 8 of them were used for analysis randomly. All samples were stored at −80 °C until analysis.

2.3. Chemical analysis

The tested NCG was determined by an Ion Chromatography, iChrom W5100, Xi'an Heb Biotech Co. Ltd, Shanxi, China). The mixing homogeneity of feed (CV) was determined following the method of ICCF Guidance #3 (ICCF, 2019). All chemical analyses of the diets 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 Kjelte 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 °C for 16 h. Gross energy was determined using an IKAC2000 Calorimeter (IKA, Staufen, Germany). The amino acids of diets were determined by an amino acid analyzer Hitachi 8900 (Tokyo, Japan) after hydrolysis in 6 mol/L HCl for 22 to 24 h at 110 °C. The free amino-acid concentrations in the plasma were analyzed by an automatic amino acid analyzer (S-433D, Sykam, Germany).

2.4. Establishment of the cell culture system

Three Japanese seabass with body weight about 90 g were anesthetized with chlorobutanol (300 mg/mL) before sampling. The adipose tissue in each abdominal cavity was isolated carefully by sterile dissection and washed 3 times with phosphate-buffered saline (PBS, pH 7.4), and then minced and digested in 0.1% Type I collagenase for 30 min (Sigma, USA). The tissue was digested, and then centrifuged (500 × *g* for 10 min). After centrifuging, the cell pellet was resuspended in fetal bovine serum (FBS)-free DMEM/F12 medium, and then the cell suspension was passed through a 200-μm nylon filter to remove large particulate material. The resulting cell suspension was then centrifuged at 500 × *g* for 10 min. The digestion medium was removed, and the cell pellet was treated with an erythrocyte lysing buffer for 5 min at room temperature to remove red blood cells. After washing twice, the cells were resuspended in a growth medium (DMEM/F12, 10% FBS, 100 U/mL penicillin/streptomycin), and the resuspended cells were seeded on a 35 mm culture dish, cultured at 28 °C and 5% CO_2. The medium was changed every 2 d to observe the proliferation and growth of the cells. At d 6, the pre-adipocytes grew to the confluence stage, and were changed to the adipogenic medium, which was composed of growth medium supplemented with 15 μg/mL insulin, 2 μmol/L Indomethacin, 1 μmol/L dexamethasone and 0.5 μmol/L IBMX referred to (Liu et al., 2015) with minor modifications, and cultured until the mature lipid droplets were induced. The morphology of cells was observed with an inverted microscope (Primovert, Carl Zeiss, Germany). Four replicates of mature adipocytes were treated with 75 mg/L NCG in the full growth medium, which was obtained by previous pre-tests.

2.5. Oil red O (ORO) staining

The cells were washed 3 times with PBS and fixed in 4% paraformaldehyde for 30 min and then rinsed with PBS. Cells were then stained with ORO for 30 min and rinse with 60% isopropanol to remove excess ORO, and rinsed with PBS 3 times. Cell morphology was observed with an inverted microscope (Primovert, Carl Zeiss, Germany) and photographed with a digital camera (Leica DM2500, Leica, Solms, Germany).

2.6. RNA isolation, reverse transcription and mRNA level analysis

Total RNA was isolated from adipose tissue and adipocytes using an miRNA easy Mini kit (QIAGEN Sciences, MD, USA), spectrophotometrically quantified using a NanoDrop2000 (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 then subjected to cDNA synthesis by reverse transcription in a 20 μL volume using an iScript 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 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. Real-time quantitative PCR (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 cDNA 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-qPCR temperature profile for all genes was 95 °C for 30 s followed by 40 cycles of 10 s at 95 °C, 30 s at annealing temperature (Tm) (Table 3) and 40 s at 72 °C. After the final cycle of RT-qPCR, 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 $(1+\text{E-values})^{-\Delta\Delta Ct}$ (Pfaffl, 2001).

2.7. Statistical analysis

Statistical differences between the 2 groups were analyzed by the unpaired student's *t*-test and homogeneity of variance was confirmed by Levene's test using SPSS Statistics 17.0 (IBM. Inc. USA). $P < 0.05$ was considered statistically significant, $P < 0.01$ was a very significant difference, and $P < 0.001$ was an extremely significant difference. The graphics were drawn using GraphPad Prism 6.0 (GraphPad Software Inc. USA).

Table 3
Primer sequences for real-time RT-qPCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Products length, bp	Tm, °C	E-value, %
ACC1	AATCAACATCCGCCTGACTCCAAC	CCTGCTTGTCTCCGTATGCTTGG	176	59.0	90.2
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/EBPα	CACGGACAACGACAGACTGA	GCCACACACCAACTCACGTA	213	60.5	93.4
C/EBPβ	TCTGATTCCAGGGTGTCCTC	TCATCTGCTCAGCCACTCTG	181	57.1	98.8
EF1α	AATCGGCGGTATTGGAACTG	TCCACGACGGATTTCCTTGA	205	58.5	102.0
FASN	AGGCATTGTGGAGGGTGTAG	CCAGTCCACCAGTGATGATG	233	56.8	97.1
HMGCR	GGAAGAGGAAGAGGACAACAAGCC	GAACCATGACCAGGCCAAGCG	80	56.6	101.0
HSL	TGATGTTTGCCAAGAAGCTG	CTGATGGACTGGTGCTCTGA	228	57.8	93.8
Leptin	TGCAACTTTTAAGTGGGGGTA	TGTTGTAACCCTCCAGCACGG	201	59.0	103.5
IL1β	CTGAACATCAAGGGCACAGA	GTTGAAGGGGACAGACCTGA	192	60.8	92.8
IL8	GAGCTGATTCCTGCCAACTC	CCGATCTGTTCAGGGTGTTC	153	55.8	98.0
LPL	AGCACCTCCAAAACCTTCCT	TCTGAGCTGCCACCACATAG	169	58.8	92.9
PPARγ	AGGCCTGCTGAATGTGAAGC	GCTGGATGAAGTGGACGTGG	170	58.0	93.3
TNF-α	GACTCCATAGGCAGCAAAGC	AGAAAGTCTTGCCCTCGTCA	205	60.8	103.2

ACC1 = acetyl-CoA carboxylase 1; *ASS* = argininosuccinate synthetase; *ATGL* = adipose triglyceride lipase; *Caspase* = cysteine-aspartic proteas; *C/EBP* = CCAAT-enhancer-binding protein; *EF1α* = eukaryotic translation elongation factor 1 alpha; *FASN* = fatty acid synthase; *HMGCR* = 3-hydroxy-3-methylglutaryl-CoA reductase; *HSL* = hormone-sensitive triglyceride lipase; *IL* = interleukin; *LPL* = lipoprotein lipase; *PPAR* = peroxisome proliferator activated receptor; *TNF* = tumor necrosis factor.

3. Results

3.1. Growth performance and the ability of Arg endogenous synthesis in Japanese seabass

The results of growth performance and morphometric parameters are presented in Table 4. No mortality occurred throughout the 10-week experimental trail. Fish in both groups had fast growth with weight gain (WG) up to 672%. There was no significant difference in WG, feeding rate (FR), feed conversion ratio (FCR), CF, VAI between the groups ($P > 0.05$). Plasma concentrations of arginine were significantly increased after NCG treatment ($P < 0.05$), but there was no effect on the concentrations of lysine ($P > 0.05$) (Fig. 1A). Compared with the N0 group, plasma ammonia in the NCG group was significantly decreased ($P < 0.05$) (Fig. 1A). Additionally, Arg synthesis related gene-argininosuccinate synthetase (*ASS*) mRNA level was up-regulated remarkedly in adipose tissue by NCG inclusion (Fig. 1B). The above results indicate that dietary NCG improved Arg metabolism and reduced the metabolic waste in Japanese seabass.

Table 4
Effects of dietary N-carbamoylglutamate (NCG) on the growth performance, morphometric parameters and whole-body composition in Japanese seabass (means ± SEM, $n = 6$)[1].

Item	N0	NCG
Growth performance		
WG[2], %	678 ± 3.71	666 ± 6.68
FR[3], % BW/d	2.09 ± 0.01	2.10 ± 0.01
FCR[4]	0.92 ± 0.00	0.93 ± 0.00
Morphometric parameters		
CF[5], g/cm^3	1.33 ± 0.02	1.26 ± 0.04
VAI[6], %	7.49 ± 0.35	7.39 ± 0.22

[1] Values in the same row with different superscript letters are significantly different ($P < 0.05$). The data for FR, FCR, CF and VAI have been published in Huang et al. (2019).
[2] WG (weight gain) = 100 × (Wf − Wi)/Wi. Wf is the final total weight (g), Wi is the initial total weight (g).
[3] FR (feeding rate) = 100 × feed intake/[(Wf + Wi)/2]/d.
[4] FCR (feed conversion ratio) = feed intake/(Wf − Wi).
[5] CF (condition factor) = 100 × (body weight, g)/(body length, cm)^3.
[6] VAI (visceral adipose index) = 100 × (visceral adipose weight, g)/(whole body weight, g).

3.2. In vivo evaluation of NCG on lipid metabolism related gene expression in visceral adipose tissue of Japanese seabass

As shown in Fig. 2, compared with N0, the NCG group showed a decreased relative mRNA level for fatty acid synthase (*FASN*) (adipogenesis), 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*) (cholesterol synthesis), peroxisome proliferator activated receptor γ (*PPARγ*), CCAAT-enhancer-binding protein β (*C/EBPβ*) (adipocytes differentiation), leptin and ipoprotein lipase (*LPL*) (fatty deposition) ($P < 0.05$). There was no significant difference in adipose triglyceride lipase (*ATGL*) and ormone-sensitive triglyceride lipase (*HSL*) (lipolysis) mRNA levels of VAT in Japanese seabass ($P > 0.05$).

3.3. Isolation and maturing induction of Japanese seabass pre-adipocytes in vitro

In the present study, we successfully established a primary adipocytes culture system and isolated pre-adipocytes in vitro of Japanese seabass for the first time. As shown in Fig. 3A, on d 2 after seeding, most of the cells were attached to the bottom of the dish. The morphology of pre-adipocytes was similar to that of fibroblasts, with a cytoplasm devoid of lipid droplets. As culture time progressed, the pre-adipocytes proliferated and reached confluence after being seeded for 6 d (Fig. 3B). At confluence, pre-adipocytes were successfully induced into mature adipocytes after 7 d of being cultured in the induction medium, in which, the lipid droplets were easily observed in the cytoplasm and were stained into a red color by ORO staining (Fig. 3C and D).

3.4. In vitro evaluation of NCG on lipid metabolism related gene expression in adipocytes

To determine the changes at the transcription level of adipocytes in vitro in comparison to adipose tissue, the expression of lipid metabolism-related genes was analyzed. As shown in Fig. 4, the NCG treatment group significantly reduced gene expression of *FASN*, *HMGCR* and fatty deposition (*LPL* and leptin) ($P < 0.05$). There was no significant difference in mRNA levels of genes related to adipocyte differentiation (*PPARγ*, *C/EBPα* and *C/EBPβ*) and lipolysis (*ATGL* and *HSL*) ($P > 0.05$).

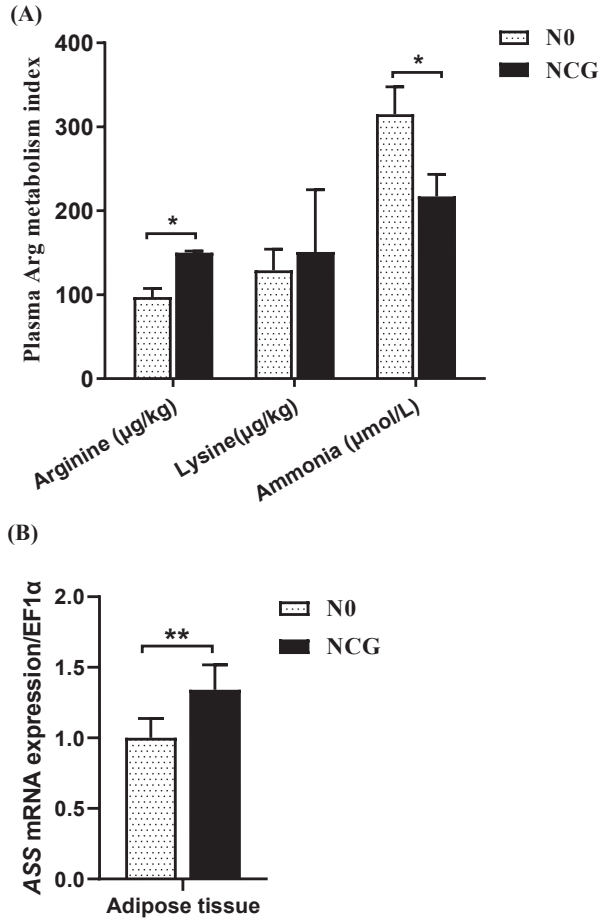

Fig. 1. Japanese seabass could utilize N-carbamoylglutamate (NCG) to promote Arg synthesis. (A) Plasma arginine and lysine level (mg/kg) and ammonia concentration (μmol/L); (B) The mRNA level of *ASS* in adipose tissue in vivo. Values marked with asterisk(s) are significantly different (*, $P < 0.05$; **, $P < 0.01$) (mean ± SEM, $n = 12$).

3.5. In vivo and in vitro evaluation of NCG on inflammation and apoptosis responses

The results of mRNA levels of inflammation and apoptosis factors in Japanese seabass adipose tissue in vivo are shown in Fig. 5. The NCG group significantly down-regulated inflammation related factors-interleukin 1β (*IL-1β*) and *IL*-10 mRNA levels in Japanese seabass adipose tissue (Fig. 5A) ($P < 0.01$). Moreover, the mRNA levels of apoptosis factors (*caspase-3* and *caspase-8*) was decreased in the NCG group compared with the N0 group (Fig. 5B) ($P < 0.05$).

The results of mRNA levels of inflammation and apoptosis factors in adipocytes in vitro are shown in Fig. 6. The pro-inflammatory factor *IL-8* was down-regulated, and the anti-inflammatory factor *IL-10* was up-regulated simultaneously in the NCG treatment group ($P < 0.01$) (Fig. 6A). There was no significant difference in the relative expression of apoptosis factors genes ($P > 0.05$) (Fig. 6B).

4. Discussion

Several studies have reported that NCG is effective in improving growth performance. Frank et al. (2007) found that 50 mg/kg body weight per 12 h for 7 d can improve body weight of piglets significantly. Hu et al. (2019) also reported that supplementation with 1,000 mg/kg NCG improved growth performance in yellow-feather broilers. A similar result was observed by Wang et al., 2019a,b, that 1,200 mg/kg dietary NCG increased WGR and protein efficiency rate in mirror carp (*C. carpio*) based on an Arg deficient diet (1.24%). However, Cheng et al. (2015) reported that 250 to 5,000 mg/kg NCG in a basal diet with 2.30% of Arg had no effect on growth performance but reduced hepatic fat deposition in Nile tilapia (*oreochromis niloticus*). In our previous study, we also did not observe a difference on specific growth rate, but an improved protein retention and lipid metabolism in the liver was found when Japanese seabass were fed the diets with 360 to 720 mg/kg NCG (Huang et al., 2019). In the present study, 720 mg/kg NCG did not affect the growth performance of Japanese

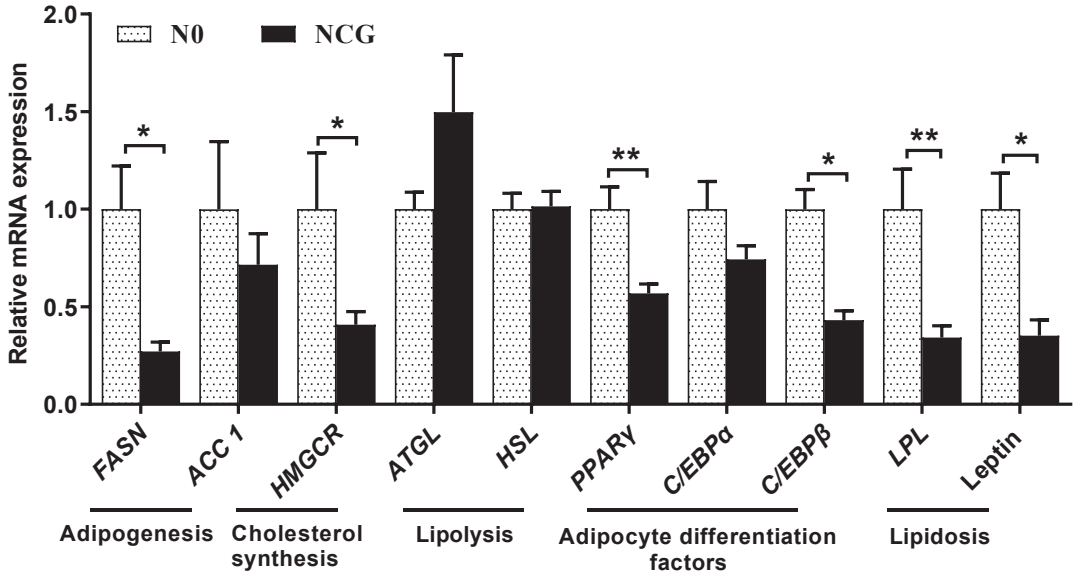

Fig. 2. Effect of dietary N-carbamoylglutamate (NCG) inclusion on the genes expression of lipid metabolism in adipose tissue in vivo of Japanese seabass. *FASN* = fatty acid synthase; *ACC1* = acetyl-CoA carboxylase 1; *HMGCR* = 3-hydroxy-3-methylglutaryl-CoA reductase; *ATGL* = adipose triglyceride lipase; *HSL* = hormone-sensitive triglyceride lipase; *PPAR* = peroxisome proliferator activated receptor; *C/EBP* = CCAAT-enhancer-binding protein; *LPL* = lipoprotein lipase. Values marked with asterisk(s) are significantly different (*, $P < 0.05$; **, $P < 0.01$) (mean ± SEM, $n = 12$).

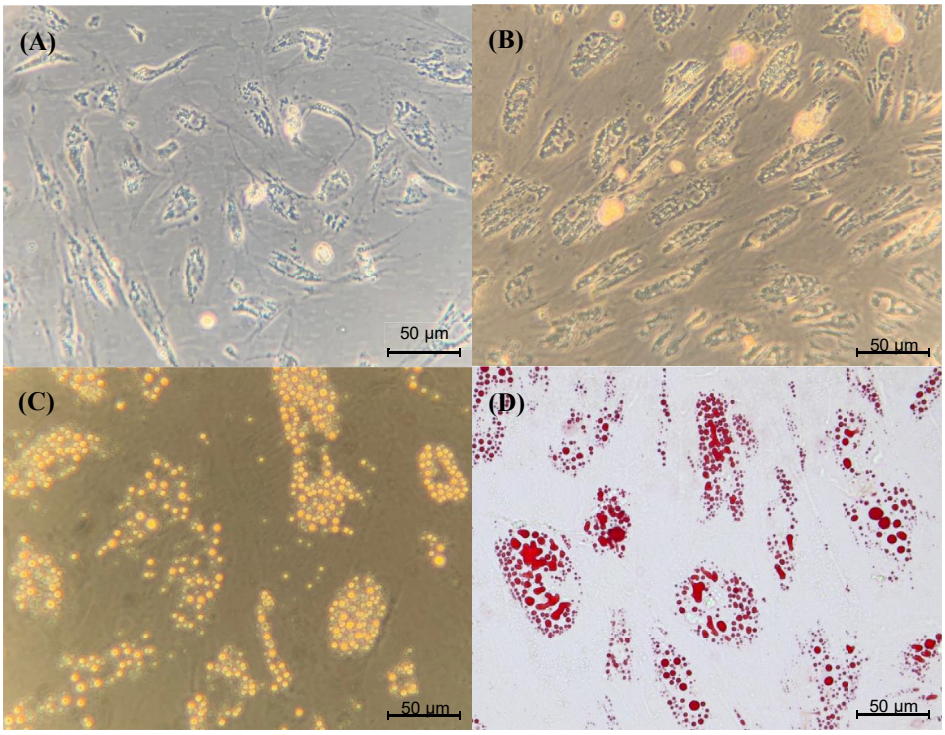

Fig. 3. Morphological changes of Japanese seabass preadipocyte during differentiation. Confluent preadipocytes were induced for differentiation with an adipogenic medium. (A) The preadipocyte on d 2; (B) preadipocyte induction after growth to confluence stage at d 6; (C) the mature adipocyte after 7 d induction; (D) the oil red O stained mature adipocyte, with the lipid drops stained in red color.

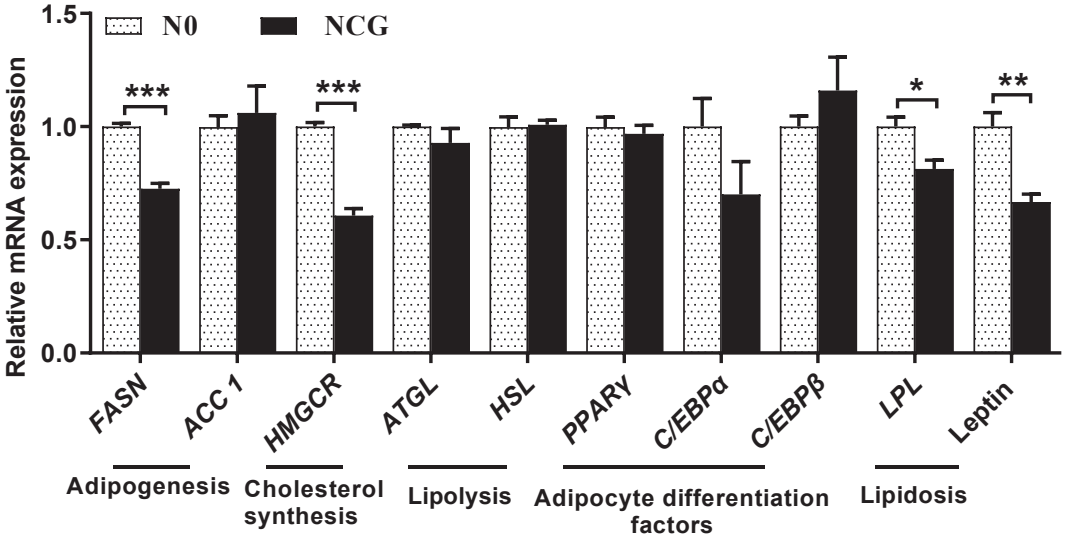

Fig. 4. Effect of N-carbamoylglutamate (NCG) treatment on the expression of lipid metabolism related genes in Japanese seabass adipocytes in vitro. *FASN* = fatty acid synthase; *ACC1* = acetyl-CoA carboxylase 1; *HMGCR* = 3-hydroxy-3-methylglutaryl-CoA reductase; *ATGL* = adipose triglyceride lipase; *HSL* = hormone-sensitive triglyceride lipase; *PPAR* = peroxisome proliferator activated receptor; *C/EBP* = CCAAT-enhancer-binding protein; *LPL* = lipoprotein lipase. Values marked with asterisk(s) are significantly different (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) (mean ± SEM, $n = 4$).

seabass, which could be related to the Arg level in the basal diet (1.90%), which might be in the range of growth requirement for most of fish (NRC, 2011).

As a metabolically stable analog of NAG, NCG has been proven to increase endogenous Arg synthesis and Arg level in circulating blood (Huang et al., 2019; Wang et al., 2019a,b; Wu et al., 2004). In the current study, we consistently found that dietary NCG inclusion increased the serum concentration of Arg. Additionally, NCG treatment had no effect on the serum lysine level in Japanese seabass, suggesting that NCG supplementation did not cause lysine-Arg antagonism. To evaluate the ability of NCG on promoting endogenous Arg synthesis, the gene expression level of ASS (the rate-limiting enzyme in providing Arg) was further determined. The results showed that dietary supplementation with NCG

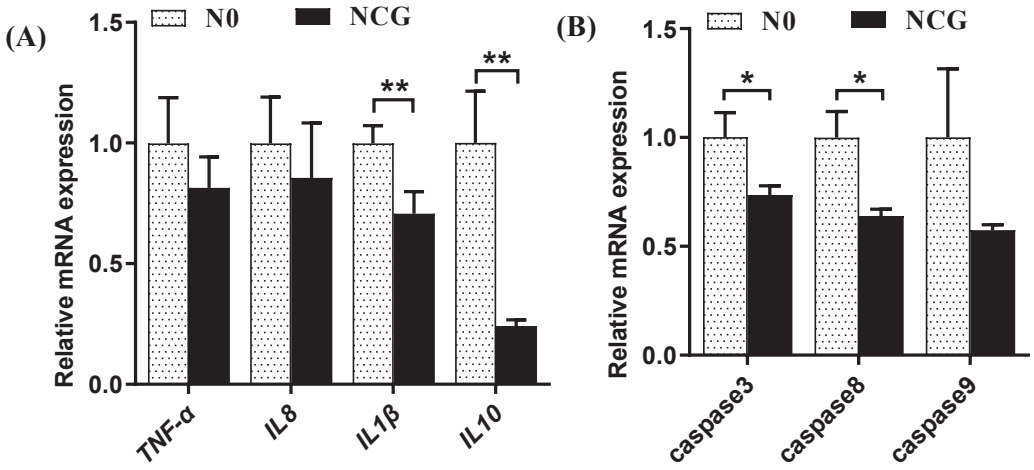

Fig. 5. Effect of N-carbamoylglutamate (NCG) on the gene expression of inflammatory and apoptosis cytokines in adipose tissue in vivo. (A) The mRNA levels of inflammatory cytokines in adipose tissue; (B) the mRNA levels of apoptosis cytokines in adipose tissue. *TNF* = tumor necrosis factor; *IL* = interleukin. Values marked with asterisk(s) are significantly different (*, $P < 0.05$; **, $P < 0.01$) (mean ± SEM, $n = 12$).

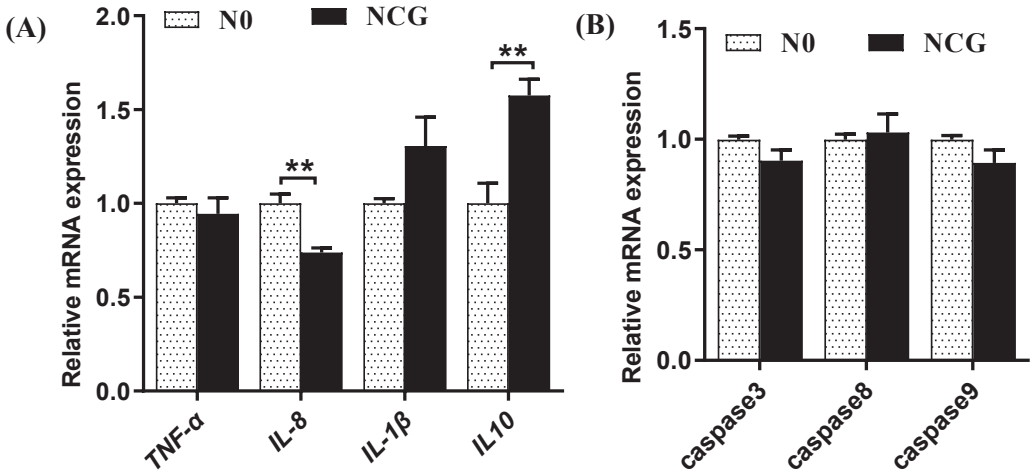

Fig. 6. Effect of N-carbamoylglutamate (NCG) on the gene expression of inflammatory and apoptosis cytokines in adipocytes in vitro. (A) The mRNA levels of inflammatory cytokines in Japanese seabass adipocytes; (B) the mRNA levels of apoptosis cytokines in Japanese seabass adipocytes. *TNF* = tumor necrosis factor; *IL* = interleukin. Values marked with asterisk are significantly different (**, $P < 0.01$) (mean ± SEM, $n = 4$).

stimulated Arg synthesis via increasing plasma Arg level and upregulating Arg synthesis related gene expression.

While there are numerous reports of potential benefits on reducing fat deposition from Arg supplementation (Fouad et al., 2013; Tan et al., 2009, 2011), information on the effects of Arg endogenous activator-NCG on fat deposition is not available. It is well-known that fat deposition is mainly affected by the course of reaction of lipid metabolism, including adipogenesis, adipocytes differentiation, deposition and lipolysis, etc (Wood et al., 2008). The balance of lipid metabolism depends both on lipogenesis and lipolysis. Fatty acid synthesis is regulated by key enzymes, including acetyl coenzyme A (CoA), ACC and FASN, whereas hydrolysis of triacylglycerols in adipose tissue is catalyzed by HSL and ATGL (Zimmermann et al., 2004; Zou and Shao, 2008). In the present study, we found that dietary NCG supplementation decreased the *FASN* mRNA level significantly, but did not affect the gene expression of *ACC*, *HSL* and *ATGL*. The downregulation of *FASN* gene expression will reduce the availability of fatty acids for esterification into triglycerides (TG) and storage in adipose tissue. In addition, we observed that the mRNA level of *HMGCR* was downregulated with dietary NCG inclusion. HMGCR is a crucial enzyme during cholesterol biosynthesis and affects local lipids uptake and deposition in pig peripheral tissues, which is indispensable for lipid metabolism (Cui et al., 2010). Besides, PPARγ, C/EBPα and C/EBPβ stimulate differentiation and proliferation of porcine adipocytes, so an increase in its expression would lead to enhanced lipogenesis in adipose tissue (Ding et al., 2000). Our results showed that NCG supplementation resulted in decreased mRNA levels of *PPARγ* and *C/EBPβ*, which indicated that NCG inhibited adipogenesis in Japanese seabass adipose tissues. LPL mediates the hydrolysis of TG-rich lipoproteins and produces nonestesterified fatty acid (NEFA) preferentially for fat storage in adipose tissue, so LPL is considered a gatekeeper in the development or progression of obesity (Wang et al., 2009). Furthermore,

studies found that leptin mRNA level in VAT is positively correlated with the degree of obesity, and with the proliferation of pre-adipocytes and hypertrophy of adipocytes, the expression of leptin mRNA in adipocytes also increased (Chen et al., 1997; Shillabbeer et al., 1998). Interestingly, mRNA levels for *LPL* and leptin were substantially decreased in response to dietary NCG supplementation. The most novel and significant result of the current study is that dietary NCG may reduce fat deposition by modulating lipid metabolism in adipose tissue of Japanese seabass.

The formation and differentiation of adipocytes is a complex process, and the adipocytes culture model in vitro provides a better condition for the study of adipogenesis. In this study, we investigated the characteristic of Japanese seabass pre-adipocytes as they developed into mature adipocytes. In the current study, similar to mammals and other fish, Japanese seabass pre-adipocytes were able to differentiate in mature adipocytes accompanied by lipid accumulation in the cytoplasm. It was found that adipocytes differentiation is characterized as lipid droplet accumulation, from a fibroblast-like cell to a mature adipocyte that is filled with a single large lipid droplet in the cytoplasm. Studies in fish have shown that lipid droplets with different size gradually filled in the cell, and that small lipid droplets fused into large lipid droplets and eventually differentiated into mature adipocytes, as shown in Atlantic salmon (*Salmo salar*) (Vegusdal et al., 2003), rainbow trout (*Oncorhynchus mykiss*) (Bouraoui et al., 2008), gilthead sea bream (*Sparus aurata*) (Salmerón et al., 2013), Nile tilapia (Wen et al., 2018) and grass carp (*Ctenopharyngodon idellus*) (Liu et al., 2015). In the current study, we successfully isolated pre-adipocytes from the visceral adipose tissue of Japanese seabass and induced them into mature adipocytes for the first time. To explore the effects of NCG on lipid metabolism-related gene expression in adipocytes in vitro, we measured the mRNA level of genes mentioned above. The results of the present study demonstrated that NCG treatment significantly inhibited *FASN, HMGCR, LPL* and leptin mRNA level in adipocytes. Besides, there was no significant difference in lipolysis (*ATGL* and *HSL*) and adipocytes proliferation and differentiation key factors (*PPARγ, C/EBPα* and *C/EBPβ*) of Japanese seabass adipocytes.

In addition to adipocytes, the most abundant cell type in adipose tissue, adipose tissue also contains pre-adipocytes (which are adipocytes that have not yet been loaded with lipids), endothelial cells, fibroblasts, leukocytes and macrophages (Tilg and Moschen, 2006). Hence, adipose is no longer considered an inert tissue mainly devoted to energy storage but is emerging as an active participant in regulating physiologic and pathologic processes, which produces and releases a variety of pro-inflammatory and anti-inflammatory factors (Fantuzzi, 2005). Furthermore, changes in the number and size of the adipocytes affect the microenvironment of expanded fat tissues, accompanied by alterations in adipokine secretion (Choe et al., 2016). Wang et al. (2017) compared the structure and metabolic functions of VAT and subcutaneous adipose tissues (SAT) of Nile tilapia, and found that VAT is the preferable lipid deposition tissue, with more inflammatory cells and lower lipid catabolic activity than SAT (Wang et al., 2017). Over-accumulated adipose tissue in fish abdomins impairs health and decreases stress resistance (Chatzifotis et al., 2010; Cheng et al., 2006). In the present study, we found that dietary NCG supplementation significantly down-regulated inflammation-related factors (*IL-1β* and *IL-10*) and apoptosis factors (*caspase-3* and *caspase-8*) mRNA level in Japanese seabass VAT in vivo. Concurrently, similar findings were observed in vitro, in which the pro-inflammatory factor *IL-8* was down-regulated, and the anti-inflammatory factor *IL-10* was up-regulated simultaneously in mature adipocytes with NCG treatment. Gao et al. (2005) reported that up-regulated gene expression of adipose inflammatory chemokines and cytokines was associated with increased fat accumulation and adipocytes hypertrophy. Primary adipocytes often do not fully reproduce in vivo results because single cells are missing many functions compared with adipose tissue. Although there was no difference in the gene expression of adipocytes proliferation and differentiation key factors in vitro, it was consistent with the in vivo results in the inhibition of adipogenesis genes and inflammatory factor mRNA levels. As described in the previous study (Huang et al., 2019), an important function of 720 mg/kg NCG inclusion was to alleviate fatty liver disease in the species. In the present study, we focused on the lipid metabolism in the VAT, and found the significantly downregulated lipogenesis gene expression by NCG inclusion in vivo and in vitro.

5. Conclusion

Dietary NCG can reduce visceral fat deposition by diminishing adipogenesis related genes expression, and further reduce the inflammatory and apoptosis responses via promoting Arg endogenous synthesis in Japanese seabass in vivo and in vitro. Moreover, we successfully established a primary adipocytes culture system, and the isolated pre-adipocytes of Japanese seabass could be a well-worked assay for lipid metabolism study.

Author contributions

Min Xue and **Haoyan Huang** designed the experiments; **Haoyan Huang**, **Xiaoran Zhang** and **Xiaofang Liang** carried out the experimental work; **Haoyan Huang** wrote the manuscript under the direction of Min Xue and Juan Han; **Xiufeng Wu** and **Xu Gu** assisted with the experimental work.

Conflict of interest

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

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