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Research Article

Effects of Adiponectin on Glucose Metabolism in the Hepatopancreas of Grass Carp (*Ctenopharyngodon idella*)

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Adiponectin plays critical roles in the regulation of energy metabolism in mammals. However, its roles in fish remain to be evaluated. In this study, we examined the tissue distribution characteristics of two adiponectin genes, namely, *adipoqa* and *adipoqb*, at transcriptional level, and detected the changes of their mRNA expressions during oral glucose tolerance test (OGTT). Furthermore, we prepared recombinant proteins of grass carp adiponectin A and adiponectin B and studied their roles in the regulation of glucose metabolism in the hepatopancreas of grass carp *in vivo*. The results showed that *adipoqa* mRNA was mainly expressed in the heart of grass carp, followed by red muscle, white muscle, and adipose tissue, while *adipoqb* mRNA was highly expressed in adipose tissue, red muscle, and white muscle. The mRNA levels of *adipoqa* and *adipoqb* in the white muscle and heart were significantly decreased at 1 h post OGTT, and their expressions in the heart were also suppressed at 3 h, whereas the transcriptional levels of *adipoqa* and *adipoqb* in the red muscle were elevated significantly at 12 h after glucose load. Intraperitoneal injection of adiponectin A or adiponectin B reduced basal serum glucose levels of grass carp and caused an increase of glycogen content in the hepatopancreas. Adiponectin A upregulated the expression of enzyme genes related to glycolysis (*gk* and *pk*) and glycogen synthesis (*gys*) in the hepatopancreas, and adiponectin B promoted the expression of *pk* and *gys*, as well as *g6pase* in the glycogenetic pathway. Besides, adiponectin A and adiponectin B have opposite regulatory effects on *glut2* mRNA expression. These results suggested that adiponectin A and adiponectin B promoted glycolysis, gluconeogenesis, and glycogen synthesis, increased glycogen content of hepatopancreas, and reduced basal blood glucose level, and they may have an antagonistic regulatory mechanism in regulating glucose uptake.

1. Introduction

Carbohydrates are important economical energy nutrient in the feed of commercial fish. Studies have shown that carbohydrates can reduce the consumption of protein used for energy and have a protein sparing effect [1]. Compared to mammals, however, fish are limited in their ability to use carbohydrates, and they are prone to persistent hyperglycemia after carbohydrate intake [2]. Furthermore, high level of dietary carbohydrates leads to hepatic accumulation of glycogen and lipid, and metabolic disorders and decreases antioxidant capacity and immunity [3, 4]. The utilization of carbohydrates and glucose metabolism regulation in fish have become one of the hot topics in the fish nutrition research in the past years. The regulation of glucose homeostasis in fish is a complex process, which is affected by multiple factors, and the endocrine system plays a key role in this process. The variability in physiological function of some endocrine factors may contribute to glucose intolerance in fish. For example, insulin classically promotes glucose disposal in mammals, whereas it has the opposite effect in some fish species, e.g., it inhibits glucose disposal in rainbow trout (*Oncorhynchus mykiss*) [5].

Cytokines derived from adipocytes play an important role in the regulation of glucose metabolism in mammals, and they have attracted extensive attention. Adiponectin, which is predominately produced by adipose tissue in mammals, is one of the most important adipocytokines, and it has high circulating levels at micrograms per milliliter [6]. Adiponectin precursor contains four domains, namely, N-

terminal signal peptide, variable domain, collagenous domain, and C-terminal globular domain [7], and the collagenous domain consists of 22 Gly-X-Y repeats [8]. Adiponectin is well known for its remarkable effect in improving insulin sensitivity and lowering blood glucose. It stimulates glucose uptake and fatty acid oxidation by binding to its receptors (typically AdipoR1 and AdipoR2) and activating AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor alpha (PPARα) in the liver and skeletal muscle [9, 10].

As one of the main target organs of insulin, the liver plays a key role in the regulation of metabolic processes in response to nutritional changes. Research has shown that adiponectin enhances hepatic glucose and lipid metabolism via the cross-talk between liver and adipose tissue [11]. In the liver of mouse, adiponectin activates AMPK signaling pathway, promotes the phosphorylation of acetyl-CoA carboxylase (ACC), and inhibits mRNA expression of gluconeogenic rate-limiting enzymes [10]. It was also found that knockout of adiponectin gene in mice inhibited mRNA expression of rate-limiting enzymes in several important pathways involved in glucose and lipid metabolism in the liver, including glycolysis, fatty acid activation, and synthesis and triglyceride synthesis [12]. Injection of recombinant adiponectin in mice triggered a decrease in basal plasma glucose levels, and this effect was due to the increased ability of insulin to suppress glucose production in the liver [13].

The roles of adiponectin in the energy metabolism of fish are not fully understood, and there are only few publications in fish species at present. The first publication about adiponectin in teleosts was reported in 2008, in which two different genes coding adiponectin A and adiponectin B were characterized in zebrafish (*Danio rerio*) [14]. Since then, adiponectin in several fish species has been reported, including rainbow trout [15, 16], ayu (*Larimichthys crocea*) [17], and large yellow croaker (*Larimichthys crocea*) [18]. Besides, adiponectin receptors (AdipoRs) were also identified in some fish species, such as zebrafish [14], rainbow trout [16], orange-spotted grouper (*Epinephelus coioides*) [19], Nile tilapia (*Oreochromis niloticus*) [20], black carp (*Mylopharyngodon piceus*) [21], and grass carp [22, 23]. In zebrafish and grass carp, AdipoRs are coded by three different genes, namely, *adipoR1a*, *adipoR1b*, and *adipoR2*, and the multiple isoforms of adiponectin and its receptors are likely resulting from gene or genome duplication events [14, 22, 23].

In teleosts, nutritional status has profound effects on the expression of adiponectin and its receptors, indicating that adiponectin/AdipoRs system plays an important role in the regulation of energy homeostasis [24]. For example, high level of dietary carbohydrate significantly promoted adiponectin receptor expression in the hepatopancreas of black carp [21]. It was found that adiponectin promotes fatty acid (FA) synthesis, oxidation, and transport through AdipoRs-APPL1 activated PPARγ in the muscle of large yellow croaker [18]. In rainbow trout, adiponectin stimulated glucose uptake in the cultured adipocytes [25]. Furthermore, adiponectin increased FA oxidation but had no effect on glucose uptake in the muscle cells of rainbow trout [16]. However, the roles of adiponectin in the hepatic glucose metabolism of fish have not been reported. High mRNA levels of adiponectin receptors were detected in the liver of fish, such as zebrafish [14], black carp [21], and large yellow croaker [18]. Our previous studies found that *adipoR1b* mRNA level was highest in the hepatopancreas of grass carp [26], and the mRNA expressions of all types of adiponectin receptors (*adipoR1a*, *adipoR1b*, and *adipoR2*) were significantly enhanced by insulin, indicating that AdipoRs might act as the targets of insulin in grass carp [22, 23]. Therefore, we speculated that adiponectin might be involved in the regulation of glucose metabolism in the hepatopancreas of grass carp.

The aim of this study was to explore the roles of adiponectin in the regulation of glucose metabolism in the hepatopancreas of grass carp. We cloned the cDNAs of *adipoqa* and *adipoqb*, which encoded adiponectin A and adiponectin B, respectively, and examined their tissue distribution and investigated the changes of their expression during oral glucose tolerance test (OGTT). Furthermore, recombinant grass carp adiponectin A and adiponectin B were prepared and then intraperitoneally injected into grass carp to evaluate their effects on glucose metabolism in the hepatopancreas. This may help us to get a better understanding about the physiological functions of adiponectin in the energy metabolism of cultured fishes.

2. Materials and Methods

2.1. Molecular Cloning. Previously, we have obtained the EST (Expressed Sequence Tag) sequences of *adiponectin A* and *adiponectin B* in grass carp, which were then assembled to obtain their cDNA sequences. In order to confirm the complete transcripts, primers (Table 1) were designed to amplify the open reading frame (ORF) sequences. PCR products were purified, subcloned into pMD™19-T, and transformed into *E. coli* DH5α, and finally, three positive monoclones were selected for sequencing. The sequences of signal peptide were predicted by SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP-4.0/). The phylogenetic tree of adiponectin from different species was constructed using the MEGA 7.0 software by neighbor-joining method based on their amino acid sequences, and the bootstrap value was 1000.The homology analysis was performed by DNAStar software with Clustal W method.

2.2. Preparation of Recombinant Adiponectin Proteins. E. coli strain BL21 was transformed with pET22b (+) or pET21a (+) vector containing the coding sequences of an N-terminal 6 × His tag, mature adiponectin A (25-258), or adiponectin B (34-290) residues, respectively. BL21 monoclonal cells containing each plasmid were cultured overnight in 5 mL LB medium with 100 μg/mL ampicillin. The culture was inoculated into fresh Amp^+ LB medium at a ratio of 1 : 50 and shaken at 37°C until the OD600 was approximately 0.6. Afterwards, the expression of His-tagged adiponectin A or adiponectin B was induced by 1 mM isopropyl β-thiogalactopyranoside (IPTG) in LB medium for 0, 1, 2, 3, and 4 h. The samples with volumes of 1 mL were taken at each time point after induction and prepared for SDS-

Table 1: Primers used for cloning and RT-qPCR of *adipoqa* and *adipoqb*.

Gene	5′-3′ sequences	Product size
Primers for ORF cloning		
adipoqa	F: GCTCAAACTACAACTCCACACAA	977 bp
	R: TAGCAGGGTGGCAGGAAGAC	
adipoqb	F: GCCATTT GCATCAAGAAGTT	1134 bp
	R: CCCAGCACCTCTCCTAGAGT	
Primers for RT-qPCR		
adipoqa	F: GGAAACCCAGGTCTGAAAGGA	225 bp
	R: CTTCCCGTTGATAGTGAGGTGAT	
adipoqb	F: CTTCTACAACGACCAGCACCAC	185 bp
	R: CCTGATCCACATTGGTTTCCTG	

PAGE analysis, and the BL21 cells transfected with empty expression vector were used as negative control. The remaining culture was centrifuged, and the cells were resuspended with binding buffer (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazoleand 6 M urea). After sonication and centrifugation, the supernatant was filtered and loaded into HisTrap Excel column (GE, USA). Purified proteins were washed with elution buffer (20 mM Tris-HCl, 500 mM NaCl, 6 M urea, and 250 mM imidazole). The purified recombinant proteins were successively dialyzed with PBS containing 4, 2, and 0 M urea at 4°C for 12 h, twice for each solution. The purified proteins were analyzed by SDS-PAGE, and the concentrations were determined by BCA assay (Solarbio, China).

2.3. Animal Experiments. Grass carp was acclimated in tanks (1500 L) connected to a freshwater recirculation system for 2 weeks, and they were fed with commercial diets four times a day (8 : 30, 11 : 30, 14 : 30, and 17 : 30). The average water temperature was 23 ± 1°C, and the photoperiod was kept at 12 h light/dark cycle. Dissolved oxygen was maintained above 5.0 mg/L by continuous aeration, total ammonia concentration is below 0.2 mg/L, and pH value is stable at 7.3 ± 0.1. All the procedures involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Henan Normal University and followed the animal welfare and ethics.

As for tissue expression analysis, three fish with body weight of 65 ± 5 g from each tank (three tanks) were randomly anesthetized with MS-222. Samples of telencephalon, mesencephalon, cerebellum, hypothalamus, pituitary gland, head kidney, kidney, heart, hepatopancreas, spleen, proximal intestine, mid intestine, distal intestine, adipose tissue, gill, white muscle, and red muscle were collected quickly, snap-frozen in liquid nitrogen, and then stored in -80°C until RNA extraction.

Oral glucose tolerance test (OGTT) was conducted according to our previous study with slight modification [27]. Briefly, grass carp (55 ± 5 g) was acclimated for two weeks and fasted for 24 h. Then, fish were anesthetized with MS-222, and oral administration was performed by means of oral gavage using a syringe with a gavage needle, which was passed through the mouth and into the esophagus. Fish were administrated with either glucose in PBS (1.67 mg/g BW) or PBS (control) [27]. Samples of heart, adipose tissue, red muscle, and white muscle from nine fish (three fish from each tank, 3 tanks per treatment at each time) were collected quickly at 1, 3, 6, and 12 h post administration. The samples were frozen in liquid nitrogen immediately and transferred to -80°C until RNA extraction.

As for injection of adiponectin, grass carp (55 ± 5 g) were acclimated for two weeks, fasted for 24 h, and then injected intraperitoneally with the prepared recombinant protein of adiponectin A or adiponectin B at concentrations of 5, 50, and 500 ng/g BW. Fish in the control group were injected with PBS according to body weight. Nine fish were injected in each group (three fish per tank, three tanks). Blood was collected from the caudal vein at 1 h post injection, and serum was collected by centrifugation and temporarily stored at -80°C. Hepatopancreas samples were also collected, snap-frozen in liquid nitrogen, and stored at -80°C.

2.4. Determination of Serum Glucose and Hepatopancreas Glycogen Content. Levels of serum glucose and hepatopancreas glycogen were determined with glucose assay kit and glycogen assay kit, respectively. All the kits were purchased from Nanjing Jiancheng Bioengineering Institute (China) and performed according to the manufacturer's protocol. Three technical duplicates were performed for each samples when testing all of the above indicators.

2.5. Real-Time Quantitative PCR (RT-qPCR). Total RNA was extracted with RNAiso Plus (TaKaRa, China) according to standard method. RNA integrity was confirmed by agarose gel electrophoresis, and RNA concentrations and purity were assessed by NanoDrop 2000C spectrophotometer (Thermo Scientific, USA). The first-strand cDNA synthesis was carried out with 1 μg total RNA using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, China) according to the manufacturer's protocol. The relative gene expression levels were determined by RT-qPCR, which was performed using LightCycler 480 II Instrument (Roche, USA). RT-qPCR was performed in a final volume of 10 μL containing 0.3 μL of each primer (10 μM), 1 μL of 10-fold diluted cDNA, 3.4 μL of sterile double-distilled water, and 5 μL of SYBR Green qPCR Mix (Dongsheng, China). RT-qPCR program contained an initial denaturation at 94°C for 3 min,

Table 2: RT-qPCR primers used for glucose metabolism-related genes.

Gene	Accession no.	Sequences ($5' \rightarrow 3'$)	Product size
gk	GU065314.1	F: GAAGAGCGAGGCTGGAAGG R: CAGAATGCCCTTATCCAAATCC	210 bp
pk	JQ951928.1	F: CCGAGAAAGTCTTCATCGCACAG R: CGTCCAGAACCGCATTAGCCAC	156 bp
g6pase	KY742725.1	F: AAGGACAGCAGGTAGAAGAGG R: ACGGAAAACAAGAAGAGCAG	131 bp
pepck	JQ898294.1	F: ATCGTCACGGAGAACCAA R: CCTGAACACCAAACTTAGCA	202 bp
gys	JQ792167.1	F: CCTCCAGTAACAACTCACAACA R: CAGATAGATTGGTGGTTACGC	277 bp
pygl	JQ782458.1	F: GGTAGAGCGCTCCAGAACA R: TCAACCTGCCAGCCATCTTT	257 bp
glut2	KY763986.1	F: GTCCAGCAGCCATAGCATTAGC R: GCACCATCTCAGCCTCTTCTTG	258 bp
18 s	EU047719.1	F: ATTTCCGACACGGAGAGG R: CATGGGTTTAGGATACGCTC	90 bp

followed by 40 cycles of 95°C for 15 s, 56°C for 15 s, and 72°C for 30 s. The relative gene expression was normalized to *18S* with $2^{-\Delta\Delta C}{}_T$ method. The sequences of primers used for RT-qPCR are shown in Tables 1 and 2.

2.6. Statistical Analysis. All the results are presented as mean ± SEM. In the oral glucose tolerance test (OGTT), independent sample *t*-test was used to compare the differences between treatment group and the corresponding control group. Statistical analysis of all other data was performed by one-way ANOVA followed by Duncan's Multiple Range tests. Value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Cloning of adipoqa and adipoqb. Grass carp *adipoqa* cDNA, which encodes adiponectin A, was 1537 bp in length, containing an ORF of 777 bp and encoding 258 amino acids (Supplementary Figure 1). The *adipoqb* cDNA were 1479 bp in length, which included an ORF of 873 bp and encoded adiponectin B with 290 amino acids (Supplementary Figure 2). Both adiponectin A and adiponectin B contain four domains, including a N-terminal signal peptide, a variable domain, a collagenous domain consisting of 22 Gly-X-Y repeats, and a C-terminal globular domain (Figure 1; Supplementary Figure 1; Supplementary Figure 2). Phylogenetic tree showed two clusters corresponded to adiponectin A and adiponectin B (Supplementary Figure 3). Grass carp adiponectin B clustered with its counterparts of other teleosts, which was separated from that of birds and mammals, and adiponectin A from grass carp clustered with zebrafish adiponectin A with high bootstrap values. The amino acid sequences of grass carp adiponectin A and adiponectin B precursor exhibit 52.2% identity (Table 3). Grass carp adiponectin A has a homology of 83.5% and 55.1% to zebrafish adiponectin A and adiponectin B, respectively, and exhibits 48.0%-54.3% amino acid identities to adiponectin from other species, while adiponectin B of grass carp share a homology of 50.0% and 85.8% to zebrafish adiponectin A and adiponectin B, respectively, and showed 57.3%-72.2% amino acid identities to adiponectin in other species.

3.2. Tissue Distribution of adipoqa and adipoqb. The relative mRNA expressions of *adipoqa* and *adipoqb* were examined in various tissues of grass carp by RT-qPCR (Figure 2). The *adipoqa* mRNA was highly expressed in the heart, red muscle, and white muscle and followed by adipose tissue (Figure 2(a)), whereas *adipoqb* mRNA was highly expressed in the adipose tissue, red muscle, and white muscle (Figure 2(b)).

3.3. Effect of Glucose Administration on adipoqa and adipoqb mRNA Expressions. Grass carp was fasted for 24 h and administrated orally with either glucose or PBS for different times, and then, *adipoqa* and *adipoqb* mRNA levels were detected in the heart, adipose tissue, red muscle, and white muscle. The results showed that *adipoqa* and *adipoqb* mRNA expressions in the heart were decreased at 3 h after glucose administration (Figures 3(a) and 3(b)). In the adipose tissue, compared to the control groups, their mRNA abundances were not altered by glucose treatment at all times selected (Figures 3(c) and 3(d)). In the red muscle, *adipoqa* mRNA levels were decreased at 3 h post OGTT but increased significantly at 12 h, and *adipoqb* mRNA level was also elevated at 12 h (Figures 3(e) and 3(f)). In addition, oral glucose administration inhibited *adipoqa* and *adipoqb* mRNA expressions in the white muscle at 1 h post treatment (Figures 3(g) and 3(h)).

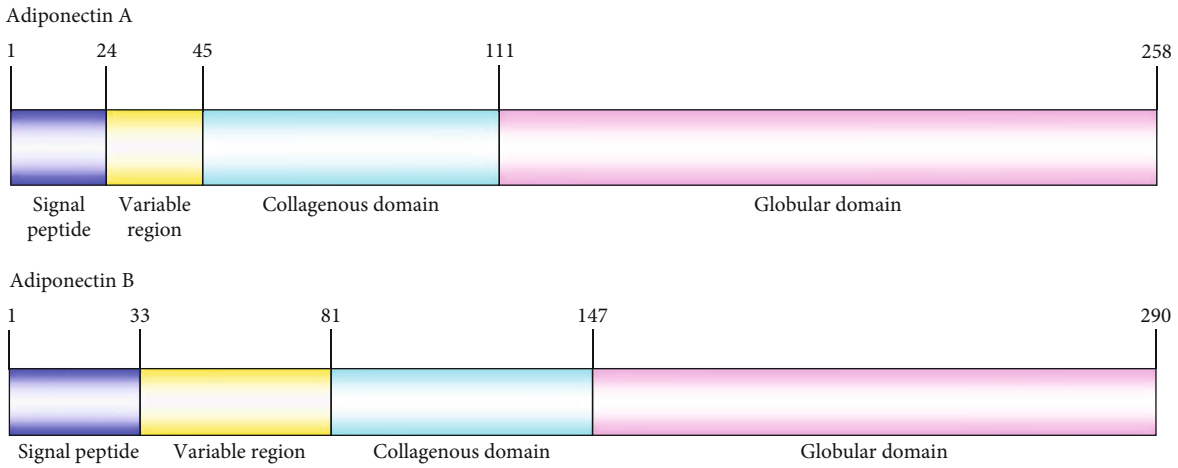

Figure 1: Schematic structure of adiponectin A and adiponectin B in grass carp.

Table 3: Amino acid identities (%) of grass carp and other species.

Adiponectin from different species	*Ctenopharyngodon idellus* adiponectin A	*Ctenopharyngodon idellus* adiponectin B
Ctenopharyngodon idellus adiponectin A	100	52.2
Ctenopharyngodon idellus adiponectin B	52.2	100
Danio rerio adiponectin A	83.5	50.0
Danio rerio adiponectin B	55.1	85.8
Salmo salar	54.3	72.2
Oncorhynchus mykiss	54.3	72.2
Larimichthys crocea	53.1	70.0
Plecoglossus altivelis	55.6	51.4
Gallus gallus	49.2	59.1
Bos taurus	50.0	59.2
Mus musculus	48.4	58.6
Homo sapiens	48.0	57.3

Accession nos. are as follows: *Danio rerio* adiponectin A (NP_001373470.1), *Danio rerio adiponectin* B (NP_001038890.1), *Salmo salar* (XP_013994730.1), *Oncorhynchus mykiss* (NP_001233281.1), *Larimichthys crocea* (QJB75263.1), *Plecoglossus altivelis* (AYQ58339.1), *Bos taurus* (NP_777167.1), *Mus musculus* (NP_033735.3), *Gallus gallus* (NP_996874.1), and *Homo sapiens* (NP_001171271.1).

3.4. Protein Expression and Purification of Recombinant Adiponectin. Recombinant expression plasmid containing the coding sequences of mature adiponectin A or adiponectin B derived from grass carp was transformed into *E. coli* BL21 cells, and the protein expression was induced by IPTG. As for adiponectin A, compared with negative control, a protein band with molecular weight of about 30 kDa appeared on the SDS-PAGE gel after induction for 1-4 h (Figure 4(a)). The recombinant protein was purified by Ni-NTA column affinity chromatography and yielded highly pure adiponectin A with a single band (Figure 4(c)). As for adiponectin B, however, two protein bands appeared after IPTG induction (Figure 4(b)), and the purified recombinant protein of adiponectin B contained two major bands with molecular weight of about 30 kDa and 60 kDa, which can be detected by SDS-PAGE (Figure 4(d)). These two bands were further verified by mass spectrometry (MS) analysis. The result showed that the molecular weights of these two bands were 30.324 kDa and identified peptides matched with the amino acid sequence of grass carp adiponectin B (Supplementary Fig. 4), suggesting that these two purified bands were indeed grass carp adiponectin B.

3.5. Effects of Adiponectin on Serum Glucose and Hepatopancreas Glycogen. Grass carp was injected intraperitoneally with different doses of adiponectin A, adiponectin B, or PBS as control, and the serum glucose levels and hepatopancreas glycogen contents were detected (Figure 5(a)). Compared to the control group, adiponectin A at dose of 500 ng/g BW significantly reduced serum glucose level (Figure 5(a)), and injection of adiponectin B at doses of 5 ng/g BW and 50 ng/g BW also lowered serum glucose levels (Figure 5(b)). The glycogen content in the hepatopancreas of grass carp was significantly higher in all the adiponectin A treatment groups compared to the control group (Figure 5(c)). Similarly, adiponectin B injection also significantly increased hepatopancreas glycogen levels, and this effect was more pronounced at dosage of 5 ng/g BW and 50 ng/g BW (Figure 5(d)).

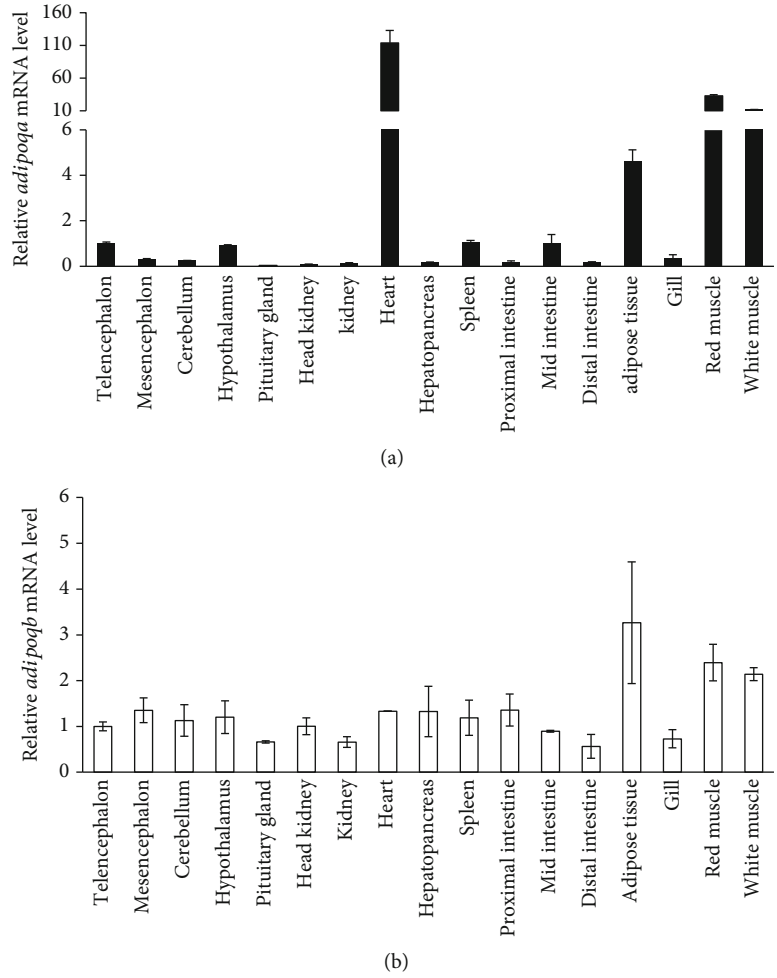

Figure 2: Tissue distribution of *adipoqa* and *adipoqb* mRNAs in grass carp. The relative mRNA expression was normalized against *18S* transcripts. The value represents the multiple of mRNA levels between different tissues and telencephalon. The data were represented as mean ± SEM ($n = 9$).

3.6. Effects of Adiponectin on Glucose Metabolism-Related Gene Expression. Adiponectin was injected intraperitoneally to study glucose metabolism-related gene expression in the hepatopancreas of grass carp (Figure 6). For glycolytic pathway, adiponectin A with dosage of 50 ng/g BW significantly promoted *gk* mRNA expression, and it also enhanced *pk* mRNA level at all concentrations used (Figure 6(a)). While the transcript abundances of *g6pase* and *pepeck* genes, which coding the key gluconeogenic enzymes, was not affected by adiponectin A. Injection with 5, 50, and 500 ng/g BW of adiponectin A promoted the expression of glycogen synthase gene *gys* and inhibited glucose transporter *glut2* mRNA expression but had no effect on the mRNA level of glycogenolysis gene *pygl*. Adiponectin B also promoted *pk* expression in the glycolytic pathway and *gys* expression in the glycogen synthesis pathway, and no significant change was observed about *pepck* and *pygl* mRNA levels in different groups (Figure 6(b)). Inconsistently, however, adiponectin B promoted *g6pase* and *glut2* mRNA expression in the hepatopancreas but had no effect on *gk* mRNA level.

4. Discussion

Carbohydrates are important ingredients in the diets of cultivated fish due to supplying energy at low cost and improving physical quality of feed. The endocrine regulatory network of glucose metabolism in fish has not been sufficiently elucidated, although the roles of pancreatic hormones insulin and glucagon in the glucose homeostasis of fish has been extensively explored [28–30]. Adiponectin and leptin are two major members of adipokines, which have a profound influence on insulin sensitivity and energy balance in mammals [31]. Recently, the roles of leptin in the regulation of energy balance were explored in Yangtze sturgeon (*Acipenser dabryanus*), and it was found that leptin involved in the adaptive regulation of changes in nutritional status, including postprandial, fasting, and food change conditions [32]. However, the role of adiponectin, another important member of adipokines, in the regulation of glucose metabolism in fish remains unclear. The present study identified two different genes, namely, *adipoqa* and *adipoqb*, coding adiponectin A and adiponectin B, respectively. The

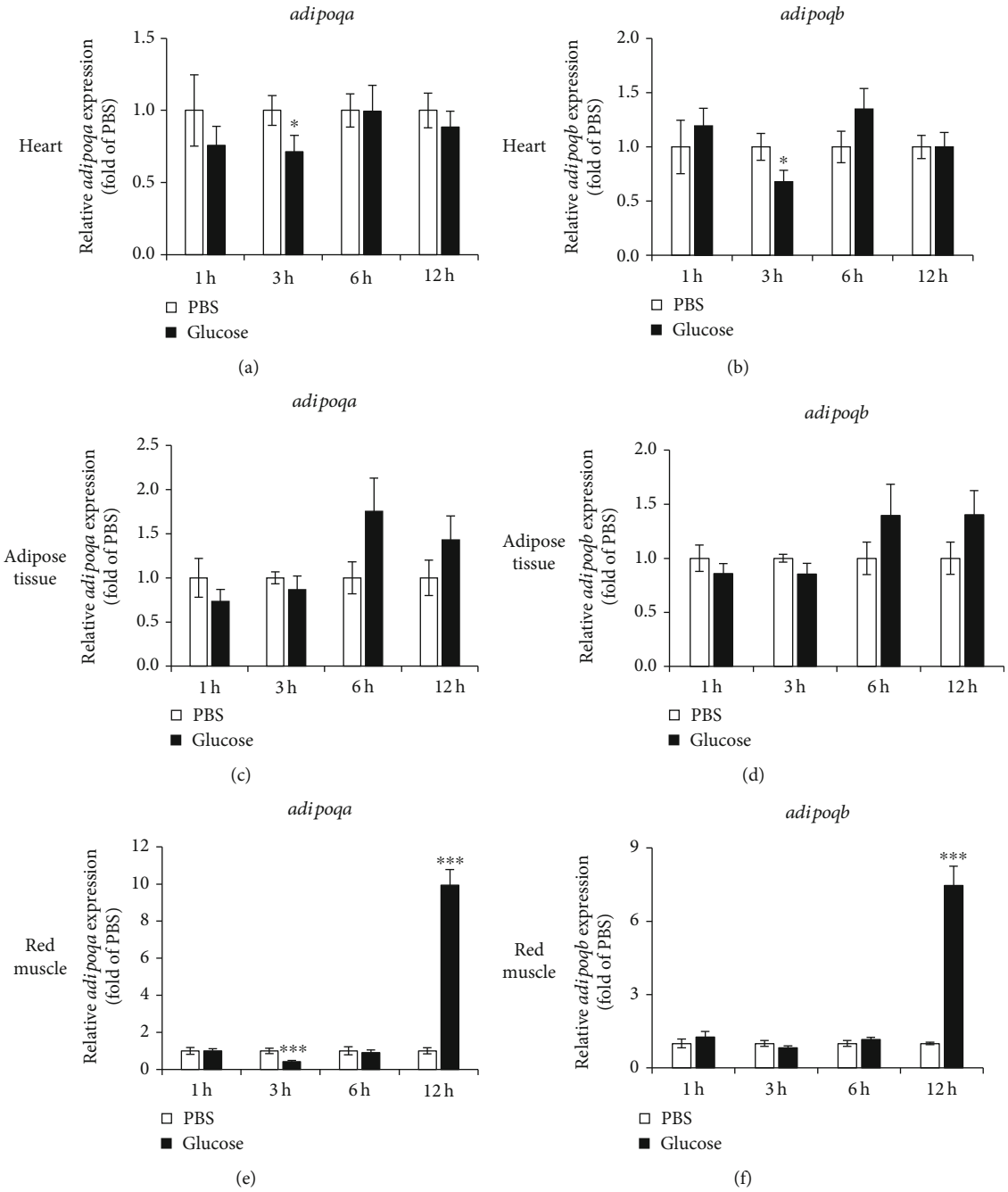

Figure 3: Continued.

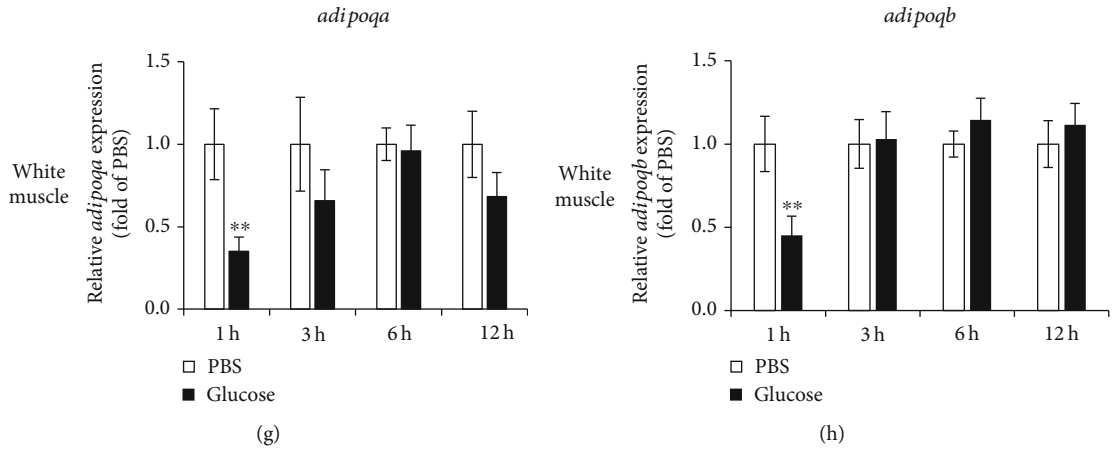

Figure 3: The mRNA expressions of *adipoqa* and *adipoqb* during oral glucose tolerance test (OGTT). Grass carp was orally administrated with either glucose in PBS (1.67 mg/g BW) or PBS as control. At 1, 3, 6, and 12 h post treatment, the *adipoqa* (a, c, e, g) and *adipoqb* (b, d, f, h) mRNA expressions in the heart (a, b), adipose tissue (c, d), red muscle (e, f), and white muscle (g, h) were detected. Results were shown as the fold of corresponding control. Data are shown as mean ± SEM ($n = 9$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

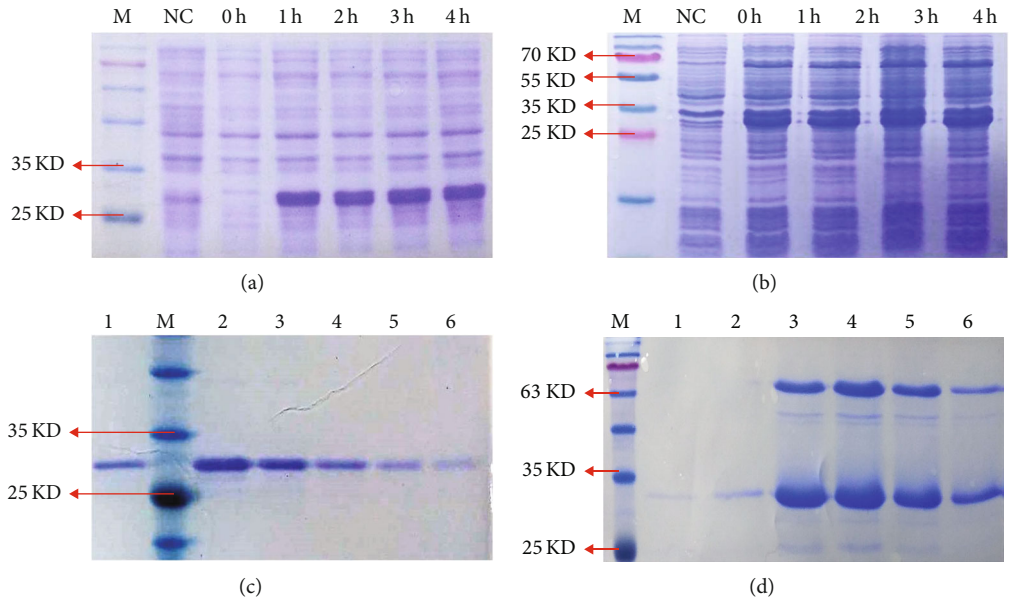

Figure 4: Preparation of recombinant adiponectin proteins. Recombinant expression vector containing histidine-tagged grass carp adiponectin A or adiponectin B was transformed into BL21 strain, respectively, and the empty plasmid was transformed as negative control. The expression of recombinant adiponectin A (a) and adiponectin B (b) was induced by IPTG for 0, 1, 2, 3, and 4 h and analyzed by SDS-PAGE. Recombinant adiponectin A (c) and adiponectin B (d) were purified with Ni^{+}–NTA resin and verified by SDS-PAGE.

paralogs of adiponectin gene were also found in other cyprinid fish, such as zebrafish [14], and this might be due to gene or genome duplication event. Structurally, four domains, including N-terminal signal peptide, variable domain, collagenous domain consisting of 22 Gly-X-Y repeats, and C-terminal globular domain, were characterized in grass carp adiponectin A and adiponectin B. This is consistent with the structure of its counterpart in mammals, suggesting that adiponectin is structurally conserved from teleosts to mammals.

Adiponectin is predominantly secreted by adipose tissue in mammals, and its mRNA expression is highly specific to adipose tissue of human [8], mouse [33], pig [34], and bovine[35]. The tissue expression pattern of adiponectin in fish is completely different from that in mammals. It is expressed more widely in fish, and the tissue with the highest level varies with different fish species. In zebrafish, *adipoqa* mRNA is mainly expressed in the kidney, while *adipoqb* mRNA is widely expressed, but its level is higher in the brain, liver, adipose tissue, and muscle [14]. The present

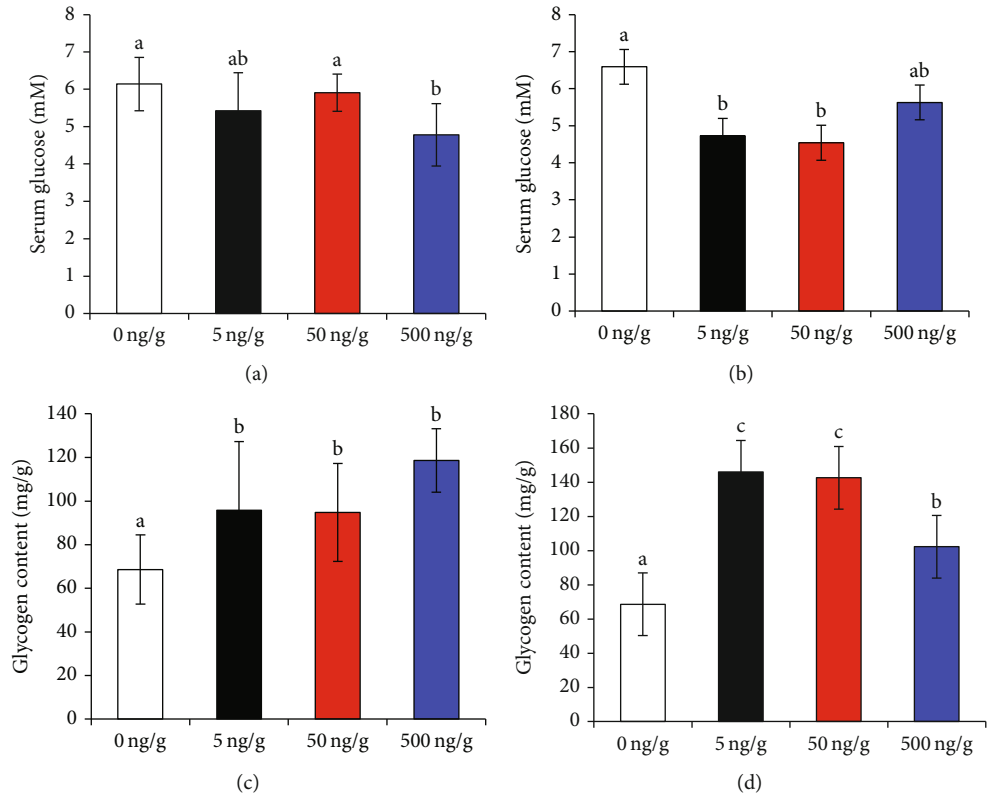

Figure 5: Effects of adiponectin on serum glucose and hepatopancreas glycogen levels of grass carp. Fish were intraperitoneally injected with adiponectin A or adiponectin B at dosages of 0, 5, 50, and 500 ng/g BW. The effects of adiponectin A (a, c) and adiponectin B (b, d) on serum glucose (a, b) and hepatopancreas glycogen levels (c, d) of grass carp were detected at 1 h post injection. All data are expressed as mean ± SEM ($n = 9$). Different lowercase letters in each group represent statistically significant differences ($P < 0.05$).

study found that *adipoqa* mRNA was mainly expressed in the heart, red muscle, white muscle, and adipose tissue of grass carp, while *adipoqb* mRNA was highly expressed in adipose tissue, red muscle, and white muscle. Consistently, high transcript level of adiponectin was also reported in the muscle of other fish such as large yellow croaker [18] and rainbow trout [15, 16], suggesting that adiponectin is more likely to be secreted by muscular tissue in some fish species. Although *adipoqa* and *adipoqb* mRNA were not predominantly expressed in the adipose tissue, especially *adipoqa* mRNA which was highly expressed in the heart and muscle, this did not mean that adiponectin was not secreted by adipocytes. This is because adipocytes are also distributed in the heart [36] and muscle [37]. Actually, it was demonstrated that high level of adiponectin in the muscle of rainbow trout is due to the adipocyte distribution in the muscle [38]. Therefore, we speculated that high levels of *adipoqa* mRNA in the heart and muscle could be related to the distribution of adipocytes in these tissues, but this needs to be further confirmed.

OGTT is a traditional method to evaluate glucose utilization. Our previous study showed that oral administration of glucose significantly elevated serum glucose levels at 1 h and 3 h post treatment, compared to the control group [27]. However, the changes of adiponectin expression during OGTT have not been detected. In this study, four tissues including heart, adipose tissue, red muscle, and white muscle, which have higher transcriptional levels of *adipoqa* and *adipoqb*, were selected to investigate adiponectin expression in response to glucose load. We found that *adipoqa* and *adipoqb* mRNA levels in the heart and white muscle of grass carp decreased at the early stage of OGTT. Consistent with this, studies in mammals have found that the mRNA expression and protein secretion of adiponectin in mature adipocytes were significantly suppressed by constant and intermittent high glucose [39], and carbohydrate-rich diet was associated with hypoadiponectinemia [40]. It should be noted that adiponectin expression in the adipose tissue of grass carp was not sensitive to glucose load. Unexpectedly, the transcriptional levels of *adipoqa* and *adipoqb* were significantly elevated in the red muscle at the late stage of OGTT, suggesting that they were involved in energy mobilization during starvation after glucose intake. Similarly, study in Atlantic salmon (*Salmo salar*) showed that feed restriction remarkably increased adiponectin mRNA level in the muscle [41]. Unfortunately, we only detected the transcription level of adiponectin in grass carp, and the detection method for plasma adiponectin level has yet to be established.

Recombinant adiponectin derived from some species has been successfully expressed in *E. coli*, such as human [42, 43] and ayu [17], which has been shown to be biologically active. In this study, we successfully obtained purified adiponectin A with molecular weight of 30 kDa through *E. coli* expression system, which was consistent with the expected value.

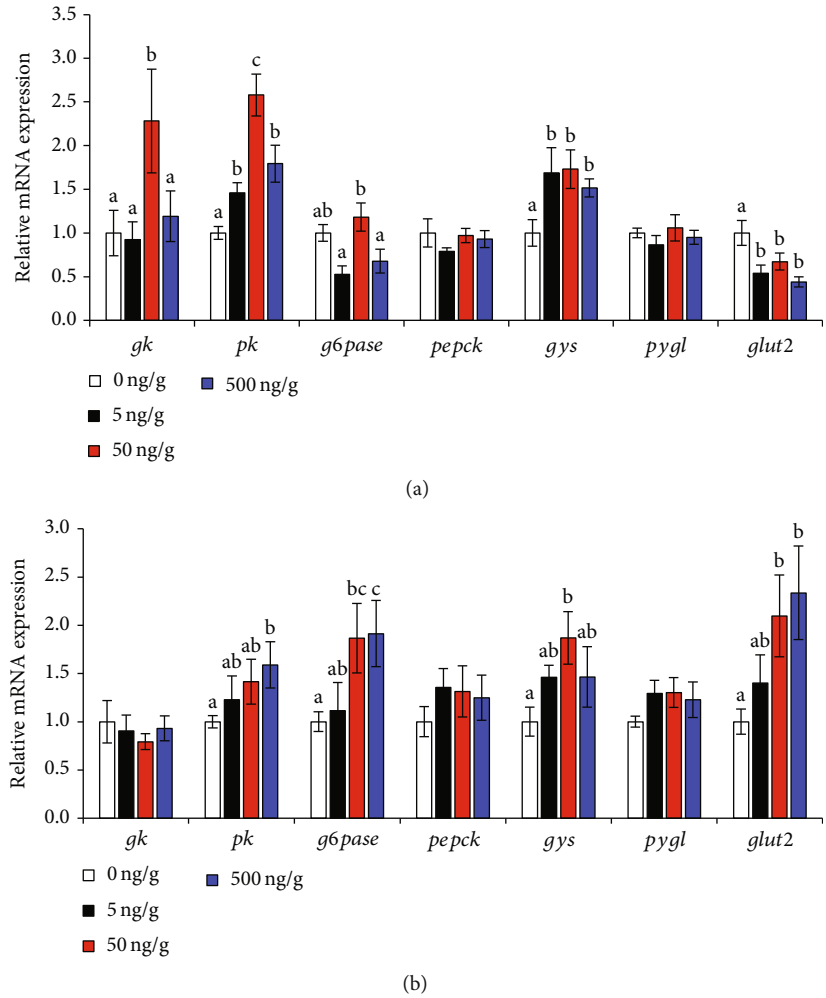

Figure 6: Effects of adiponectin on glucose metabolism-related gene expression in the hepatopancreas of grass carp. Fish were intraperitoneally injected with 0, 5, 50, and 500 ng/g BW adiponectin A or adiponectin B. The mRNA levels of glucose metabolism-related genes in the hepatopancreas were quantified at 1 h post injection by RT-qPCR and normalized against *18S* transcripts. Results are shown as the fold of corresponding control and expressed as mean ± SEM ($n = 9$). Different lowercase letters in each genes represent statistically significant differences ($P < 0.05$).

However, SDS-PAGE analysis showed that the purified adiponectin B had two protein bands with molecular weights of about 30 kDa and 60 kDa, respectively, which was not in line with our expectation. MS analysis showed that the molecular weights of these two protein bands were the same value, both of which were 30.324 kDa (Supplementary Figure 4), which was consistent with the expected value. Furthermore, the identified peptides of these protein bands by MS were consistent with amino acid sequence of grass carp adiponectin B. This suggested that the 60 kDa protein might be the dimer of adiponectin B, which was dissociated into monomer during MS analysis. Study has shown that two adiponectin monomers are bound by disulfide bonds to form dimers [44]. However, this type of dimer is weakly resistant against reduction and boiling during SDS-PAGE analysis. The formation of special dimer of grass carp adiponectin B remains to be studied.

Adiponectin is known for its key role in improving insulin sensitivity and lowering plasma glucose. The levels of plasma adiponectin are negatively correlated with plasma glucose [45]. Injection of adiponectin in mice resulted in a reduction in basal blood glucose levels [13]. In the present study, acute injection of adiponectin A or adiponectin B reduced the blood glucose level of grass carp, respectively, which is in agreement with results in mammals. The liver, one of the main target organs of adiponectin, plays an important role in preventing hyperglycemia. Regarding the effect of adiponectin on glycogen content, conflicting results have been reported in mammals. Results from earlier studies showed that adiponectin had no effect on liver glycogen content [13, 46]. However, recent studies found that adiponectin and adiponectin-derived active peptide ADP355 promoted glycogen accumulation [47, 48]. In this study, we found that both adiponectin A and B increased hepatopancreas glycogen content of grass carp, and these results are consistent with the recent findings.

Glycolysis is the main route for glucose catabolism. Previous study in mice showed that intravenous infusion

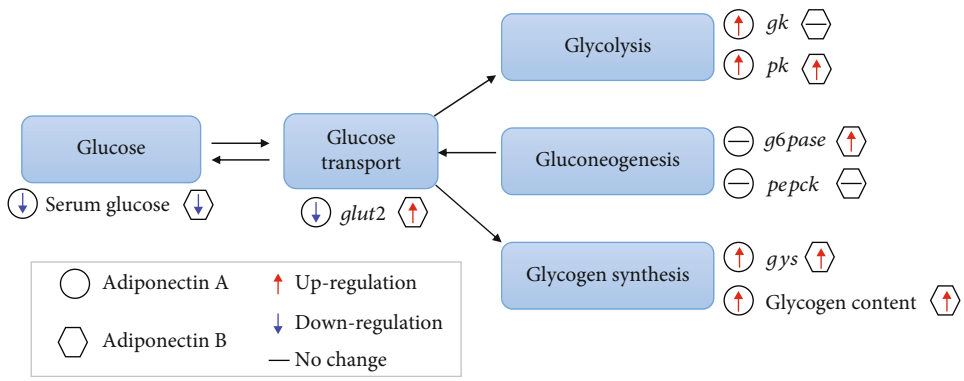

Figure 7: Schematic diagram showing the roles of adiponectin in the regulation of glucose metabolism in the hepatopancreas of grass carp under fasting conditions. Adiponectin A and adiponectin B reduced basal serum glucose levels of grass carp, but they have opposite regulatory effects on *glut2* mRNA expression. Adiponectin A upregulated the expression of enzyme genes related to glycolysis (*gk* and *pk*) and glycogen synthesis (*gys*) in the hepatopancreas, while adiponectin B promoted expression of *pk*, *g6pase*, and *gys*. Finally, adiponectin A and adiponectin B caused an increase in hepatopancreas glycogen content.

of adiponectin had no effect on glycolysis in the liver [46]. However, adiponectin KO mice exhibited decreased mRNA level of glycolysis related enzyme genes in the liver, including *Gk* and phosphofructokinase (*Pfkfb1*), which suggested that adiponectin promoted hepatic glycolysis [12]. Consistent with this result, we also found that adiponectin A upregulated mRNA levels of enzyme genes involved in glycolysis (*gk* and *pk*) in the hepatopancreas of grass carp, and adiponectin B also promotes *pk* mRNA expression but failed to induce *gk* mRNA expression. Gluconeogenesis is an important process mainly responsible for glucose production in the liver. Studies in rodents have confirmed that adiponectin suppresses hepatic glucose production by downregulation of rate-limiting enzymes *Pepck* and *G6pase* expression [10, 46, 49]. However, our results showed that both adiponectin A and adiponectin B had no effect on *pepck* expression in the hepatopancreas of grass carp and adiponectin B upregulated *g6pase* mRNA level. These results differ from previous reports, showing that the different effects of adiponectin on hepatic gluconeogenesis between mammals and teleosts.

In addition, we found that both adiponectin A and B increased the transcriptional level of glycogen synthase gene *gys* but had no effect on glycogenolysis enzyme gene *pygl* expression. In accordance with the changes of hepatopancreas glycogen content, this result provided another evidence to the point that adiponectin promoted hepatopancreas glycogen synthesis in grass carp. Aligns with our result, Duval et al. found that adiponectin induced glycogen synthase gene expression, while had no effect on transcriptional level of glycogen phosphorylase gene [47]. Glucose transportation into the cells is the first step for glucose utilization, and GLUT2 is the major glucose transporter in the hepatopancreas of grass carp [50, 51]. Geng et al. found that adiponectin treatment stimulated *Glut2* mRNA expression in HepG2 cells and improved glucose uptake [52]. This study showed that *glut2* mRNA expression was also promoted significantly by adiponectin B in the hepatopancreas of grass carp, but adiponectin A showed completely opposite effect. Possibly, these two kinds of adiponectin cooperatively regulate glucose transport in the hepatopancreas of grass carp. The mechanism of adiponectin regulating glucose transport through GLUT2 in the liver of fish remains to be further studied. Teleosts underwent an extra event of whole-genome duplication, and the evolutionary fates of the duplicated genes are suggested as non-, neo-, or subfunctionalization, which means silencing of gene function, gaining of novel function, or partitioning of function, respectively [53]. The present study showed that both adiponectin A and adiponectin B were involved in the regulation of glucose metabolism. However, the different roles were observed, which could be the result of subfunctionalization of duplicated adiponectin genes.

5. Conclusions

In conclusion, different from mammals, in which adiponectin is predominantly expressed in adipose tissue, two adiponectin genes (i.e., *adipoqa* and *adipoqb*) were highly expressed in the heart, muscle, and adipose tissue of grass carp. At the early stage of OGTT, *adipoqa* and *adipoqb* mRNA levels in the heart and white muscle decreased, and their expression level in the red muscle increased significantly at the late stage. Both of the two adiponectin reduced blood glucose level, increased hepatopancreas glycogen content, and promoted the expression of genes related to glycolysis, gluconeogenesis, and glycogen synthesis in hepatopancreas (Figure 7). Besides, they have opposite effect on *glut2* mRNA expression in the hepatopancreas grass carp.

Data Availability

All data generated or used during the study appear in the submitted article.

Conflicts of Interest

The authors declare no conflict of interest, financial or otherwise.

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Supplementary Materials

Supplementary 1. Nucleotide and deduced amino acid sequences of grass carp adiponectin A. The solid line below represents the predicted signal peptide, the dashed line below represents the variable region, the red letters represent the collagen domain, and the rectangular box represents globular domain.

Supplementary 2. Nucleotide and deduced amino acid sequences of grass carp adiponectin B. The solid line below represents the predicted signal peptide, the dashed line below represents the variable region, the red letters represent the collagen domain, and the rectangular box represents globular domain.

Supplementary 3. Phylogenetic analysis of adiponectin from grass carp and other vertebrates.

Supplementary 4. MS analysis of two protein bands of purified recombinant grass carp adiponectin B.

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