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ORIGINAL ARTICLE

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# Effects of glutamine and its precursors on the growth performance and relevant protein synthesis pathway of mirror carp *Cyprinus carpio*

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**Abstract** This study aimed to investigate the effects of Gln and its precursors on Gln anabolism and ammonia excretion to determine the role of Gln in protein synthesis in *Cyprinus carpio*. The growth performance, glutamine synthetase (GS) activity, blood ammonia level, and gene expression of GS, rhesus glycoprotein (Rhag, Rhbg and Rhcg), TOR and 4E-BP1 of fish were measured. Seven diet treatments including glucose (control), glutamine (Gln), glusate (Glu), α-ketoglutarate (AKG), L-ornithine-α-ketoglutarate (OKG), L-arginine-α-ketoglutarate (AAKG), and α-ketoglutarate sodium (2Na-AKG) were conducted. All were substituted for glucose at 1.5% of the dry diet. The results showed the feed conversion ratios (FCRs) of the AKG group and AAKG group were significantly lower ($P < 0.05$) than that of the control group. The expression levels of the Rhbg gene in the gills of the AKG, AAKG and 2Na-AKG groups were significantly higher than that in the control group ($P < 0.05$). The expression levels of the TOR gene in the gut of the fish in the AKG group and the Glu group were significantly higher than that in the control group ($P < 0.05$). Therefore, the addition of AAKG in feed can significantly reduce the FCR of *Cyprinus carpio* and significantly improve the weight gain rate (WGR) and protein efficiency of the fish. Gln can reduce ammonia release in gills, and AKG can effectively promote the excretion of ammonia. The addition of Gln, Glu, AKG and AAKG in diets can effectively promote protein synthesis. The Gln, Glu, AKG and AAKG can significantly up-regulate GS gene expression in the gut; however, the expression level of the GS gene is not significantly correlated with GS activity.

**Keywords** Glutamine · Precursors · *Cyprinus carpio* · Growth performance · Nitrogen metabolism · Protein synthesis pathway

## Introduction

Glutamine (Gln) is the most abundant amino acid in animal blood [1]. It is an important precursor in the synthesis of proteins, pyrimidine and purine nucleotides, nicotinamide adenine dinucleotides, and amino sugars. Glutamine is also the main energy source for rapidly dividing cells, such as lymphocytes, and further functions in transamination and as a source of nitrogen in metabolic processes [2, 3]. In general, Gln is introduced into the body from the intake of external sources and from endogenous synthesis. However, under stress or pathological conditions, the amount of glutamine produced via endogenous synthesis cannot meet the body demands [4]. Studies have confirmed that Gln additives in feed can promote intestinal development in carp, improve intestinal structure and function, and effectively prevent oxidative stress induced by hydrogen peroxide on intestinal epithelial cells [5, 6]. However, these exogenous sources of Gln are very unstable and can easily decompose into toxic pyroglutamic acid and ammonia [7]. Due to these drawbacks, scholars have

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performed in-depth studies on potential Gln substitutes, especially glutamine dipeptide. Brito et al. [8] showed that alanyl-glutamine (Ala-Gln) inhibited the induction of *Clostridium difficile* toxin on intestinal epithelial apoptosis and injury [8]. Kim et al. [9] used Ala-Gln to promote the in vitro maturation and embryonic development of porcine oocytes [9].

As the precursors of Gln, glusate (Glu), the function and nutritional support α-ketoglutarate (AKG) and L-ornithine-α-ketoglutarate (OKG) have been well documented in mammals [10–13]. As an arginine salt, AAKG can promote nutrition and energy absorption in liver cells to protect the liver function. 2Na-AKG as the organic intermediate can provide the AKG for the organism, and promote AKG conversion [14]. Mirror carp *Cyprinus carpio* is one of the most extensively cultured fish species in China because of its ease of breeding, fast growth, tolerance to a wide range of environmental conditions, and resistance to diseases [15, 22]. This study aimed to investigate the effects of Gln and its precursors on Gln anabolism and ammonia excretion to determine the role of Gln in protein synthesis in mirror carp, and further provided a theoretical basis for a scientific formulation of carp feed.

## Materials and methods

### Experimental fish and stocking

The mirror carps were obtained from the Hulan fishing ground, Heilongjiang Fisheries Research Institute (Harbin, China) and transferred to an indoor aquarium for a 2-week acclimation. During acclimation, fish were fed to satiation 3 times a day with the basal diet. The water quality was monitored under the conditions of temperature 25 ± 2 °C, pH 7.5–8.2 and over 5.0 mg $l^{-1}$ dissolved oxygen level.

After acclimation, 35 circular polyethylene tanks, each of 500 l, were connected to a closed recirculation system and supplied with aerated freshwater and filtered through zeolite, corallite and activated carbon. Healthy carps with an average initial weight of 40.27 ± 3.96 g were randomly assigned to 35 aquaria with an initial stocking density of 30 fish per aquarium and cultured for 56 days. The experimental units were maintained under a natural light and dark cycle. Each diet was fed to five randomly assigned aquaria. Throughout the entire experiment period, fish were fed to satiation 3 times a day (07:30, 12:30 and 14:30). During this period, aeration was provided continuously, and 20% of tank water (dechlorinated tap water) was replaced daily. The water quality was monitored under the conditions of 25 ± 2 °C, pH 7.5–7.9, > 6.0 mg $l^{-1}$ dissolved oxygen and < 0.5 mg $l^{-1}$ total ammonia nitrogen.

### Diets and sampling

Seven isoproteic and isolipidic diets were formulated to meet the protein and energy requirements of the mirror carp, using fish meal and soybean meal as protein sources, and fish oil, soybean oil and phospholipid as lipid sources. The glucose (1.5%) in the basal diet was substituted for Gln, Glu, AKG, OKG, AAKG, and 2Na-AKG, respectively. AKG (A, purity ≥ 98%) was obtained from Sigma-Aldrich Trading Co., Ltd., Shanghai. China. Gln, Glu, OKG (L-ornithine: α-ketoglutarate = 1:1), AAKG (L-arginine: α-ketoglutarate = 1:1), and 2Na-AKG were obtained from Shanghai drum Biotechnology Co., Ltd., Shanghai, China. All diets were individually blended in a mixer and then homogenized after fish oil and soybean oil were added. The mixture was made into pellets (2 mm in diameter) and air-dried at room temperature, and then stored in a refrigerator at −20 °C until further use. The composition and nutrient levels of the basal diet (control) are shown in Table 1.

At the end of the culture period, all of the fish were deprived of feed for 24 h. Then, the final body weights of the carps were collected. The growth indices, including the weight gain rate (WGR), feed conversion rate (FCR), condition factor (CF) and protein efficiency ratio (PER), were calculated using the following equations. A total of ten fish from each treatment group (two fish from each aquarium) were netted randomly and anesthetized with tricaine methanesulfonate (Sigma, USA). The whole fish and the viscera were weighed to calculate the viscerosomatic index (VSI).

Weight gain rate (WGR, %) = 100 × (final weight−initial weight)/initial weight,

Feed conversion rate (FCR) = dry feed intake (g)/wet weight gain (g),

Protein efficiency ratio (PER) = wet weight gain (g)/total protein fed (g),

Condition factor (CF) = 100 × body weight (g)/(body length)$^3$,

Viscerosomatic index (VSI, %) = 100 × viscera weight (g)/whole body weight (g).

Blood samples taken from the caudal vein were centrifuged at 3000*g* for 10 min at 4 °C, and the serum was stored at −80 °C for assays. The intestinal, liver and gill tissues were quickly collected and stored in liquid nitrogen for analyses of glutamine synthetase (GS) and rhesus glycoprotein (Rh) gene expression. The intestinal tissues (foregut, midgut and hindgut) were collected and stored at −20 °C for analyses of the activity of GS.

### Measurement of the activity of GS

The intestine was homogenized in 10 volumes (w/v) of ice-cold physiological saline and centrifuged at 4000×*g* for 10 min at 4 °C, and the supernatant was conserved at

**Table 1** Composition and nutrient levels of the basal diet (dry matter basis)

| Ingredients | Content (%) |
|---|---|
| Fish meal | 5.00 |
| Soybean meal | 38.10 |
| Corn starch | 2.00 |
| Wheat middling | 43.40 |
| Fish oil | 1.00 |
| Soybean oil | 3.30 |
| Phospholipid | 1.00 |
| Vitamin premix[a] | 0.30 |
| Mineral premix[b] | 0.20 |
| *L*-Lys | 0.54 |
| *L*-Met | 0.56 |
| *L*-Thr | 0.43 |
| Choline chloride | 0.20 |
| $Ca(H_2PO_4)_2$ | 2.47 |
| Glucose | 1.50 |
| Total | 100.00 |
| Nutrient levels[c] | |
| Crude protein | 29.22 |
| Ether extract | 6.75 |
| Lys | 2.20 |
| Met | 1.00 |
| Thr | 1.50 |
| Total phosphorus | 1.21 |

[a]The vitamin premix provided the following per kg of the diet: VA 8000 IU, VC 500 mg, $VD_3$ 3000 IU, VE 60 mg, $VK_3$ 5 mg, $VB_2$ 30 mg, $VB_6$ 15 mg, $VB_{12}$ 0.5 mg, choline chloride 5000 mg, nicotinic acid 175 mg, D-biotin 2.5 mg, inositol 1000 mg, folic acid 5 mg, pantothenic acid 50 mg

[b]The mineral premix provided the following per kg of the diet: Zn 25 mg, Cu 3 mg, Fe 25 mg, Mn 15 mg, I 0.6 mg, Co 0.1 mg, Se 0.4 mg

[c]The crude protein and ether extract values were measured, while other values were calculated

−80 °C until analyzed. The activity of GS was determined by a kit from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China) based on the chemical colorimetry method. The GS activity of intestinal, liver and serum was expressed in U $mg^{-1}$ protein. One unit of GS activity was defined as a 37 °C reaction generated by 1 μmol of γ-glutamine oxime acid oxygen per hour. The protein concentration of the samples was measured using the method of Coomassie light blue [16].

### Quantitative analysis of GS, Rh, TOR and 4E-BP1 glycoprotein gene expression levels

Fish were netted to extract the total RNA from the intestine and gill tissue using the SV total RNA Isolation System (Promega, Madison, USA) according to the manufacturer's instructions. To test the RNA quality, the purity of all of the RNA samples was measured using Thermo Scientific Evolution 260 Bio (Massachusetts, USA), and the integrity of the RNA samples was analyzed using 1% agarose gel electrophoresis. According to the conservative regions of cyprinid fish cDNA published by the National Centre for Biotechnology Information (NCBI), the degenerate primers were designed for a homologous clone (Table 2). The expression primers were designed according to the complete open reading frames of the GS, Rh (Rhag, Rhbg and Rhcg), TOR and 4E-BP1 glycoprotein genes. The β-actin and 18s rRNA genes were selected as endogenous genes. Relative quantification analysis was performed using a 7500 real-time PCR system (ABI, USA). The reaction volume was 20 μl, including 10 μl of 2 × SYBR Premix ExTaqTM (Takara, ID: DRR081A), 0.4 μl of 50 × ROX Reference Dyell, 0.4 μl of each primer (10 mM), 1.0 μl of cDNA solution and 7.8 μl of sterilized water. The program was as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 57.5 °C for 34 s, and the dissociation stage. Two repeats were performed for each sample. The results were analyzed using 7500 software 2.0.6 (ABI) using double endogenous genes and the ddCt method [17].

### Statistical analyses

All of the data are presented as the mean ± standard error (X ± SE) and were analyzed using SPSS 17.0 for

**Table 2** Fluorescence quantitative primer sequences

| Positive primer | Primer sequence (5′–3′) | Reaction primer | Primer sequence (5′–3′) |
|---|---|---|---|
| GS-EF | TCGTGGGTACCGACCGATGGACAT | GS-ER | CTACCCACAAGTGATCTCCC |
| Rhag-EF | TAATCCTGGAAGCCGTGACC | Rhag-ER | GTTGATGCCCACACTGCTGA |
| Rhbg-EF | ATGCAGGGCTTTTTCCACGG | Rhbg-ER | TGGATCCGCCAGCATCATTG |
| Rhcg-EF | TGCAGATTTCTGTGTGGCGG | Rhcg-ER | CTCTCATCCAAATCAGGCCG |
| TOR-EF2 | CCACAACGCAGCCAACAA | TOR-ER2 | GCCACAGAATAGCAACCCT |
| 4E-BP1-EF | GCTACCTCACGACTATTGC | 4E-BP1-ER | TTCTTGCTTGTCACTCCTG |
| 18S-F | GGAGGTTCGAAGACGATCAG | 18S-R | GTGAGGTTTCCCGTGTTGAG |
| Actin-F | GATCGGCAATGAGCGTTTCC | Actin-R | ACGGTGTTGGCATACAGGTC |

Windows. Data were analyzed by one-way ANOVA after homogeneity of variance test. When significant differences were found, Duncan's multiple comparison tests were used to identify differences among experimental groups. Differences were considered significant at the level $P < 0.05$.

## Results

### Growth performance

Compared with the control group, the feed conversion ratios (FCRs) of the AKG group and AAKG group were significantly lower ($P < 0.05$), whereas the FCR of the AAKG group was significantly lower than that of the AKG group ($P < 0.05$) (Table 3). The weight gain rate (WGR), protein efficiency ratio (PER, and condition factor (CF) of the AAKG group were significantly higher than those of the control group ($P < 0.05$), whereas the viscerosomatic indices (VSIs) of the fish body in the Glu group, the AAKG group, and the 2Na-AKG group were significantly lower than that of the control group ($P < 0.05$). There were no significant differences in the growth parameters between the Gln group and the control group ($P > 0.05$).

### Rh gene expression and blood ammonia levels

The expression level of the Rhag gene in the gills of the fish in the 2Na-AKG group was significantly higher than that in the control group ($P < 0.05$), whereas no significant differences were observed between the other groups and the control group ($P > 0.05$) (Table 4). The expression levels of the Rhbg gene in the gills of the AKG, AAKG and 2Na-AKG groups were significantly higher than that in the control group ($P < 0.05$), whereas the expression level of the Rhbg gene in the gills of the Gln group was significantly lower than that of the control group ($P < 0.05$) (Table 4). The expression levels of the Rhcg gene in the gills of the AKG and OKG groups were significantly higher than those of the control group and the other groups ($P < 0.05$) (Table 4). Compared with the control group, the blood ammonia level of the fish in the Gln group was significantly higher ($P < 0.05$) (Table 4); there were no significant differences between the other groups and the control group ($P > 0.05$).

### Protein synthesis pathway

The expression levels of the TOR gene in the gut of the fish in the Glu group and the AAKG group were significantly higher than those in the other groups ($P < 0.05$) (Table 4). The expression levels of the TOR gene in the gut of the fish in the AKG group and the Glu group were significantly

**Table 3** Effects of Gln and its precursors on growth performance of mirror carp

| Items | FCR | WGR (%) | PER | CF | VSI (%) |
|---|---|---|---|---|---|
| Control | $1.48 \pm 0.04^{a}$ | $275.33 \pm 4.62^{bc}$ | $245.67 \pm 2.52^{bc}$ | $1.8 \pm 0.20^{b}$ | $12.55 \pm 1.31^{a}$ |
| Gln | $1.44 \pm 0.04^{ab}$ | $262.33 \pm 21.20^{c}$ | $245.67 \pm 7.64^{bc}$ | $1.93 \pm 0.12^{b}$ | $11.91 \pm 0.96^{ab}$ |
| Glu | $1.50 \pm 0.05^{a}$ | $264.33 \pm 5.86^{c}$ | $236.67 \pm 7.77^{c}$ | $1.96 \pm 0.10^{b}$ | $10.24 \pm 0.51^{c}$ |
| AKG | $1.38 \pm 0.04^{b}$ | $282.00 \pm 8.89^{abc}$ | $260.00 \pm 3.46^{ab}$ | $1.97 \pm 0.11^{b}$ | $13.07 \pm 1.84^{a}$ |
| OKG | $1.44 \pm 0.02^{ab}$ | $289.00 \pm 8.00^{ab}$ | $245.33 \pm 4.16^{bc}$ | $1.96 \pm 0.11^{b}$ | $11.03 \pm 1.21^{bc}$ |
| AAKG | $1.25 \pm 0.03^{c}$ | $298.67 \pm 7.23^{a}$ | $274.67 \pm 17.90^{a}$ | $2.23 \pm 0.23^{a}$ | $10.18 \pm 0.75^{c}$ |
| 2Na-AKG | $1.47 \pm 0.05^{a}$ | $294.00 \pm 13.89^{ab}$ | $234.8 \pm 11.17^{c}$ | $2.25 \pm 0.31^{a}$ | $10.08 \pm 1.16^{c}$ |

Values are mean ± SE ($n = 5$). Data with different letters in the same line mean significant difference from others ($P < 0.05$)

**Table 4** Effects of Gln and its precursors on gene expression and blood ammonia of mirror carp

| Items | Rhag-gill | Rhbg-gill | Rhcg-gill | TOR-gut | 4E-BP1-gut | GS-gut | GS-liver | Blood ammonia (mol $l^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| Control | $1.621 \pm 0.288^{bc}$ | $3.193 \pm 0.887^{c}$ | $0.018 \pm 0.004^{b}$ | $1.251 \pm 0.145^{c}$ | $2.005 \pm 0.580^{b}$ | $0.689 \pm 0.180^{d}$ | $0.639 \pm 0.208^{c}$ | $548.67 \pm 64.20^{b}$ |
| Gln | $0.502 \pm 0.067^{c}$ | $1.410 \pm 0.274^{d}$ | $0.017 \pm 0.007^{b}$ | $3.529 \pm 0.430^{b}$ | $14.005 \pm 0.327^{a}$ | $14.842 \pm 3.363^{ab}$ | $0.742 \pm 0.307^{bc}$ | $747.35 \pm 90.68^{a}$ |
| Glu | $2.211 \pm 0.248^{bc}$ | $4.030 \pm 0.273^{bc}$ | $0.027 \pm 0.005^{b}$ | $5.748 \pm 0.323^{a}$ | $14.725 \pm 0.088^{a}$ | $17.321 \pm 1.033^{a}$ | $0.896 \pm 0.281^{bc}$ | $611.00 \pm 23.74^{ab}$ |
| AKG | $3.958 \pm 1.127^{ab}$ | $7.128 \pm 0.094^{a}$ | $0.174 \pm 0.092^{a}$ | $3.548 \pm 0.376^{b}$ | $15.985 \pm 1.116^{a}$ | $18.608 \pm 0.453^{a}$ | $1.412 \pm 0.178^{ab}$ | $493.12 \pm 31.28^{b}$ |
| OKG | $1.10 \pm 0.330^{bc}$ | $2.999 \pm 0.304^{c}$ | $0.240 \pm 0.010^{a}$ | $1.578 \pm 0.027^{bc}$ | $4.348 \pm 1.114^{b}$ | $2.166 \pm 0.057^{d}$ | $0.755 \pm 0.255^{bc}$ | $517.81 \pm 40.43^{b}$ |
| AAKG | $1.439 \pm 0.013^{bc}$ | $4.770 \pm 0.357^{b}$ | $0.028 \pm 0.008^{b}$ | $6.086 \pm 1.153^{a}$ | $16.704 \pm 3.889^{a}$ | $9.352 \pm 0.682^{bc}$ | $1.780 \pm 0.113^{a}$ | $558.86 \pm 38.14^{b}$ |
| 2Na-AKG | $5.420 \pm 1.736^{a}$ | $7.168 \pm 0.065^{a}$ | $0.028 \pm 0.007^{b}$ | $2.470 \pm 0.077^{bc}$ | $19.878 \pm 4.864^{a}$ | $6.476 \pm 0.483^{cd}$ | $0.42 \pm 0.051^{c}$ | $583.49 \pm 57.66^{b}$ |

Values are mean ± SE. Data with different letters in the same line mean significant difference from others ($P < 0.05$)

higher than that in the control group ($P < 0.05$). Compared with the control group, the expression levels of the 4E-BP1 gene in the gut of the fish in all groups except the OKG group were significantly higher ($P < 0.05$) (Table 4).

### GS gene expression and contents

Compared with the control group, the expression levels of the GS gene in the gut of the fish in the Gln, Glu, AKG and AAKG groups were significantly higher ($P < 0.05$) (Table 4). The expression levels of the GS gene in the liver of the fish in the AKG and AAKG groups were significantly higher than that in the control group ($P < 0.05$), whereas no significant differences between the other groups and the control group were observed ($P > 0.05$) (Table 4).

Except for the Gln group, the GS activities in the hindgut of all other groups were significantly lower than that of the control group ($P < 0.05$) (Table 5). The GS activities in the foreguts and midguts of all other groups showed no significant differences when compared with those of the control group ($P > 0.05$). The GS activities in the liver of the AAKG group were significantly higher than those of the control group and the other groups ($P < 0.05$), whereas no significant differences were observed between the other groups and the control group ($P > 0.05$). Compared with the control group, the GS level in the serum of the Glu group was significantly higher ($P < 0.05$) while the GS level in the serum of the 2Na-AKG group was significantly lower ($P < 0.05$).

## Discussion

### Effects of Gln and its precursors on the growth performance of *Cyprinus carpio*

To date, the effects of Gln precursors on the growth performance of aquatic animals have rarely been investigated. The few relevant reports mainly focused on Gln itself. Lin et al. [6] showed that the WGR of juvenile *Cyprinus carpio* significantly increased in proportion with the level of Gln additives in feed [6]. However, no significant impacts of Gln additives on growth were found in juvenile rainbow trout *Oncorhynchus mykiss*, hybrid tilapia, large yellow croaker *Larimichthys crocea* and juvenile *Pelteobagrus fulvidraco*, although they had a significant impact on feed utilization in hybrid tilapia and juvenile rainbow trout [7, 18–20]. In this study, the addition of 1.5% Gln in the basal diet showed no significant effects on growth parameters, including the growth, FCR, and protein efficiency of the feed, indicating that the effects of Gln on the growth performance of fish may primarily depend on interspecific differences of fish.

Wei et al. [21] found that the addition of AKG in the feed of different protein sources had a certain impact on the WGR, FCR and protein efficiency of *Cyprinus carpio*, but the differences were not significant [21]. Li et al. [24] examined glutamine substitutes and showed that the addition of AKG in plant protein feed significantly reduced the FCR of *Cyprinus carpio*, while it did not significantly increase the WGR, which was consistent with the findings in this study [24]. Chen [23] showed that AKG could significantly increase the WGR and protein efficiency of juvenile *Acipenser schrenckii* ♀ × *A. baeri* ♂ and significantly reduce the FCR [23], suggesting that there may be species differences in the effects of AKG on the growth performance and feed utilization of fish. In the present study, the addition of Gln precursor AAKG significantly enhanced the growth performance of carp, indicating that arginine may have a positive effect with AKG, resulting in a more significant effect of AAKG on the growth of the fish.

### Effects of Gln and its precursors on Rh gene expression and ammonia content in the gills of *Cyprinus carpio*

Ammonia is a natural metabolite and plays an important role in maintaining the acid–base metabolic balance in the body [25, 26]. However, excessive levels of ammonia are toxic. To date, numerous studies on ammonia transport in the body have been conducted [27–30]. As the numbers of studies on Rh genes in animals have increased, the important roles of Rh glycoproteins in ammonia transport processes of aquatic

**Table 5** Effects of Gln and its precursors on GS activity of mirror carp (U/mg protein)

| Items | Foregut | Midgut | Hindgut | Liver | Serum |
|---|---|---|---|---|---|
| Control | $9.86 \pm 0.65^{ab}$ | $1.72 \pm 0.12$ | $15.95 \pm 1.48^{a}$ | $4.77 \pm 0.46^{bcd}$ | $75.31 \pm 4.37^{bc}$ |
| Gln | $9.57 \pm 0.87^{b}$ | $2.14 \pm 0.19$ | $17.72 \pm 1.80^{a}$ | $5.01 \pm 0.46^{bcd}$ | $94.29 \pm 6.85^{b}$ |
| Glu | $15.06 \pm 1.57^{a}$ | $2.29 \pm 0.23$ | $4.28 \pm 0.39^{b}$ | $6.61 \pm 0.59^{b}$ | $125.49 \pm 11.72^{a}$ |
| AKG | $9.14 \pm 0.59^{b}$ | $3.09 \pm 0.30$ | $7.21 \pm 0.54^{b}$ | $3.13 \pm 0.30^{d}$ | $77.19 \pm 5.69^{bc}$ |
| OKG | $6.20 \pm 0.48^{b}$ | $2.53 \pm 0.17$ | $5.74 \pm 0.25^{b}$ | $3.75 \pm 0.38^{cd}$ | $103.30 \pm 7.28^{ab}$ |
| AAKG | $9.87 \pm 0.63^{ab}$ | $2.04 \pm 0.16$ | $6.46 \pm 0.22^{b}$ | $9.30 \pm 0.74^{a}$ | $53.19 \pm 3.39^{cd}$ |
| 2Na-AKG | $8.83 \pm 0.77^{b}$ | $2.13 \pm 0.21$ | $6.57 \pm 0.40^{b}$ | $5.87 \pm 0.44^{bc}$ | $43.08 \pm 4.23^{d}$ |

Values are mean ± SE ($n = 10$). Data with different letters in the same line mean significant difference from others ($P < 0.05$)

animals have been confirmed [31, 32]. Correspondingly, the model of ammonia transport in fish has also been proposed [30]. Dong et al. [33] successfully cloned the full-length cDNA sequences of Rhag, Rhbg, and Rhcg1 of the Rh glycoprotein family in carp and confirmed that the expression levels of these three genes in the gills of carp were significantly higher than those in other tissues [33]. Furthermore, the expression of Rhbg was significantly higher than the other two glycoproteins.

Dong et al. [34] suggested AKG, Glu and Gln significantly up-regulated the expressions of Rhag and Rhbg in the gills of Songpu mirror carp, and OKG had no significant impact on Rhbg and Rhcg but resulted in a downward trend for Rhag [34], which were consistent with those of this study. The results of this study also showed that all the substitutes had certain effects on the ammonia excretion of *Cyprinus carpio*. Gln down-regulated the gene expression of Rhag in the gills and inhibited the release of ammonia in red blood cells. Gln also down-regulated the expressions of the Rhbg and Rhcg genes and inhibited the excretion of ammonia. AKG up-regulated gene expression in all three groups and promoted ammonia excretion but did not significantly reduce ammonia content and was not conducive to the deposition of nitrogen. Further studies are needed on their effect mechanisms.

### Effects of Gln and its precursors on the protein synthesis pathway in *Cyprinus carpio*

TOR is a key regulator of cell growth. Through the integration of intracellular nutrients and extracellular signals of growth, TOR regulates translation, transcription, autolysis and other physiological events in cells [35]. TOR can mediate cell growth through 4E-BP1 signaling [36]. 4E-BP1 can bind to the eukaryotic initiation factor 4E in the ternary eukaryotic initiation factor 4F to inhibit the initiation of translation. TOR can also phosphorylate 4E-BP1, enabling 4E-BP1 to leave the eukaryotic initiation factor 4E, leading to the initiation of the translation process [37, 38]. In the study by Xiao et al. [39], the addition of Gln in the diet of weaned piglets significantly improved the intestinal expression of the TOR gene at the 30th day [39]. Wang [40] found that the addition of 1% AKG in diet significantly improved the phosphorylation of mTOR in pigs and promoted protein synthesis and growth [40]. Jiang [41] found that the addition of Gln alone in culture media for carp intestinal epithelial cells enhanced the expression of the TOR gene by 47% and increased the protein synthesis of the intestinal epithelial cells by 125% [41], consistent with the results of this study. The results of this study showed that the expression of the TOR and 4E-BP1 genes in the gut had similar trends; Gln, Glu, AKG and AAKG significantly up-regulated the expression of TOR and 4E-BP1 in the gut and promoted protein synthesis.

### Effects of Gln and its precursors on the expression of GS gene and its activity in *Cyprinus carpio*

In many animals, the main source of energy for intestinal mucosal cells and other rapidly growing cells is Gln, not glucose. Gln can be supplied exogenously, but because of its instability, Gln can be easily thermally decomposed into toxic pyroglutamic acid and ammonia. These drawbacks have limited the utilization of Gln. Gln can also be endogenously synthesized but not at the levels required to meet the body's demands. GS is an in vivo catalyst for the conversion of Glu to Gln. In recent years, many researchers have explored the function of GS in plants and animals [42–44]. The existence of GS is conducive to the in vivo conversion of glutamate and ammonia into Gln and to the reduction of ammonia toxicity. Dong et al. [45] demonstrated that the gut is the most important organ of Gln synthesis in carp, and the addition of Glu, OKG and Gln in feed significantly up-regulated the expression of the GS gene in the foregut, midgut, and hindgut; furthermore, GS activity significantly increased with the addition of AKG and Glu [45]. Jiang [41] added Gln to culture media for carp intestinal epithelial cells and observed up-regulated GS gene expression, consistent with the results of this study [41]. Chen [23] added AKG in sturgeon feed and observed enhanced GS activity and Glu content in the gut [23]; these results differed from those of this study. These conflicting results were likely caused by differences in the digestive tract structures of sturgeon and carp. In the present study, the GS activities in the serum were different from those in the gut and liver among treatments, indicating that there are different GS activities in different tissues of the fish. The results of this study showed that Gln, Glu, AKG and AAKG could significantly up-regulate the expression of the GS gene in the gut. However, the expression of GS in the liver was not significantly up-regulated, likely because the gut is the main site of Gln synthesis. In this study, GS gene expression was up-regulated, but GS activity was not significantly enhanced. This indicated that the amount of the GS enzyme and its activity were not significantly correlated and that other regulatory mechanisms may control GS activity.

The addition of AAKG in feed can significantly reduce the FCR of *Cyprinus carpio* and significantly improve the WGR and protein efficiency of the fish. Gln can reduce ammonia release in gills, and AKG can effectively promote the excretion of ammonia. The addition of Gln, Glu, AKG and AAKG in diets can effectively promote protein synthesis. Gln, Glu, AKG and AAKG can significantly up-regulate GS gene expression in the gut; however, the expression

level of the GS gene is not significantly correlated with GS activity.

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