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Research Article

Irisin Regulates Hepatic Glucose Metabolism via AMPK and PI3K/Akt Activation

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Irisin plays a crucial role in the glucose metabolism of mammals and fish. However, the mechanism by which irisin regulates hepatic glucose metabolism is not entirely known. To clarify irisin's function and underlying mechanisms in common carp glucose metabolism, the *FNDC5* expression patterns were detected after insulin/glucagon injection, long-term high-glucose, and high-fat diet feeding. In vivo, the activity of PFK and the synthesis of liver glycogen were significantly increased, while the enzyme activity of PEPCK was reduced after intraperitoneal injection with irisin. In contrast, irisin siRNA attenuated the brain and liver *FNDC5a* and *FNDC5b* expression, and the serum glucose was increased. *Glut2*, *gs*, *hk*, and *pfk* were considerably downregulated in the brain and liver, while *pygl*, *pepck*, and *g6pase* were remarkably upregulated after siRNA treatment. In vitro, irisin activates the phosphorylation level of AMPK and PI3K/Akt in primary hepatocytes. Compound C and wortmannin inhibit AMPK and PI3K/Akt, reversing irisin-induced expression of *g6pase*, *fbpase*, *pepck*, *gk*, *gs*, *pfk*, *hk*, and *gsk3b*. At the same time, compound C attenuated irisin-induced expression of *pi3k* and *akt*. Our research reveals the mechanisms of irisinA and irisinB as glucose metabolism regulators in teleost for the first time.

1. Introduction

The balance of glucose metabolism in fish has always been an interesting question [1]. The utilization capacity of glucose in fishes is different, and the time to relieve the glucose load state is longer than that of mammals [2]. Endocrine regulation is an essential tool in maintaining glucose homeostasis in the body [3]. Irisin, a cytokine discovered in 2012, was widely expressed in various body tissues [4]. Irisin is produced by digesting the membrane protein expressed by its precursor gene *FNDC5* (fibronectin type III domain-containing 5) [5]. Human's irisin comprises 112 amino acids and has 100% homology with rats and mice [6]. In mammals, irisin has critical physiological functions, such as promoting the conversion of white fat to brown fat and promoting glucose and lipid uptake and utilization [7]. A study reported that irisin enhances postprandial stimulated insulin secretion and promotes insulin biosynthesis [8].

Studies have shown that irisin can, through the AMPK (5′- AMP-activated protein kinase) and PI3K (phosphoinositide 3-kinase)/Akt (protein kinase B), regulate body metabolism [9, 10]. AMPK is a crucial sensor in the homeostasis of liver and fat glucose metabolism [11]. AICAR and its allosteric can activate AMPK, and the specific inhibitor of AMPK is compound C [11]. In rats, GLUT4 (glucose transporter 4) was transported from intracellular storage vesicles to the plasma membrane after AICAR continuous infusion [12]. The glycogen synthase *gs1* (glycogen synthase) and *gs2* could be regulated by AMPK to prevent glycogen storage, and the gluconeogenesis enzymes PEPCK and G6Pase (glucose-6-phosphatase) were regulated to maintain glucose metabolism [13]. In myoblasts, glucose uptake and glycolysis were

promoted, and glycogen synthesis was inhibited by the activation of AMPK [14]. Studies have shown that compound C can reverse the effect of *FNDC5* in reducing adipose tissue inflammation and insulin resistance [15]. At the same time, irisin can phosphorylate AMPK through LKB1, regulate acetyl-CoA carboxylase, and improve the body's glucose tolerance, glucose uptake, and hepatic glucose and fat metabolism [16, 17]. The PI3K/Akt is a regulator to maintain energy homeostasis [18]. A study has shown that irisin can inhibit gluconeogenesis and increase glycogen production through the PI3K/Akt pathway [9]. Irisin activates the Akt signaling pathway in C2C12 cells, which lowers the inhibitory impact of palmitate on insulin signaling [19].

The common carp *Cyprinus carpio* L. is an essential freshwater economic fish. Although its production value reached 2.896 million tons in 2020, it is still in short supply [20]. Carp is a frequently used model in glucose metabolism research [21, 22]. Currently, irisin has been relatively well studied in mammals, but studies on the mechanisms of irisin regulate glucose metabolism in fish that have not been reported. The *FNDC5*/irisin system in fish is limited. To date, the physiological functions of irisin in fish are limited to the areas of cardiovascular system development, feeding regulation, and growth [23–26]. FNDC5s in zebrafish were coded by two genes, namely, *FNDC5a* and *FNDC5b* [27]. In this study, based on our previous research about the cloning of irisin [28], we hypothesized that irisin promotes glucose homeostasis in carp hepatocytes through AMPK and PI3K/Akt signaling pathways and verified by (1) detecting the expression patterns of *FNDC5* and irisin in different nutrition states, (2) examining the effect of irisin on glucose metabolism in vivo and in vitro, and (3) investigating the roles of AMPK and PI3K/Akt signaling pathways in mediating irisin which regulates glucose metabolism. This study would provide a theoretical basis for researching the physiological effects and mechanisms of irisin in fish and enrich the endocrine physiological regulation mechanism of teleost glucose metabolism.

2. Materials and Methods

2.1. Ethics Statement. This study conformed to the guidance of ethical animal treatment for the care and use of experimental animals and was approved by the Institutional Animal Care and Use Committee of Henan Normal University.

2.2. Animals. The Aquaculture Experimental Center in Yanjin County, Henan Province, China, provided carps *Cyprinus carpio* L. (male and female) weighing 20 to 65 g. The carps were transferred to Henan Normal University and domesticated for two weeks before being transferred to a recirculating aquaculture system (300-L plastic tank). 30 fish per tank were grown on a photoperiod (light/darkness = 12/12 h). The carps were fed with a commercial diet (Tongwei, China) at 8 : 30, 13 : 30, and 18 : 30 until satiety, and the water quality indicators and environment were as follows: DO (5.8-6.2), temperature (27°C), pH (7.2-7.4), and ammonia (<0.02 mg/L). The fishes were anesthetized with MS-222 (55 mg/L) (Aladdin, China) before being euthanized, and all efforts were made to minimize suffering.

2.3. Proteins. The proteins were prepared according to the previous experimental method [28]. In short, the cDNA of irisin was cloned by PCR. The 6 × His tag, *Not I*, and *EcoRI* were introduced to construct the plasmid. The sequences were sequenced by ABI 3730 DNA sequencer (Thermo, USA). The plasmid was induced into BL21 (Solarbio, China) stimulated by thermal. The expression of irisin protein was induced by IPTG (1 mmol/L). The product was purified by Ni^{+}-NTA resin column (GE, Pittsburgh, USA) and dialyzed against PBS buffer. The SDS-PAGE and western blot were used to verify the protein.

2.4. Long-Term High-Glucose and Fat Feeding Experiments. A total of 108 carp (five-month-old male and female) weighing 20 g were randomly divided into nine tanks (three groups) of 12 fish each. The control group was fed conventional feed (25% glucose and 5% lipid), the high-glucose group was fed a 50% glucose feed, and the high-fat group was fed a 10% fat feed. Samples were taken after an 8-week feeding trial, and the liver, gut, and muscle were sampled and stored at -80°C.

2.5. Insulin and Glucagon Challenge. A total of 144 carp were separated into three groups at random (12 fish in each group). After 24-h fasting, fish were injected 100 ng/g B.W. of human insulin (ProSpec, Israeli) or glucagon (ProSpec, Israeli) intraperitoneally. In the control group, an equal volume of PBS was injected into the carps. Samples were taken 1, 3, 6, and 12 h after injection. The liver and gut were stored at -80°C.

2.6. Intraperitoneal Injection Experiment. Intraperitoneal injection was performed as follows [28]. In brief, according to different concentrations (0, 10, 100, 1000 ng/g B.W.) and different times (3, 6, and 12 h), PBS, irisinA, or irisinB was injected into 216 carps for 24 groups. The liver and muscle were collected on ice. The samples were tested in a phosphofructokinase (PFK) test kit, phosphoenolpyruvate carboxylase kinase (PEPCK) test kit, and liver/muscle glycogen assay kit following the instructions (Nanjing Jiancheng, China).

2.7. RNA Interference. In this part, we performed intraperitoneal injection and intracerebroventricular (*i.c.v*) injection of siRNA (small interfering RNA). The siRNAs and scrambled siRNA sequence were designed based on the irisin sequence and shown in Table 1 (GenePharma, China) [28]. A total of 216 carp were used for the experiment. The experiment groups were injected with irisinA siRNA, and irisinB siRNA (10 ng/g B.W.), PBS, or scramble was given to the control group.

To screen the effectiveness and optimal time of siRNA, an *i.p.* experiment was performed. Fish were randomly divided into scramble, irisinA1 siRNA, irisinA2 siRNA, irisinA3 siRNA, irisinB1 siRNA, irisinB2 siRNA, and irisinB3 siRNA groups and sampled at 12, 24, and 36 h after injection. For *i.c.v.* experiment, 60 carp were randomly divided into PBS, scramble, irisinA siRNA, irisinB siRNA, irisinA, and irisinB siRNA groups ($n = 12$) and sampled at 24 h after *i.c.v.* Blood samples were obtained from the caudal

Table 1: RT-qPCR Primers and siRNA sequences used in this study.

Name	Sequences (5′-3′) (sense)	Sequences (3′-5′) (antisense)	GenBank ID
qFNDC5a	AACCGCTGCGTTTCAAAACC	CATGGTCACTTCGTCTTTGCTC	MT361875
qFNDC5b	ATGACAGTTTATCTGCTCCAC	TCCTCCAAGTCCCACAA	MT361876
ucp1	TCATTTCGTGTCTGCGTTCG	TTCATGTAGCGCGTCTTCAC	AY461434.3
ucp2	AAGGAACTGGCCCAAACATC	ACCAGCTCCAAATGCAGATG	AJ243486.1
ucp3	TACAACGGCACAATGGATGC	CGCGTTCCTTGTGATGTTTG	AY505343.1
glut2	GAGGGTCTTTGTGGGAACTATG	GTTTCAGGTACACGCAAGTAGA	XM_019072653.1
gs	TTTTGGCCGCTGGTTGATTG	ATAGGGTAGTCCAATGCTGCAC	XM_019090903.1
gsk3β	ATCAGGTGCATGAGGAGTTGAG	AGCAGAGCATTTCCAAACGC	XM_042774357.1
pygl	TGGTTGACGACGATGCTTTC	ACTGCGCAAACTTCAGCTTG	XM_019125106.1
g6pase	GAGGCCTTCAACAGACAGAAA	GAGCTTTGAGAAGCAGGTACAA	AF427863.1
fbpase	CCTGCCATCGGTGAGTTTAT	CCATCCTCTGGGAATTTCTTCT	AF427864.1
gk	TGGAGTACGACCGTGTTATTG	CGCAAGCTCTCCCATATACTT	AF053332.2
pfk	CGTTCGACAGGAATTTTGGC	TTCATGCCGATCACACAAGC	XM_019074709.1
hk	CGTTGTAGCCGTAGTGAATGA	GCTACCCGTTCCTGCAATTA	AF119837.1
pepck	ACTCTTTGGGCAGACCTTTAC	CTGTCTGGTGTCAGGAAGATG	KP250869.1
ampkα1	TGCGAGAAGTTTGAGTGCAC	TTCATAATGCGCCGGTTGTC	XM_019104472.2
ampkα2	AAATGTGTGCCAGCCTCATC	ATGTTTGTGGCGCTACTGTG	XM_042777568.1
pi3k	GAAGATGACGACTGGAGAG	GCCTGTAGTGACTGATGAG	XM_019085737.2
akt	ACTGTTATTGAGCGCACCTT	TCCATTGGCTCCTCCTCTTC	XM_042774924.1
18s	GAGACTCCGGCTTGCTAAAT	CAGACCTGTTATTGCTCCATCT	FJ710826.1
siRNA-A	GCAAAGACGAAGUGACCAUTT	AUGGUCACUUCGUCUUUGCTT	
siRNA-B	GCUGCGAUUCAUCCAGGAATT	UUCCUGGAUGAAUCGCAGCTT	
Scramble	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT	

vein of each fish after injection using a 22-gauge needle attached to a 1-mL heparinized syringe. Subsequently, samples were centrifuged at 3500 *g* for 10 minutes, and the serum was collected. The brain and liver were sampled and stored at -80°C.

2.8. Primary Hepatocytes Experiment and Western Blot. The common carp primary hepatocytes were isolated as previously described [28]. In short, the carp hepatocytes were taken out in precooled HBSS. Subsequently, hepatocytes were treated with collagenase 4 (0.5 mg/mL, Sigma, USA) and DNase 2 (100 U/mL, Sangon Biotech, China). These cells were added at a density of 8×10^5 cells/mL in 24-well plates and cultured at 27°C. After 12-h starvation, cells were treated with irisin (10, 100, and 1000 nmol/L) for 3, 6, 12, and 24 h and collected by TRIzol (Takara, Japan). On the other hand, hepatocytes were treated with 1000 nmmol/L irisinA or irisinB for 0, 5, 10, 15, 30, and 60 minutes. The protease inhibitor and phosphatase inhibitor were added to RIPA Lysis Solution (100 : 1, Beyotime, China). The samples were collected on ice. After sonication, the protein concentration was detected, the protein concentration was adjusted to 1.5 μg/μL, and the western blot was performed using phospho-AMPKa, AMPKa, phospho-PI3K, and PI3K (1 : 1000, Cell Signaling Technology, USA). In order to detect the downstream target genes of irisin, compound C (10 μmmol/L, Selleck, USA) and wortmannin (1 μmmol/L, Selleck, USA) were pretreated for 1 h before being incubated with 1000 nmmol/L irisin. The cells were collected by TRIzol (Takara, Japan) and stored at -80°C.

2.9. Real-Time Quantitative PCR. According to TRIzol instruction for total RNA extraction (Takara, Japan), agarose gel electrophoresis was used to assess the quality of RNA. The purity and yield of RNA were evaluated by spectrophotometer (NanoDrop 2000, Thermo, USA). The first-strand cDNA was synthesized according to the instruction of PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan). The qPCR protocol as before in Roche LightCycler 480 [28]. The $2^{-\Delta\Delta Ct}$ method was calculated for gene expression [29]. Primers were assessed by serial dilutions method and with close to 100% amplification efficiency. The *18s* and *b-actin* were used as housekeeping genes in all experiments. All of the trials were carried out in duplicate.

2.10. Statistical Analysis. The values were represented as mean ± S.E.M. after normalization with the control group. The statistical differences were assessed using the SPSS 20.0 (SPSS Inc., USA). Firstly, normality and homoscedasticity tests were used to analyze data. Subsequently, when the statistical assumptions were fulfilled, between two groups, the *T*-test was performed, and between three and more groups, the one-way ANOVA analysis and the least significant difference (LSD) test determined the difference in each sample.

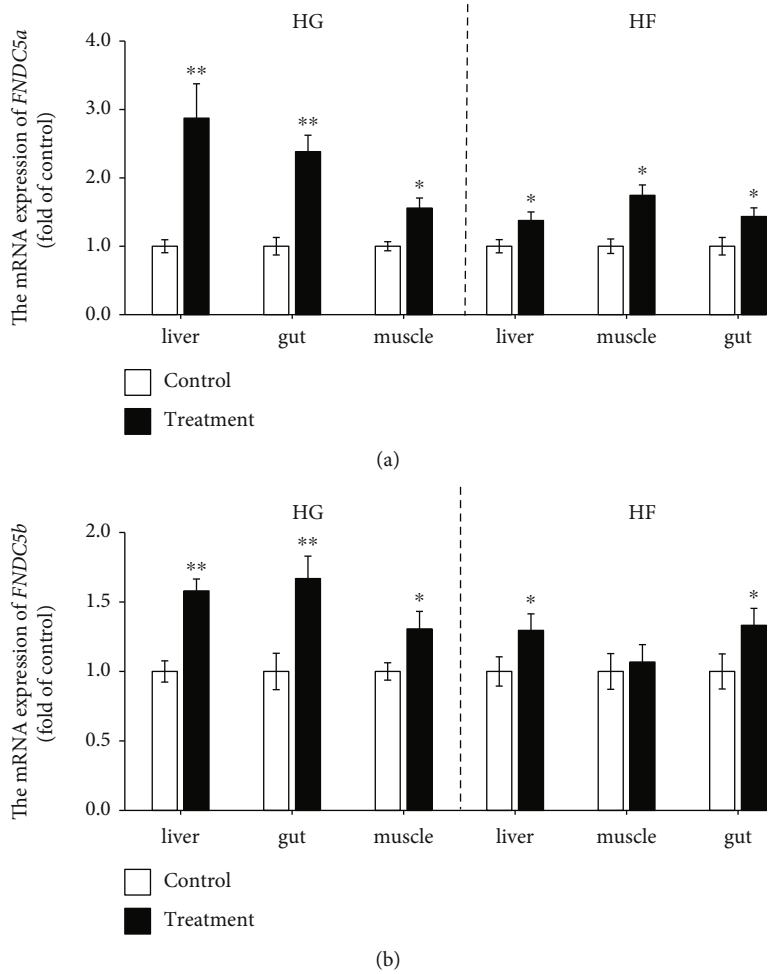
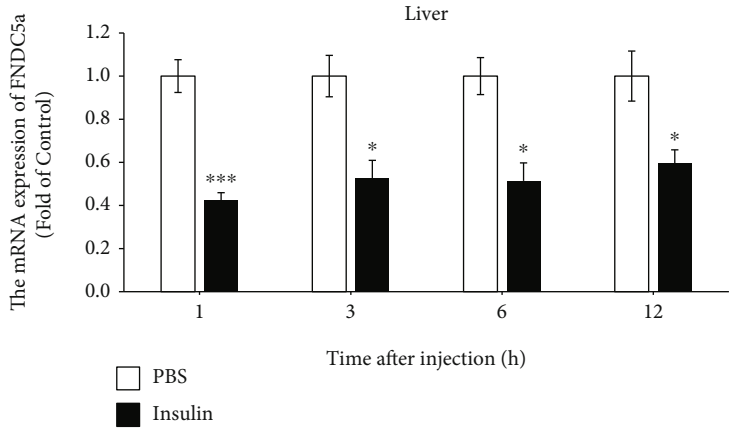


Figure 1: The effect of long-term high-glucose or high-fat feeding on common carp *FNDC5*. The common carps were fed with high-glucose (HG) or high-fat (HF) diet. The (a) *FNDC5a* and (b) *FNDC5b* mRNA relative expression in the liver, gut, and muscle. The results were represented as the fold of control. All data were shown as the mean ± S.E.M. ($n = 12$). The probability less than 0.05 indicated a significant difference in *T*-test. ∗P < 0.05, ∗∗P < 0.01, and ∗∗∗P < 0.001.

3. Results

3.1. The Expression Pattern of Irisin Is Related to Glucose and Lipid Homeostasis in Common Carp. To explore the effect of long-term high-glucose and high-fat on *FNDC5* in carp, high-glucose and high-fat feeding trials were carried out. After feeding, the liver, gut, and muscle *FNDC5a* were significantly increased ($P < 0.05$) (Figure 1(a)). And after high-glucose feeding, the liver, gut, and muscle *FNDC5b* were upregulated ($P < 0.05$). After high-fat feeding, the liver and muscle *FNDC5b* were dramatically increased ($P < 0.05$) (Figure 1(b)). Insulin and glucagon injection experiments were used to assess the effects of insulin and glucagon on *FNDC5* mRNA expression. After insulin injection, the liver *FNDC5a* was significantly decreased ($P < 0.05$) (Figure 2(a)). The *FNDC5a* in carp gut was significantly decreased after the injection 1 h ($P < 0.05$) (Figure 2). After glucagon injection, the liver *FNDC5a* was significantly upregulated ($P < 0.05$) (Figure 2(e)).

3.2. Irisin Induces Glucose Uptake and Glycogen Synthesis, Promotes Glycolysis, and Inhibits Gluconeogenesis in Common Carp. In order to explore the function of irisin on common carp glucose metabolism, the intraperitoneal injection experiment was performed. Different concentrations of irisinA or irisinB can significantly reduce the enzymatic activity of PEPCK, enhance the enzymatic activity of PFK, and promote the synthesis of liver glycogen ($P < 0.05$) (Figure 3). At the same time, to interfere with the production of endogenous irisin, siRNA (10 ng/g B.W.) was intraperitoneally injected at 12, 24, and 36 h. In the brain, the *FNDC5a* mRNA expression was decreased by irisinA1 siRNA at 12 h ($P < 0.05$) but increased by irisinA1 siRNA, irisinA2 siRNA, and irisinA3 siRNA at 24 h ($P < 0.05$)(Supplementary Figure 1 A). The *FNDC5b* was down-regulated by irisinB2 siRNA at 36 h (P < 0.05) (Supplementary Figure 1B). The liver *FNDC5a* was downregulated by irisinA1, A2, and A3 siRNA at 12, 24, and 36 h (P < 0.05) (Supplementary Figure 2A). The *FNDC5b*



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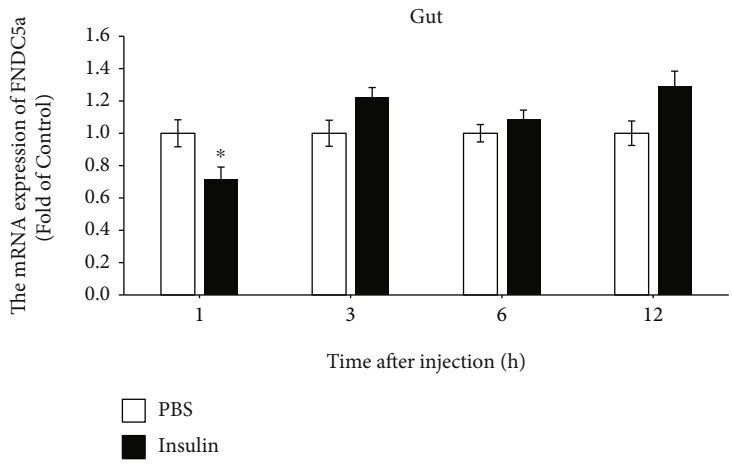

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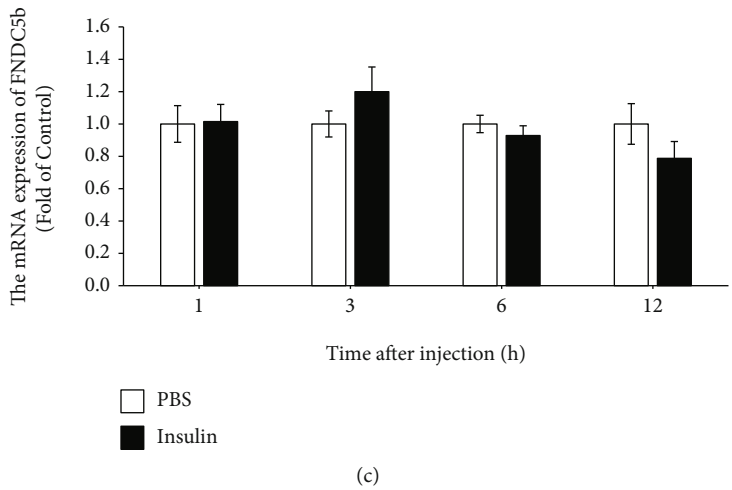

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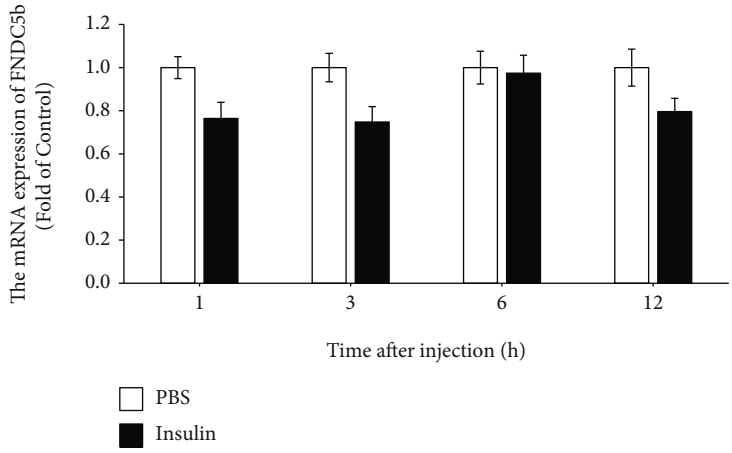

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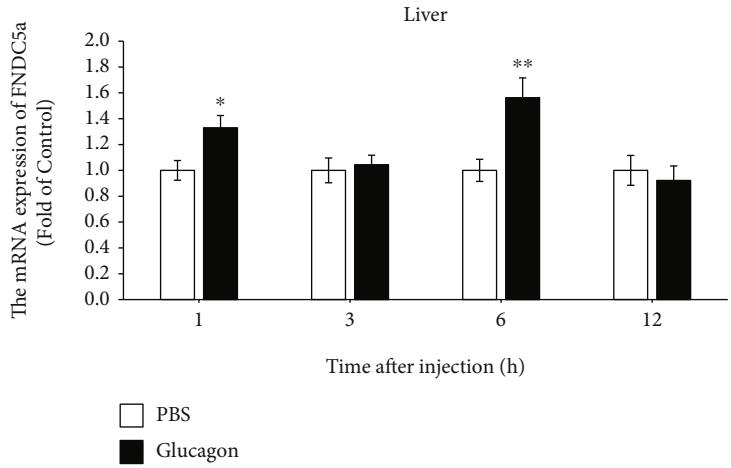

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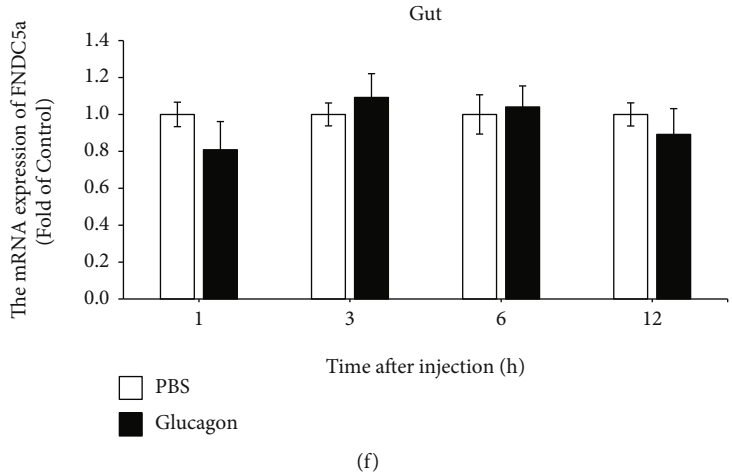

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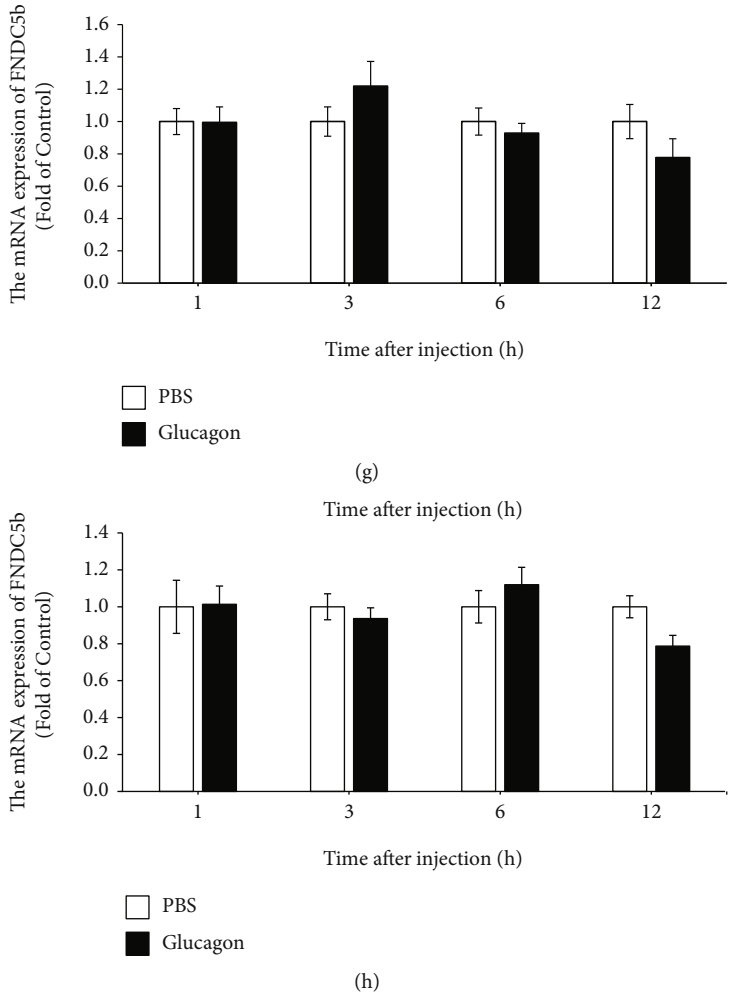
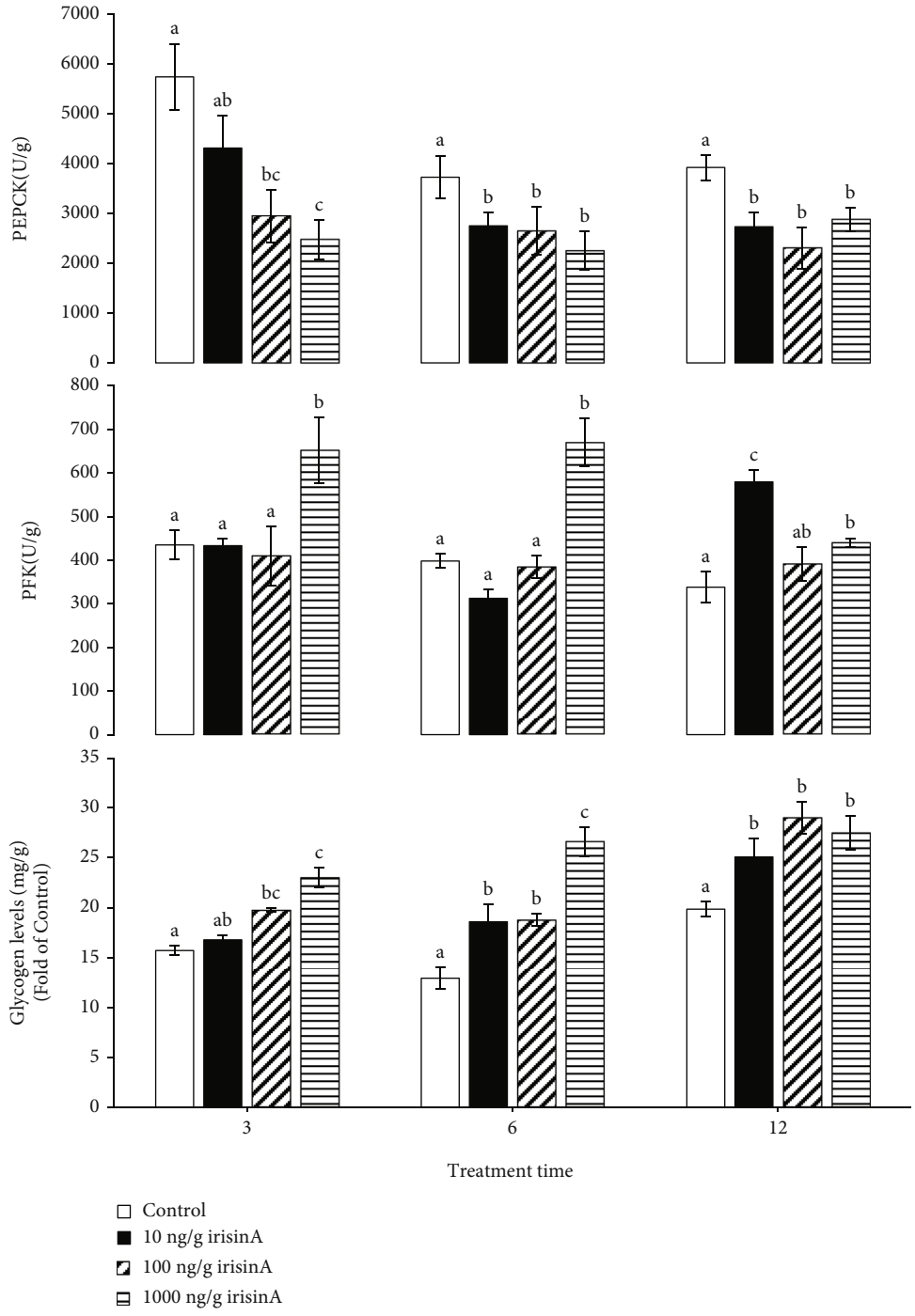


Figure 2: The effect of insulin or glucagon injection on *FNDC5* mRNA expression of common carp. The common carps were injected with insulin. The *FNDC5a* mRNA relative expression (a) in the liver and (b) in the gut. The *FNDC5b* mRNA relative expression (c) in the liver and (d) in the gut. The common carps were injected with glucagon. The *FNDC5a* mRNA relative expression (e) in the liver and (f) in the gut. The *FNDC5b* mRNA relative expression (g) in the liver and (h) in the gut. These results were represented as the fold of control. All data were shown as the mean ± S.E.M. ($n = 12$). The probability less than 0.05 indicated a significant difference in T-test. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

was markedly decreased by irisinB1, B2, and B3 siRNA at 24 h ($P < 0.05$) (Supplementary Figure 2B). The irisinA3 siRNA and irisinB1 siRNA were chosen for the subsequent experiment. The operation process of *i.c.v* injection was shown in Supplementary Figure 3, and this method was improved from previous research [30]. In the brain, *FNDC5a* and *glut2* were significantly decreased after irisinA siRNA treatment ($P < 0.05$) (Figure 4(a)). After irisinB siRNA injection, *FNDC5b* and *glut2* were significantly decreased ($P < 0.05$) (Figure 4(a)). The expression of *FNDC5a*, *FNDC5b*, and *glut2* were significantly reduced by irisinA and B siRNA ($P < 0.05$) (Figure 4(a)). The liver *FNDC5a* and *FNDC5b* were downregulated by irisinA siRNA, irisinB siRNA, and irisinA and irisinB siRNA ($P < 0.05$) (Figure 4 B). The *ucp1*, *ucp2*, and *ucp3* were decreased by irisinA siRNA, irisinA, and irisinB siRNA ($P < 0.05$) (Figure 4(c)). The *ucp1* and *ucp2* were reduced by irisinB siRNA ($P < 0.05$), but *ucp3* was not changed ($P > 0.05$) (Figure 4(c)). After injection, the serum glucose was significantly increased by irisin siRNA ($P < 0.05$) (Figure 4(d)). The expression of *hk* and *pfk* in the liver were considerably reduced by irisinA siRNA ($P < 0.05$), and the *pygl* and *g6pase* were markedly upregulated ($P < 0.05$) (Figures 4(e) and 4(f)). After irisinB siRNA, irisinA, and irisinB siRNA injection, the mRNA expression of *glut2*, *gs*, *hk*, and *pfk* were significantly decreased ($P < 0.05$), and the *pygl*, *g6pase*, and *pepck* were increased ($P < 0.05$) (Figures 4(e) and 4(f)). Enzyme activity analysis showed that RNAi (RNA interference) could increase the enzyme activity of PEPCK ($P < 0.05$) and depress the enzyme activity of PFK ($P < 0.05$) (Figures 4(g) and 4(h)). The glycogen levels were downregulated after RNAi ($P < 0.05$) (Figure 4(i)).

3.3. AMPK and PI3K/Akt Pathway Mediate Irisin Regulate Common Carp Glucose Metabolism. To elucidate the mechanism of irisin on common carp glucose metabolism, firstly,



(a)

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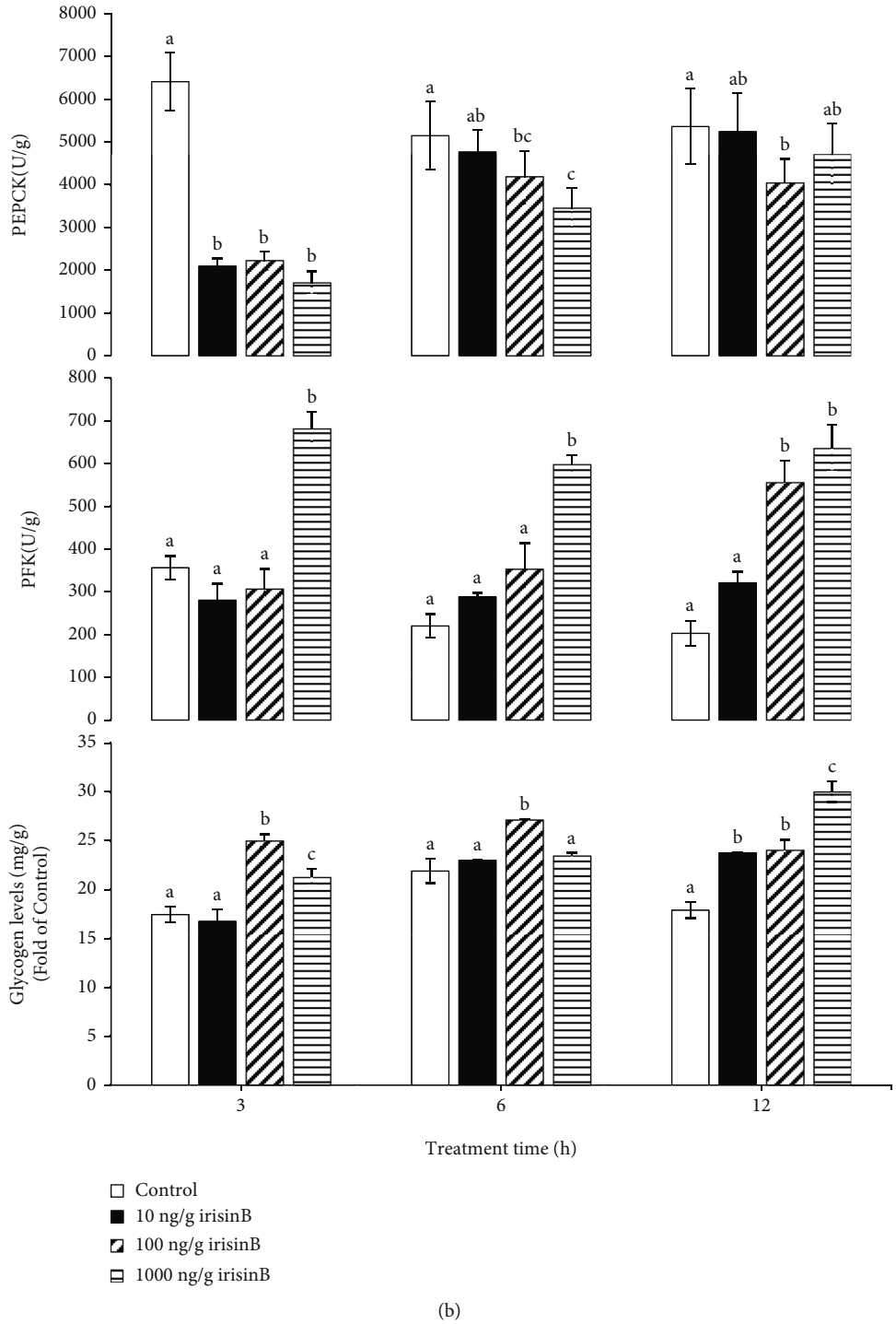
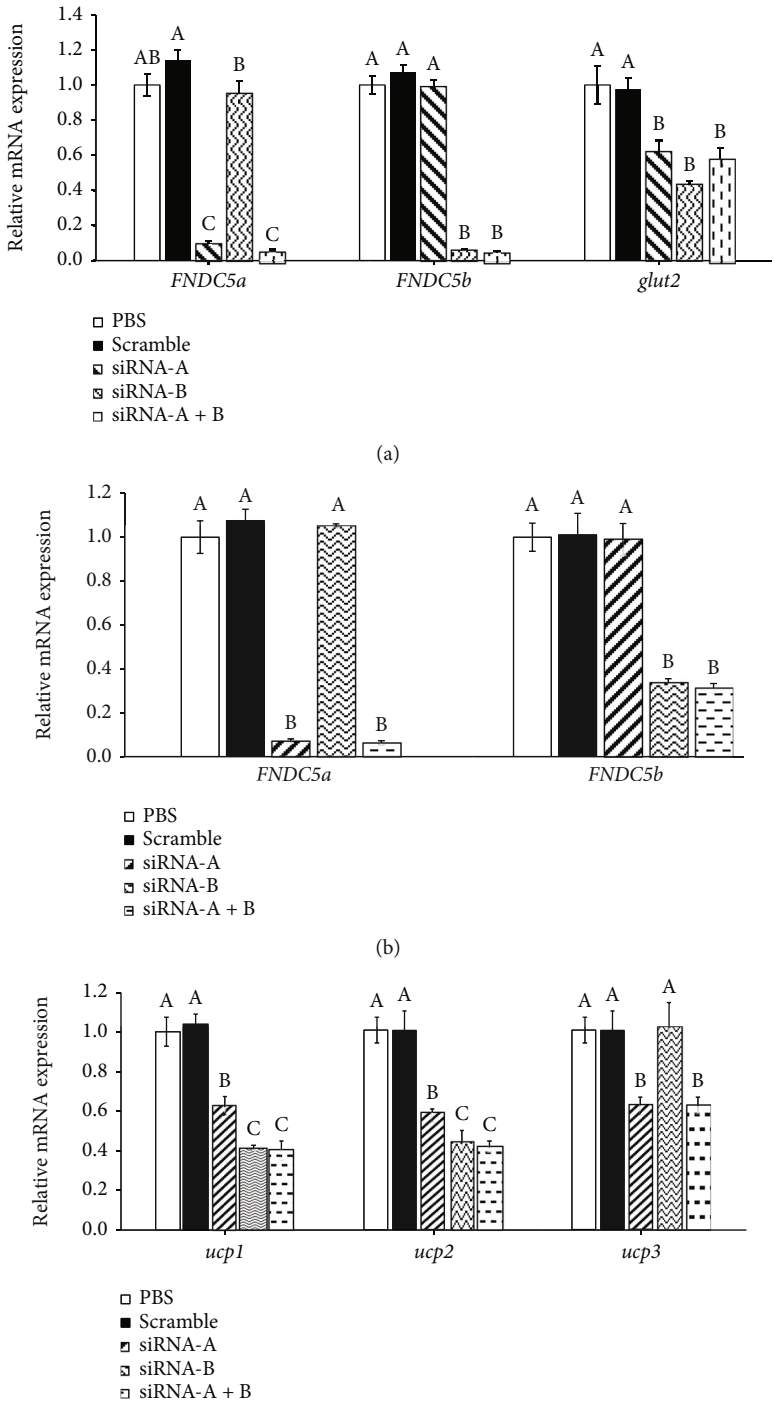


(b)

Figure 3: Effect of intraperitoneal injection of irisin on common carp glucose metabolism. Fish were injected intraperitoneally with vehicle (PBS) or irisin (10 ng/g B.W., 100 ng/g B.W., and 1000 ng/g B.W.) for 3, 6, or 12 h. After injection of common carp irisin, the enzymatic activity of glycolysis and gluconeogenesis were detected, and the synthesis of liver glycogen was investigated. The value of PEPCK, PFK enzymatic activity, and glycogen content in the liver after injected with (a) irisinA and (b) irisinB. In the data presented, the mean ± S.E. M. ($n = 9$). The difference of the one-way ANOVA analysis was represented by different superscript letters.



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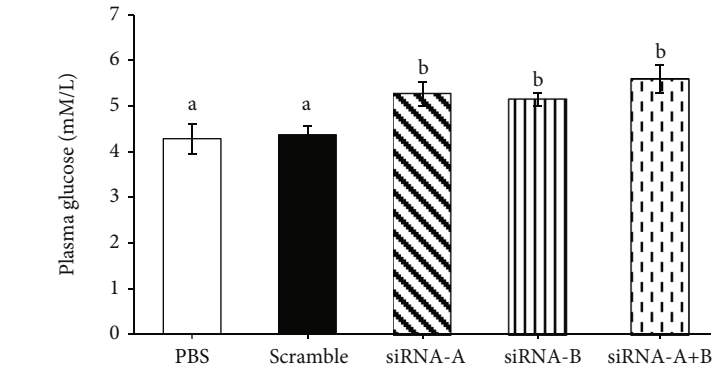

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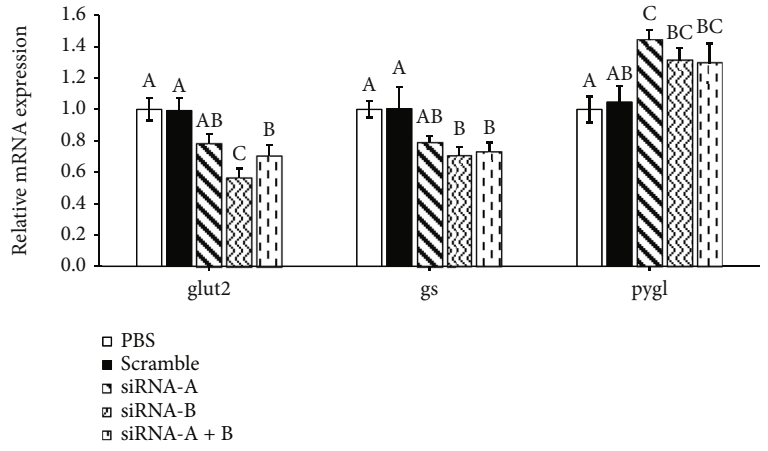

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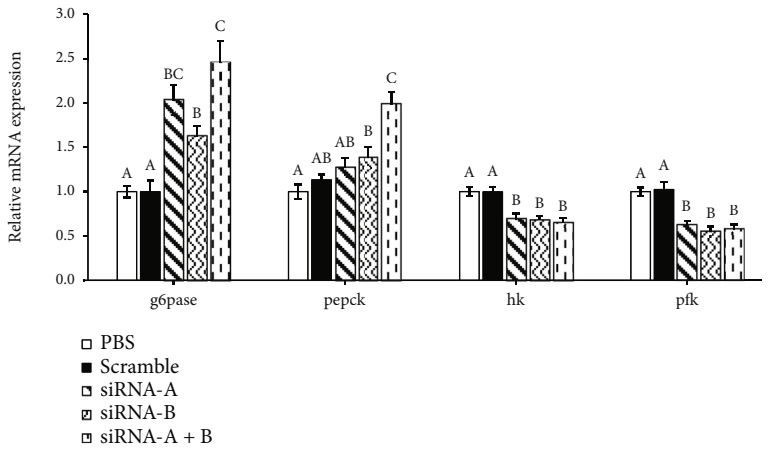

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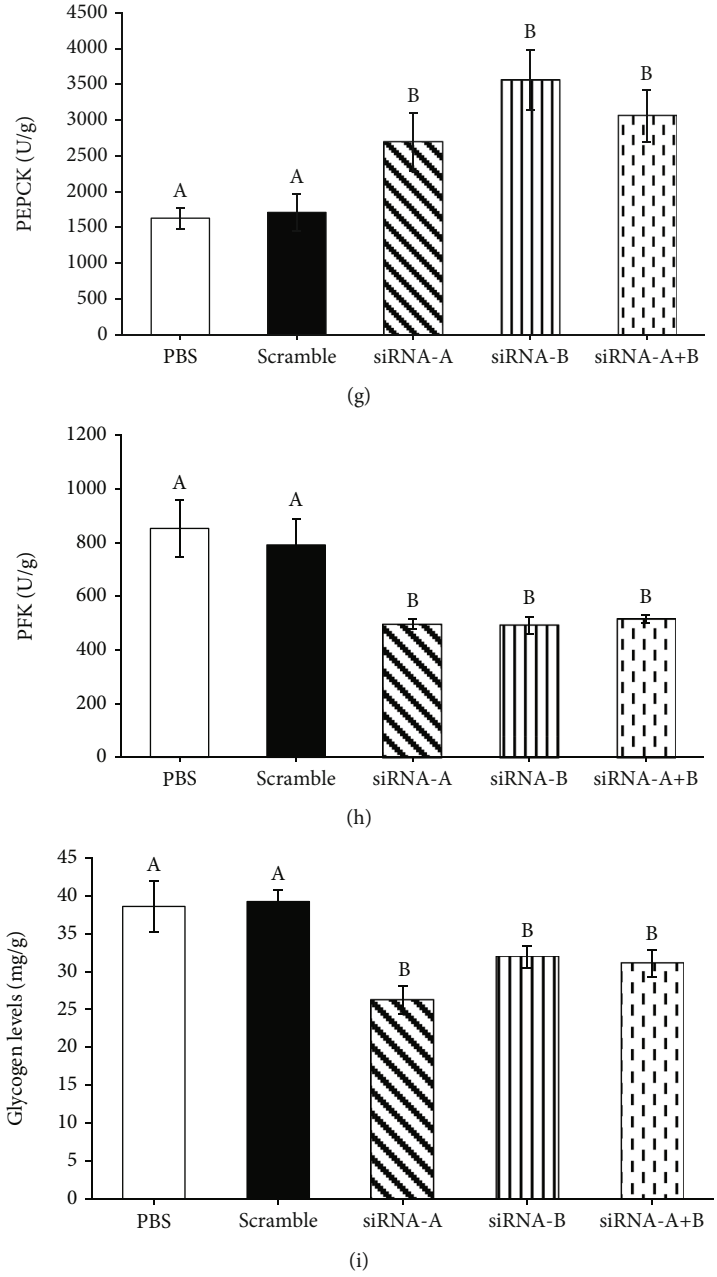
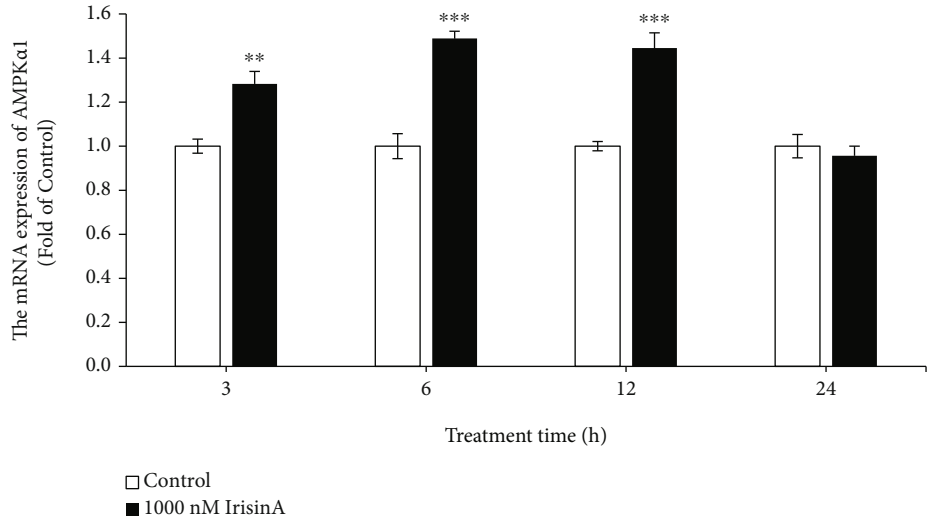


Figure 4: Effect of *i.c.v* injection of siRNA on common carp glucose metabolism. Fish were *i.c.v* injected with vehicle (PBS), scramble, irisinA siRNA, irisinB siRNA, or irisinA and irisinB siRNA (10 ng/g B.W.) for 24 h. The mRNA expression levels of (a) brain *FNDC5* and *glut2*, (b) liver *FNDC5*, and (c) liver *ucp1*, *ucp2*, and *ucp3* after *i.c.v* injection of siRNA. (d) The common carp serum glucose. (e) The expression of genes related to glucose transport, glycogen synthesis, and decomposition in common carp. (f) The expression of genes related to glycolysis and gluconeogenesis in common carp. The enzymatic activity of (g) PEPCK and (h) PFK. (i) The liver glycogen levels. The gene expression levels were represented as the fold of control. All data were shown as the mean ± S.E.M. ($n = 12$). The difference of the one-way ANOVA analysis was represented by different superscript letters.

through treatment with 1000 nmmol/L irisin, the *ampka1* mRNA expression was markedly activated for 3, 6, and 12 h ($P < 0.05$) (Figures 5(a) and 5(b)). The *ampka2* was upregulated by irisinA after 6 and 12 h ($P < 0.05$) and significantly promoted by irisinB after 3 and 6 h ($P < 0.05$) (Figures 5(c) and 5(d)). Then the hepatocytes were incubated with different concentrations of irisin. After 3, 6, and 12 h of irisinA or irisinB administration, *ampka1*levels rose in a dose-dependent manner ($P < 0.05$) (Figures 5(e) and 5(f)). The *ampka2* was markedly upregulated after 3 and 6 h of irisinA exposure ($P < 0.05$) and



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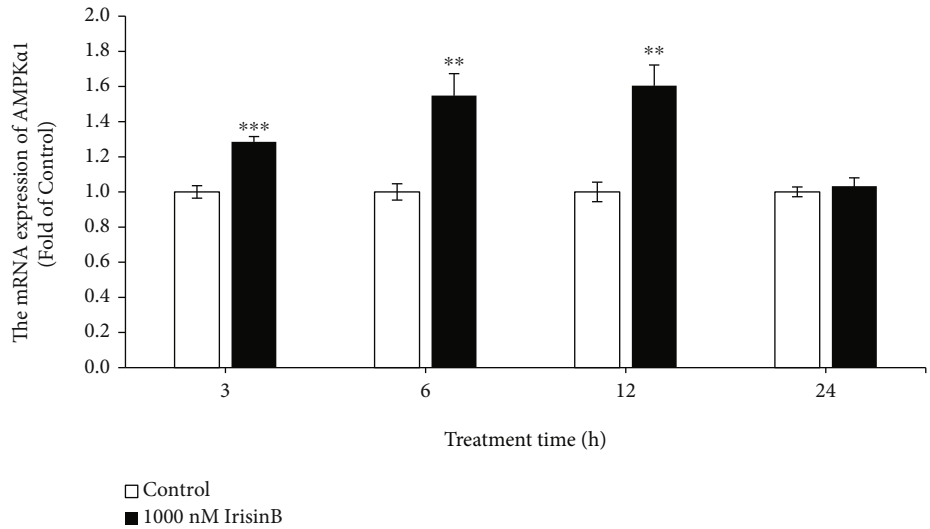

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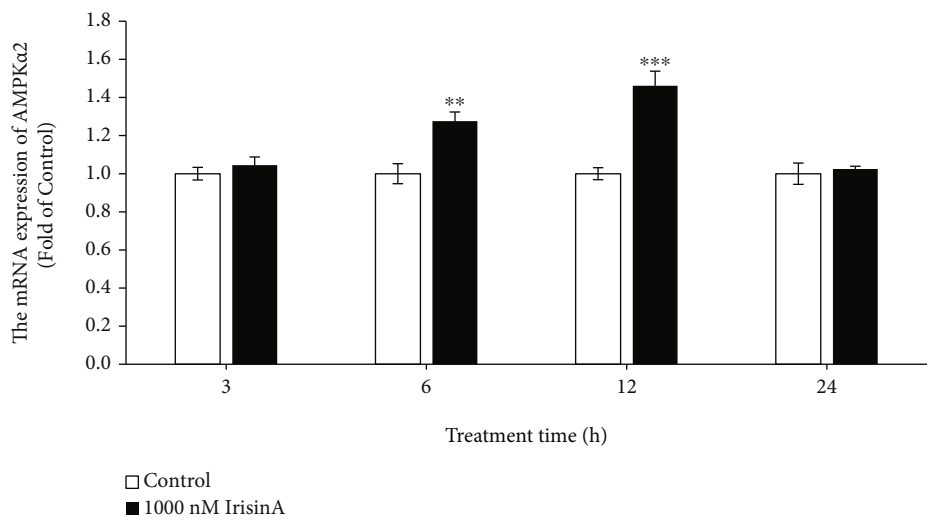

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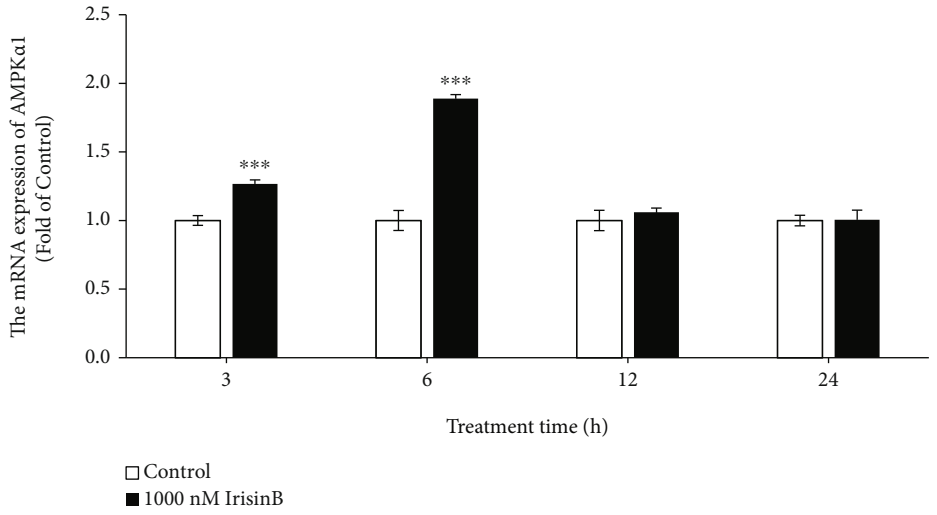

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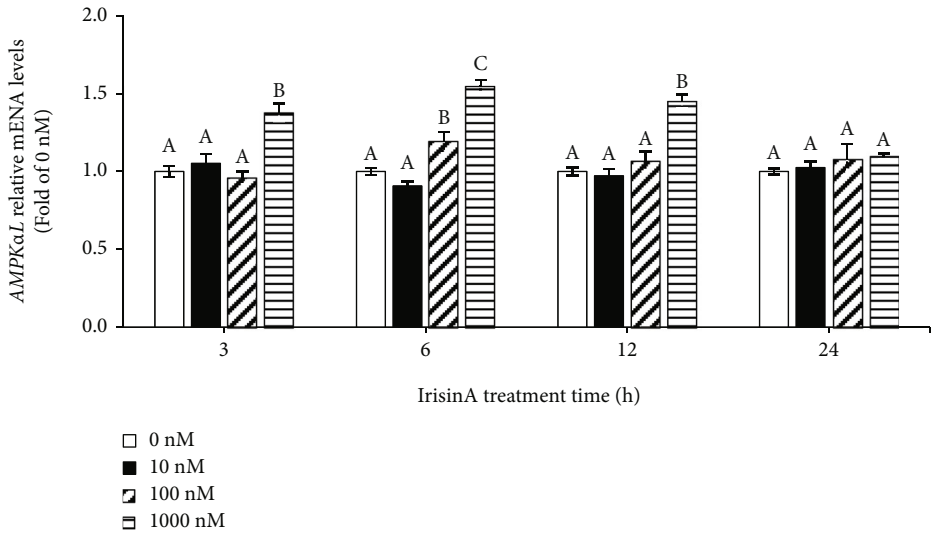

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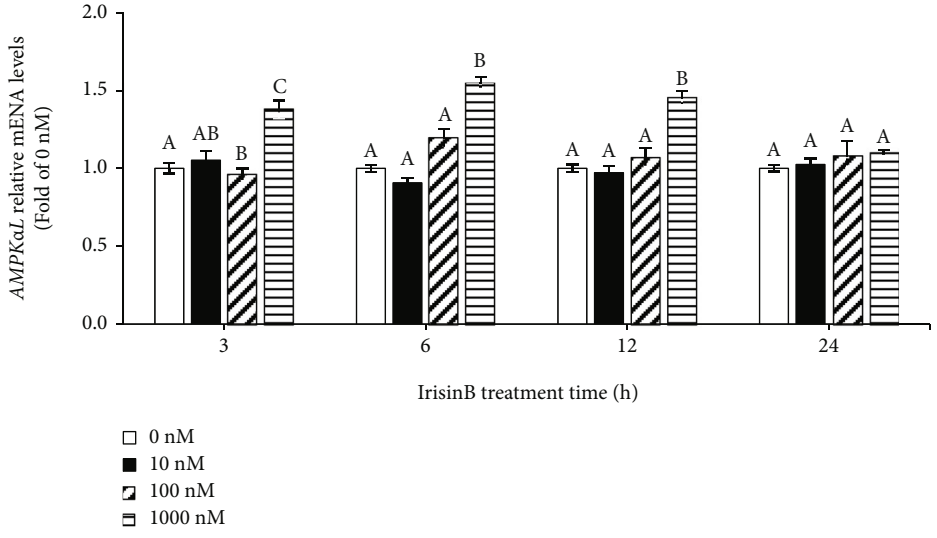

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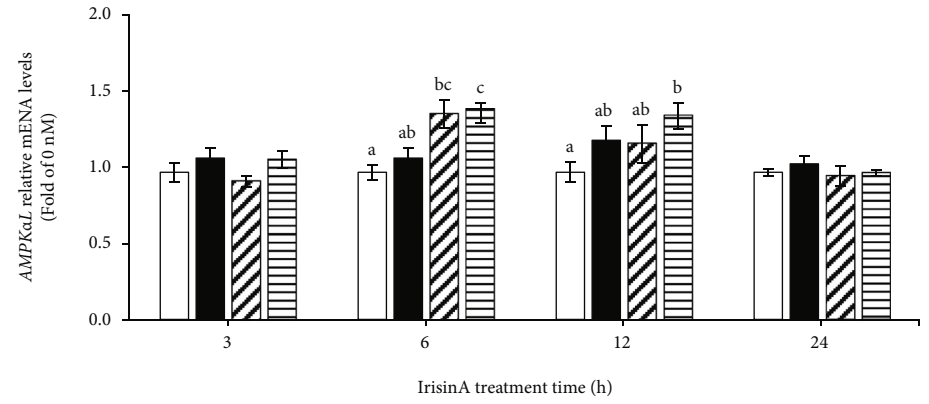

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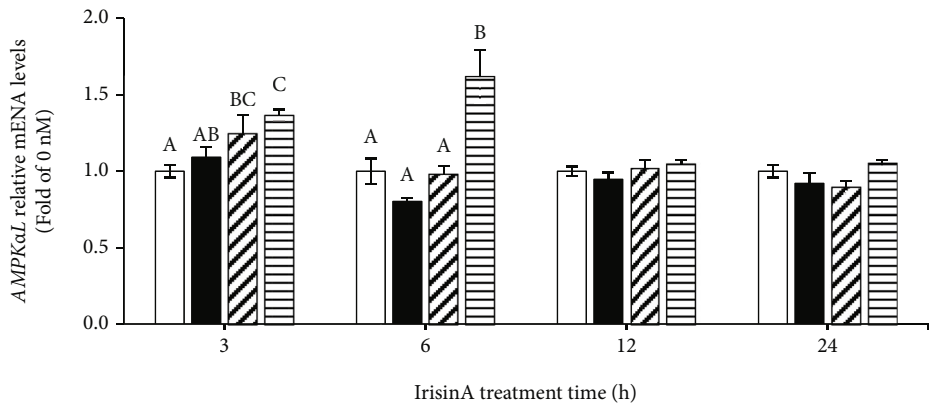

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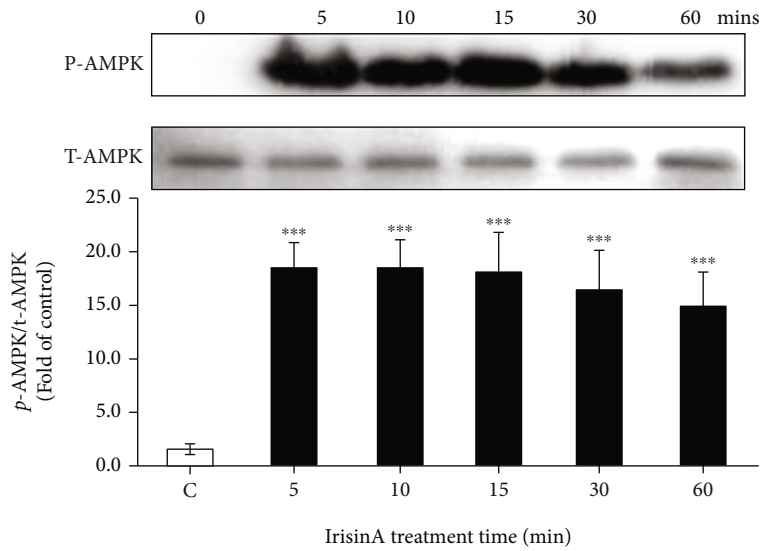

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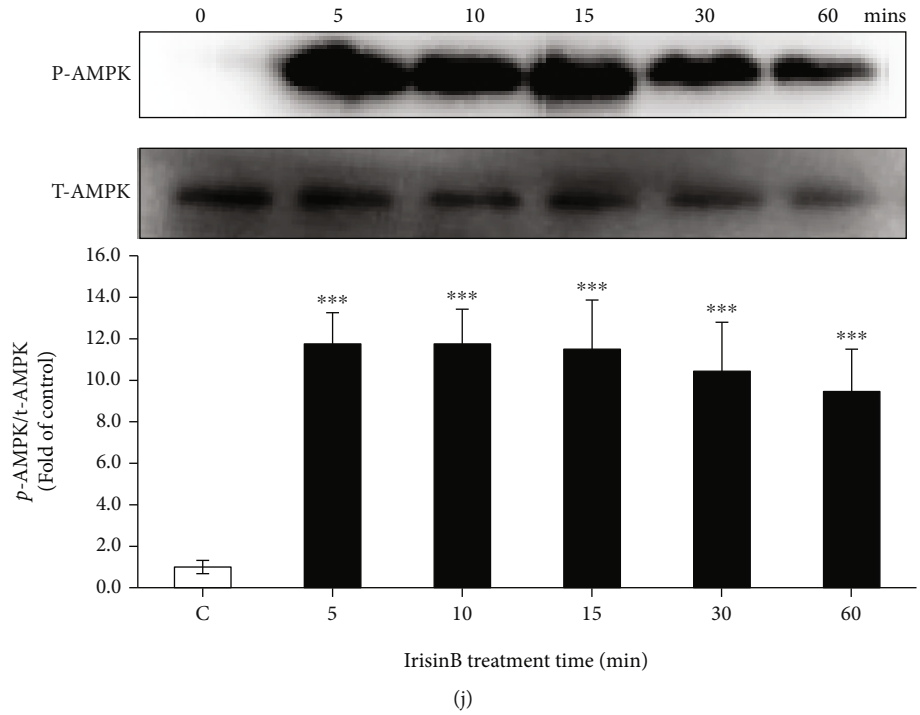
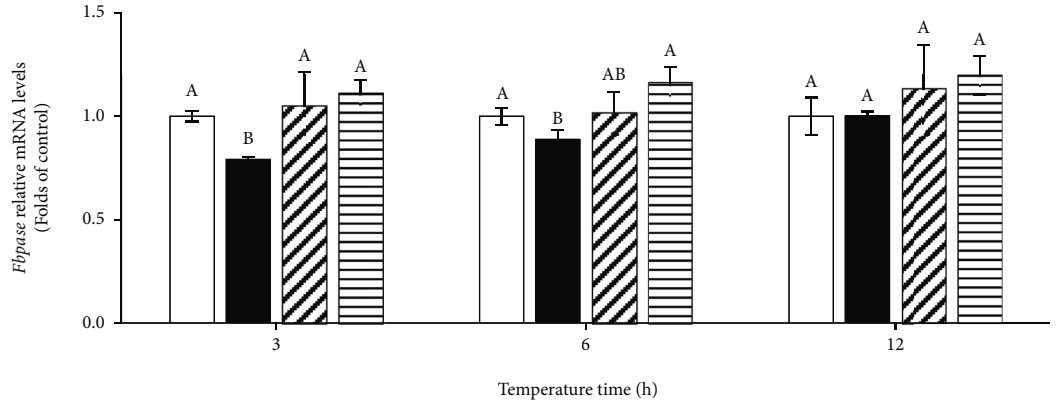


Figure 5: The hepatocytes AMPK signaling pathway was activated by irisin. The common carp hepatocytes were incubated with irisin. The mRNA expression of *ampka1* after incubation with 1000 nM (a) irisinA and (b) irisinB. The mRNA expression of *ampka2* after incubation with 1000 nM (c) irisinA and (d) irisinB. The mRNA expression of *ampka1* after incubation with 0, 10, 100, and 1000 nM (e) irisinA and (f) irisinB. The mRNA expression of *ampka2* after incubation with 0, 10, 100, and 1000 nM (g) irisinA and (h) irisinB. The phosphorylation degree of AMPK after stimulating the cells with 1000 nM of (i) irisinA and (j) irisinB for various intervals of time. The gene expression levels were represented as the fold of control. All data were shown as the mean ± S.E.M. ($n = 6$). The signals of target proteins were quantitated by Image J and expressed as a ratio of P/T-form in the bar graphs ($n = 4$). The probability less than 0.05 indicated a significant difference in *T*-test. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. The difference of the one-way ANOVA analysis was represented by different superscript letters.

increased by irisinB of different concentrations after 6 and 12 h ($P < 0.05$) (Figures 5(g) and 5(h)). We further examined the phosphorylation of AMPK after stimulating the cells with 1000 nM of irisinA or irisinB at various times ($P < 0.05$). As shown in Figure 5(i), increased phosphorylation of AMPK peaked at 5 min after irisinA treatment. After irisinB incubation, the AMPK phosphorylation peaked at 10 min (Figure 5(j)). Besides, the effect of irisinA at *fbpase*, *gk*, *g6pase*, and *pepck* was nullified by cotreatment with the AMPK inhibitor compound C (10 μmmol/L, Figure 6(a)–6(d)). And the effect of irisinB at *gk*, *g6pase*, and *pepck* was nullified by cotreatment with the AMPK inhibitor compound C (10 μmmol/L, Figure 6(e)–6(g)). The hepatocytes were incubated with 1000 nmmol/L irisinA, and the *pi3k* was markedly increased after 3, 6, and 12 h ($P < 0.05$) (Figure 7(a)). At the same time, the *pi3k* can be significantly activated by irisinB after treatment with 3, 6, 12, and 24 h ($P < 0.05$) (Figure 7(b)). The *akt* was upregulated by irisinA after 12 and 24 h ($P < 0.05$), and irisinB significantly increased the *akt* mRNA expression levels at 6 and 12 h ($P < 0.05$) (Figures 7(c) and 7(d)). After the hepatocytes were exposed to different doses of irisinA or irisinB, the *pi3k* and *akt* were increased by irisinA or irisinB ($P < 0.05$) (Figure 7(e)–7(h)). We further explored the phosphorylation of Akt after incubating with irisinA or irisinB at different times. Western blot results showed that the phosphorylation peak of Akt was 10 min after irisinA or irisinB treatment (Figures 7(i) and 7(j)). In addition, the effect of irisinA or irisinB at *gs*, *pfk*, *g6pase*, *hk*, and *gsk3b* was nullified by cotreatment with the PI3K inhibitor wortmannin (10 μmmol/L, Figure 8(a)–8(j)). And the *ampka1* was not changed by wortmannin compared to the irisinA or irisinB incubated group ($P > 0.05$) but significantly reduced *ampka2* after 6 h ($P < 0.05$) (Figures 9(a) and 9(b)). Moreover, the *pi3k and akt* were significantly decreased by compound C compared to the irisinA or irisinB incubated group ($P < 0.05$) (Figures 9(c) and 9(d)).

4. Discussion

Irisin was found as a crucial factor in regulating the body's metabolism, including that glucose and lipid homeostasis and growth ([16, 17, 24]). Irisin was the protein encoded by precursor gene *FNDC5*, which was produced by protease hydrolysis and cleavage [31]. In fish, the paralogs of *FNDC5* have only been identified in a few species, such as Nile tilapia



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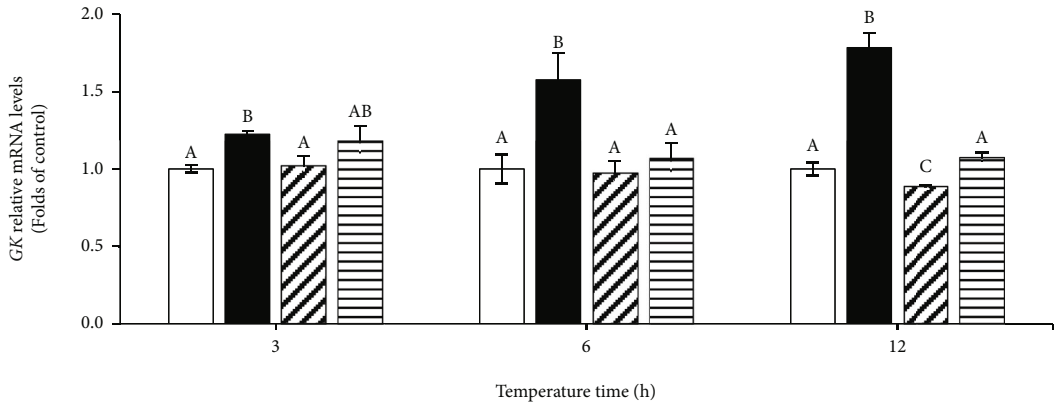

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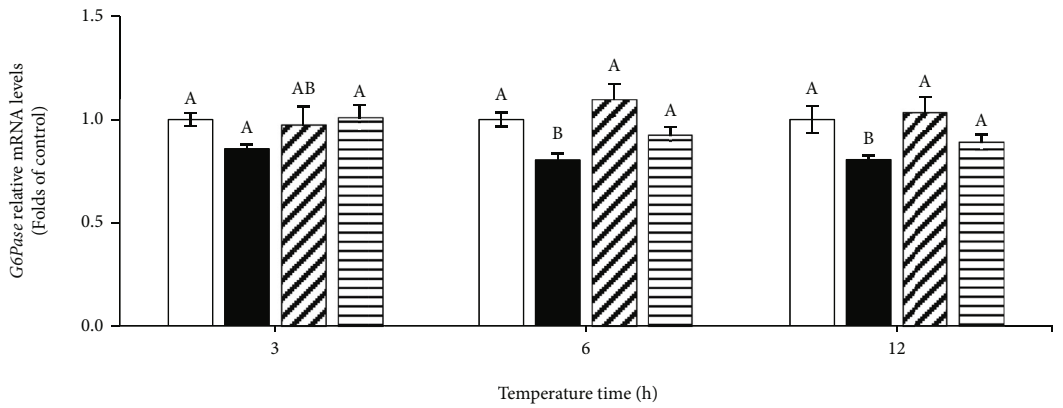

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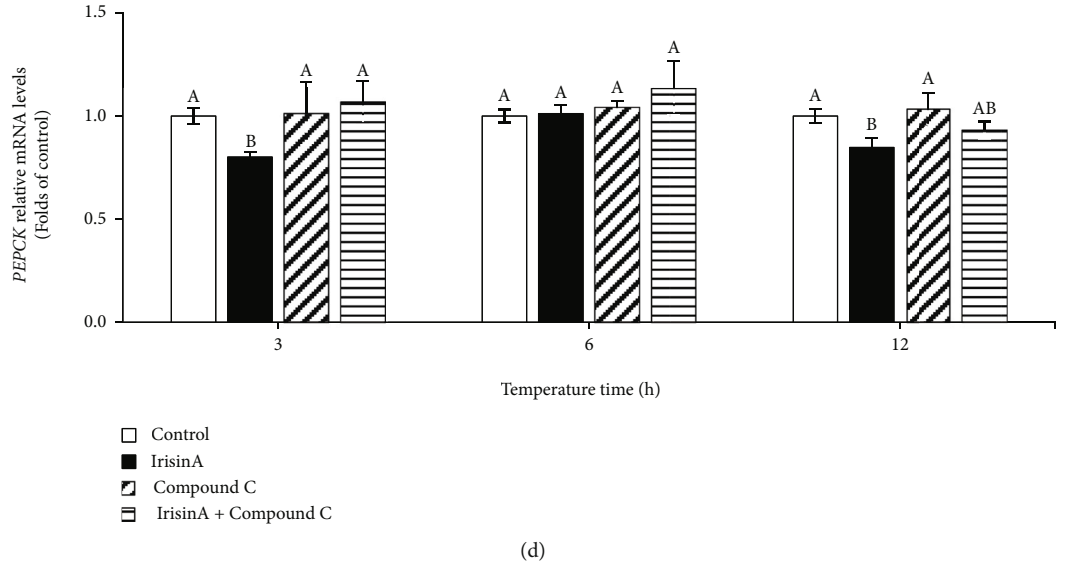

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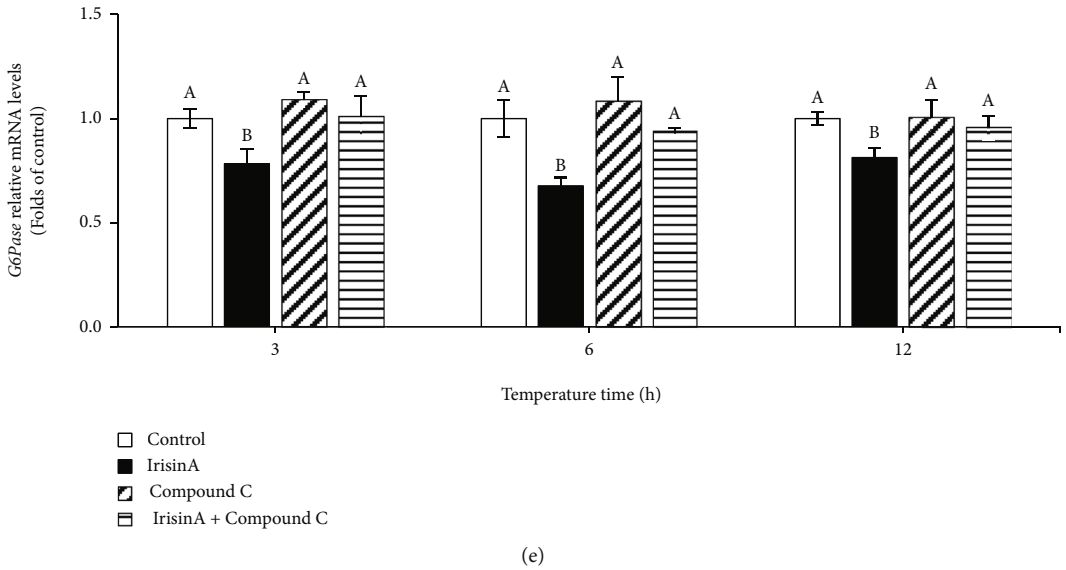

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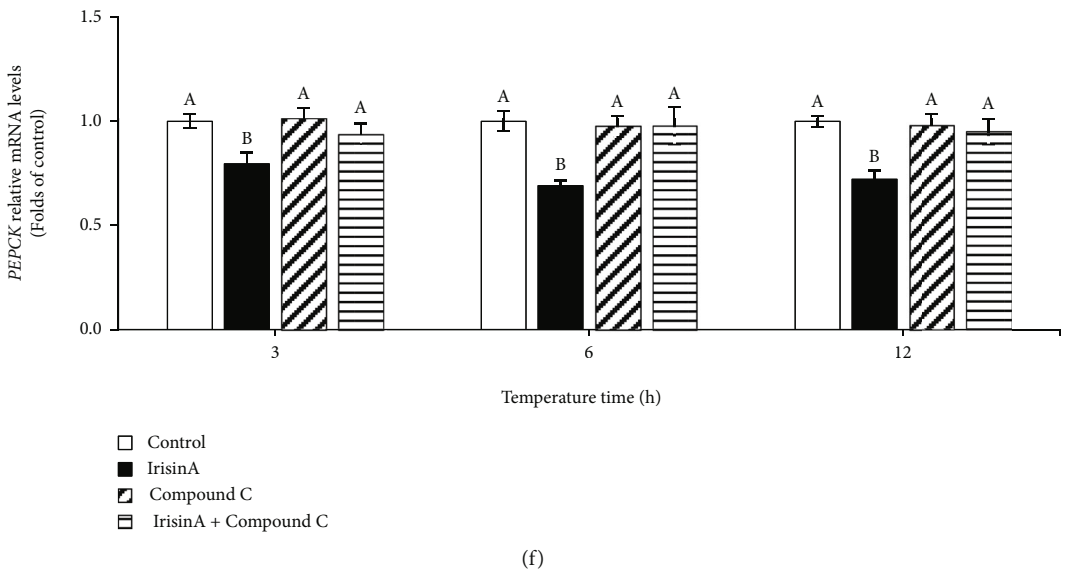

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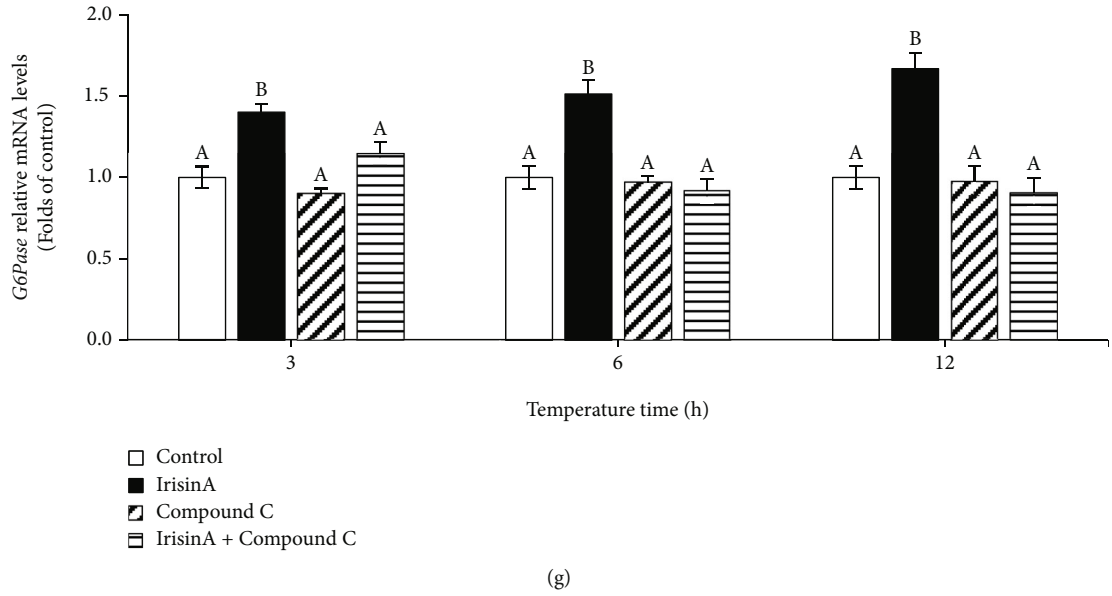
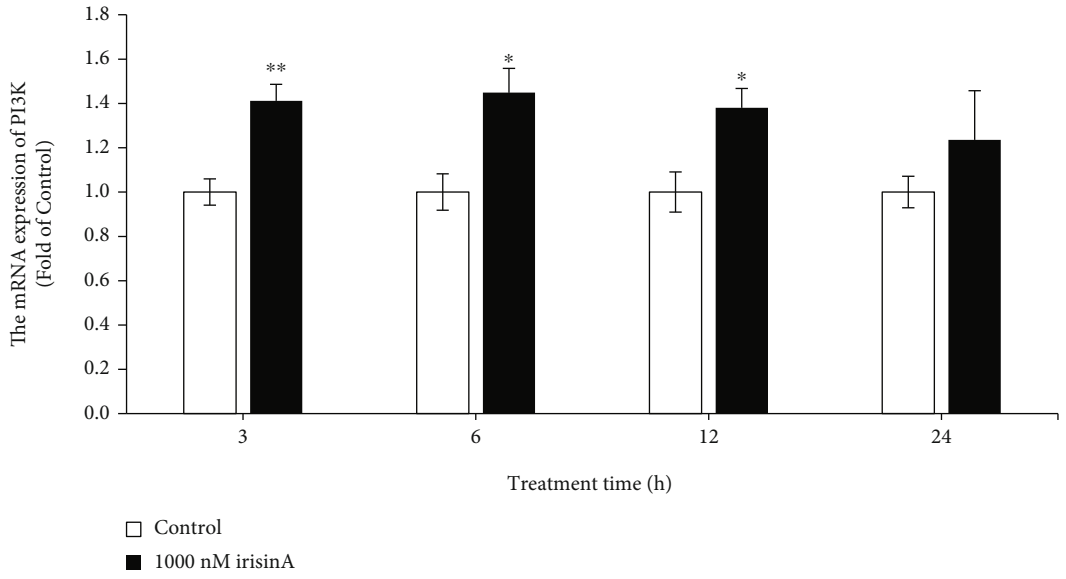


Figure 6: Irisin regulates downstream genes through AMPK to improve common carp glucose metabolism. The common carp hepatocytes were incubated with irisinA (1000 nM), irisinB (1000 nM), and compound C (10 nM). The mRNA expression of (a) *fbpase*, (b) *gk*, (c) *g6pase*, and (d) *pepck* after incubated with irisinA and compound C. The mRNA expression of (e) *g6pase*, (f) *pepck*, and (g) *gk* after incubated with irisinB and compound C. The gene expression levels were represented as the fold of control. All data were shown as the mean ± S.E.M. ($n = 6$). The difference of the one-way ANOVA analysis was represented by different superscript letters.

[24], zebrafish [27], and *Cyprinus carpio* [28]. Previous studies have shown that irisin was mainly produced by skeletal muscle and adipose tissue in mammals [32]. In rats, *FNDC5* was expressed in the skin, liver, and muscle membrane [33]. In fish, *FNDC5* was detected in the brain, gut, kidney, liver, and muscle of goldfish and *Nile Tilapia* [23, 24]. These data point to irisin distribution as tissue-specific.

Previous studies have shown that glucose could significantly increase the mRNA levels of *FNDC5a* in the brain, midgut, red muscle, and white muscle and decrease the *FNDC5a* in the liver and *FNDC5b* expression in the brain, liver, and midgut [28]. However, in mice, *FNDC5* of myoblasts was significantly downregulated after being affected by glucose [34]. In our study, after long-term feeding experiments, the *FNDC5* of muscle and gut were significantly upregulated by a high-glucose or high-fat diet. Marrano et al. have shown that the secretion of irisin was significantly increased after being fed with a high-glucose and high-fat diet [8]. A high-fat diet causes a fast and persistent rise in blood irisin content and mRNA expression in skeletal muscle and adipose tissue in wild-type mice [35, 36]. Previous studies have determined that circulating irisin was related to the function of pancreatic b-cells [10]. One study demonstrated that low *FNDC5* gene expression in muscles and low circulating irisin levels were related to the decrease in endogenous insulin production [37]. Meanwhile, both continuous insulin infusion and insulin injection could increase the plasma irisin content in patients [38]. In our study, insulin could significantly reduce the expression of *FNDC5a* in common carp liver and midgut, but *FNDC5b* levels were not changed. And the *FNDC5a* mRNA levels in common carp liver were significantly upregulated after glucagon injection. These results provide an experimental basis for further research on the function of irisin.

The reports on the biological functions of fish irisin were limited to the growth and reproduction of tilapia [24, 39]. To clarify the effect of irisin on common carp glucose metabolism, this study carried out the intraperitoneal injection of irisin and RNAi. The *i.p.* experiment showed that the enzyme activity of glycolysis-related enzyme PFK and the liver glycogen content were significantly increased after irisin injection. The enzyme activity of gluconeogenesis-related enzyme PEPCK was significantly decreased after irisin injection. GLUT2 (glucose transporter 2) was the primary glucose transporter of liver cells [40]. Studies have shown that in HepG2 cells, irisin could improve the reduction of *glut2* mRNA expression induced by palmitate [41]. In vivo experiments found that the intracerebroventricular injection of irisinA siRNA or irisinB siRNA significantly reduced the liver *glut2* expression. Hepatic glycogen acts as a buffer store for glucose to maintain a constant blood glucose level. GS is a critical enzyme in glycogen synthesis. When treated with siRNA, we observed that the mRNA expression of *gs* was significantly decreased. GSK3b (glycogen synthase kinase 3 beta) was a glycogen synthase kinase, and irisin could reduce glucose production by inhibiting GSK3b and then activating GS [9]. Glycogen phosphatase (PYG) could catalyze



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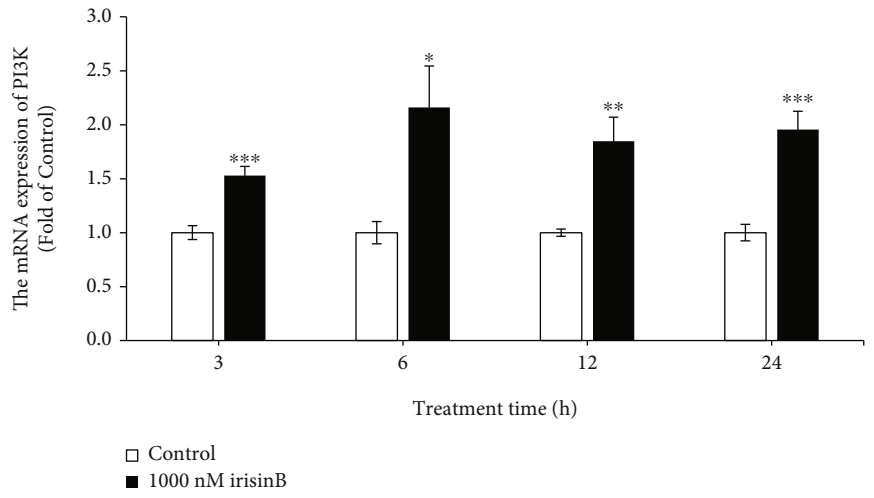

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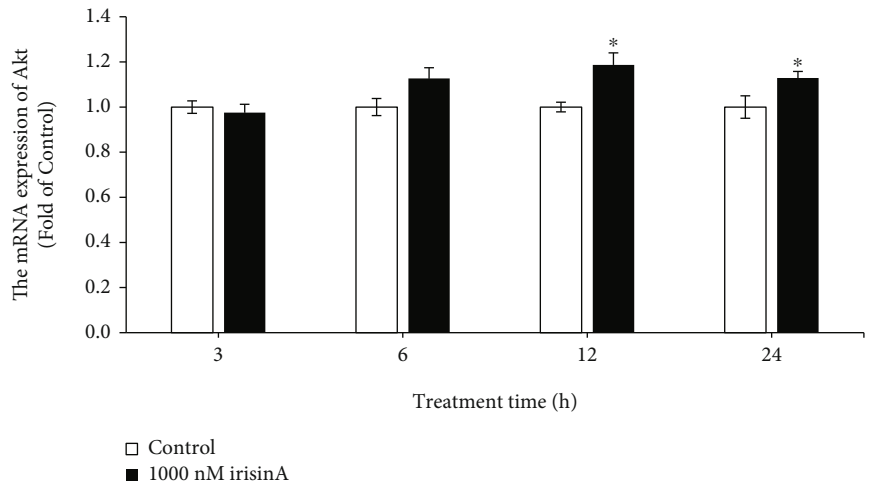

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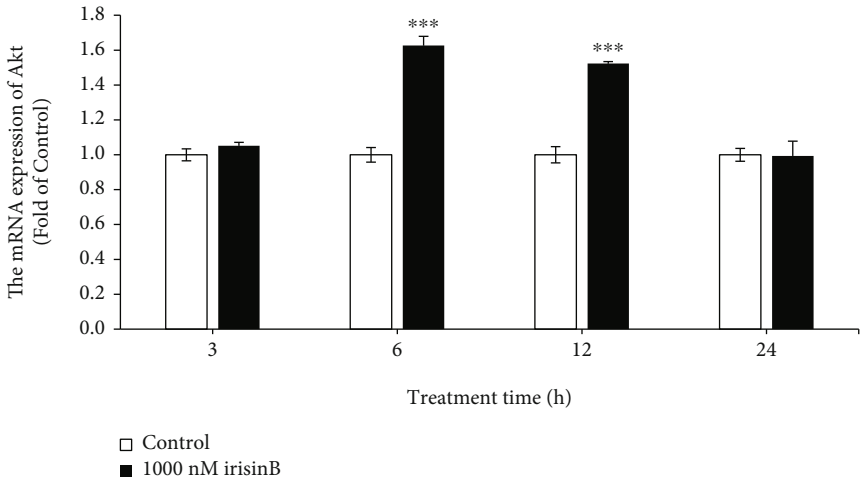

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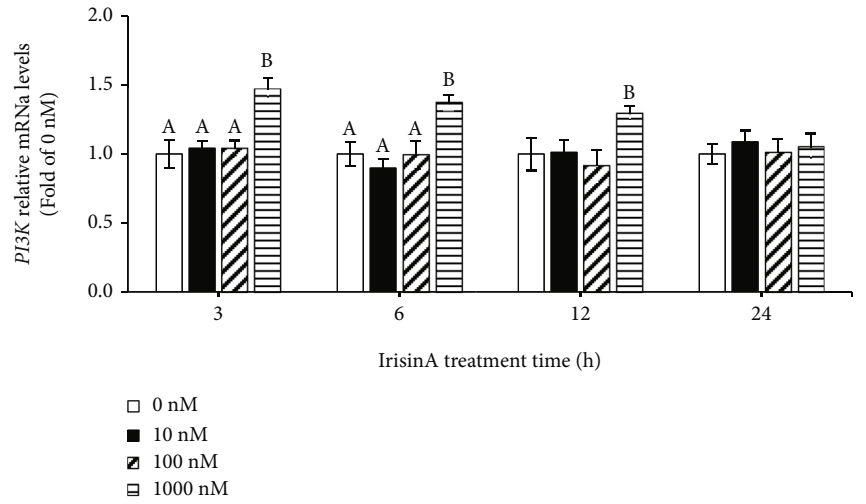

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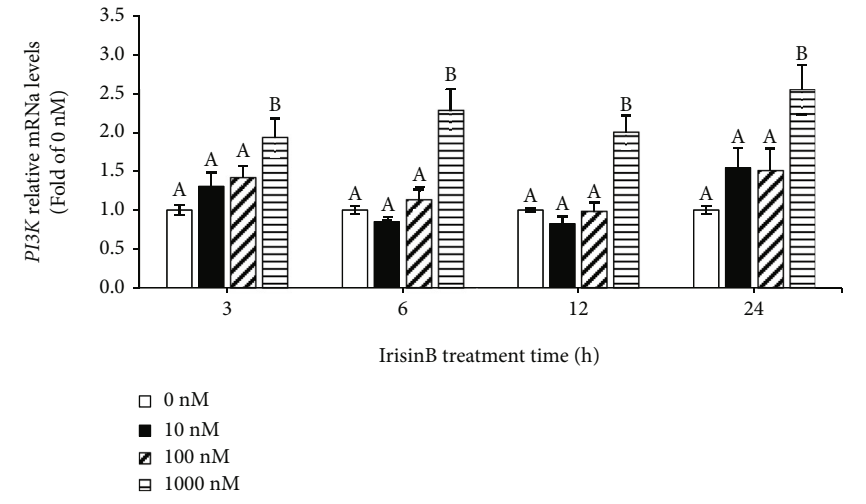

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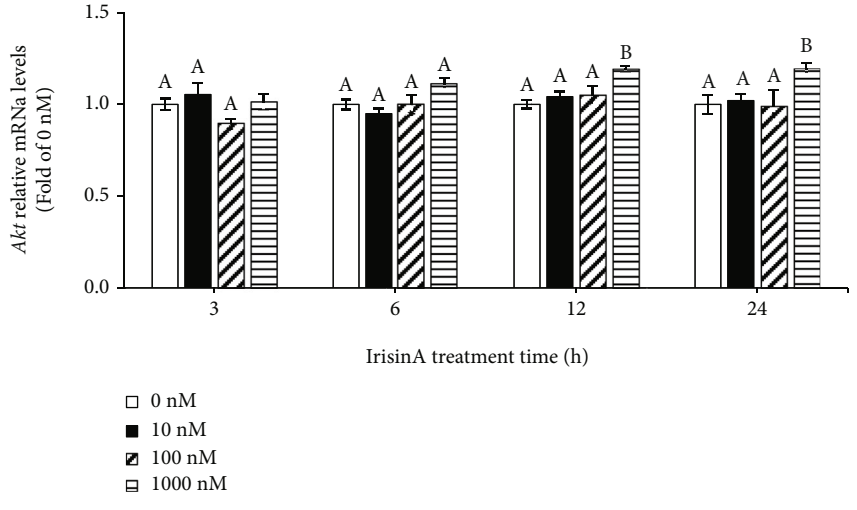

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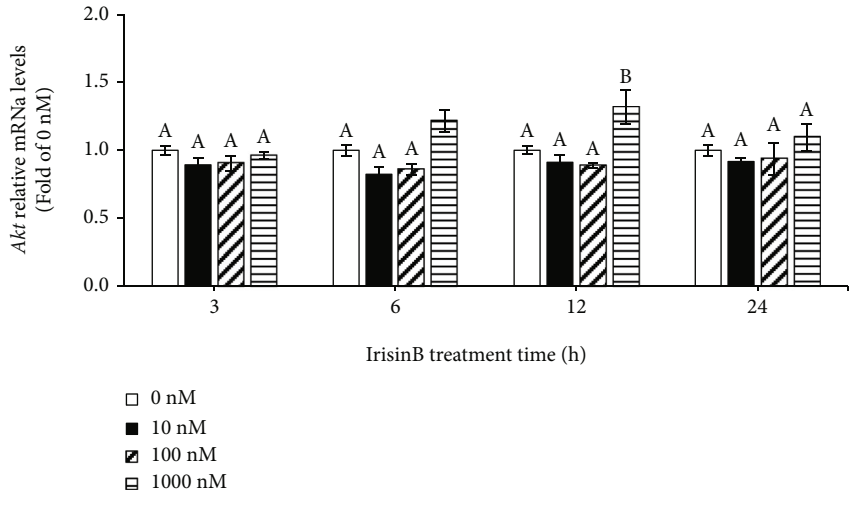

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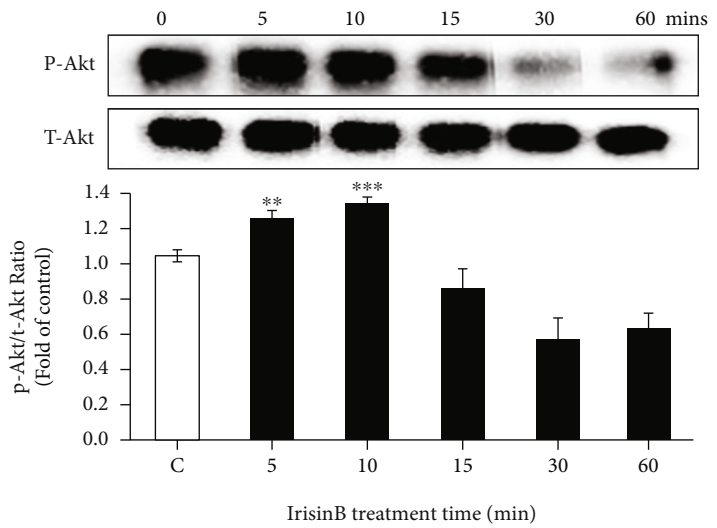

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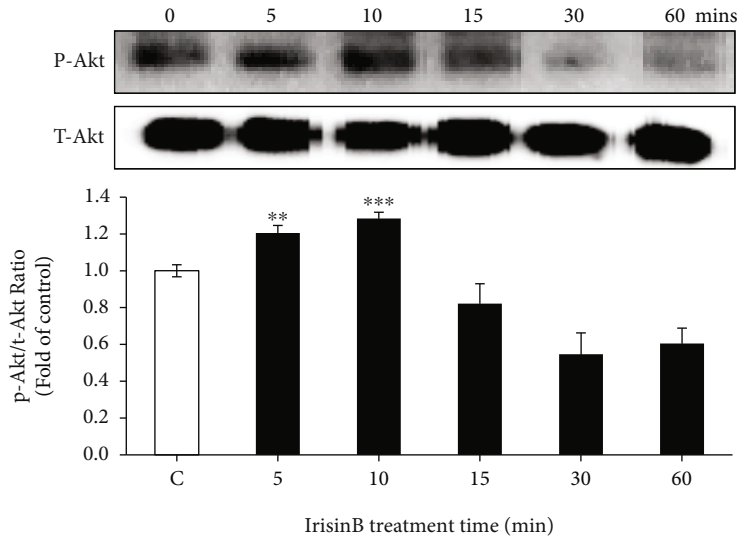
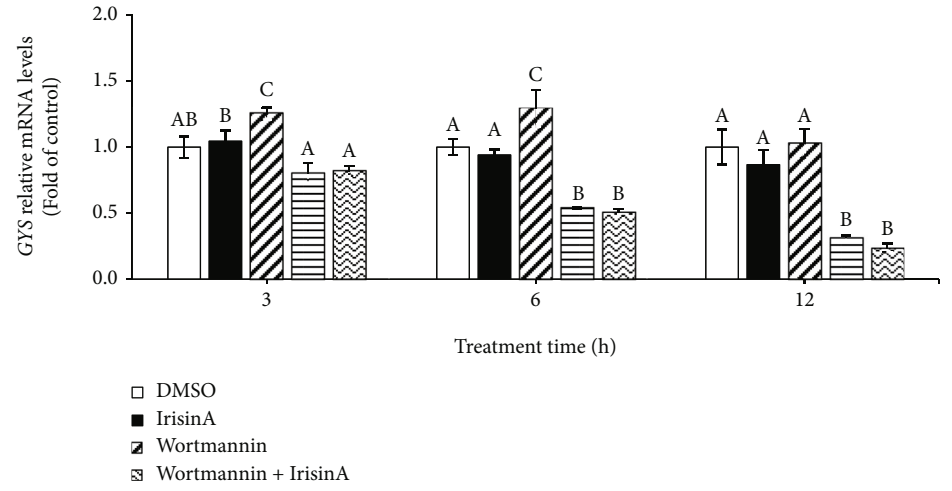


Figure 7: The hepatocytes PI3K/Akt signaling pathway was activated by irisin. The common carp hepatocytes were incubated with irisin. The mRNA expression of *pi3k* after incubation with (a) irisinA and (b) irisinB (1000 nM). The mRNA expression of *akt* after incubation with (c) irisinA and (d) irisinB (1000 nM). The mRNA expression of *pi3k* after incubation with (e) irisinA and (f) irisinB (0, 10, 100, and 1000 nM). The mRNA expression of *akt* after incubation with (g) irisinA and (h) irisinB (0, 10, 100, and 1000 nM). The phosphorylation degree of Akt after stimulating the cells with 1000 nM of (i) irisinA and (j) irisinB for various intervals of time. The gene expression levels were represented as the fold of control. All data were shown as the mean ± S.E.M. ($n = 6$). The signals of target proteins were quantitated by Image J and expressed as a ratio of P/T-form in the bar graphs ($n = 4$). The probability less than 0.05 indicated a significant difference in T-test. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. The difference of the one-way ANOVA analysis was represented by different superscript letters.

the conversion of glycogen to glucose [42]. In this study, the *pygl* (liver glycogen phosphorylase) was markedly upregulated after siRNA injection, which means that the absence of irisin could upregulate the glycogen phosphorylase mRNA levels, leading to the excessive decomposition of glycogen and increased blood glucose. From the above results, we could know that irisin could control the absorption or excretion of glucose by changing the expression of *glut2* and control the synthesis of liver glycogen by *gs*, *gsk3b*, and *pygl*. The liver regulates glucose utilization and glucose production to maintain glucose homeostasis, and the key enzymes G6Pase, PEPCK, and FBPase (fructose-1,6-bisphosphatase) in the process gluconeogenesis play critical roles in glucose production [43]. Previous research has revealed that the *pepck* was significantly increased caused by the downregulation of *FNDC5* in human HepG2 cells and mouse primary hepatocytes [44]. Our research found that after treatment with siRNA, the levels of *pepck* and *g6pase* mRNA were significantly increased in the liver. In addition, in diabetic mice with a continuous intraperitoneal injection of irisin for 14 days, the expression of *pepck* and *g6pase* were reduced after in hepatocytes by activating AMPK [17]. In diabetic mice, irisin could stimulate the PI3K/Akt signaling pathway and downregulate *pepck* and *g6pase* through FOXO1 [9]. Fructose-1,6-bisphosphatase (*fbpase*) maintains glucose homeostasis by modulating the glycogen pathway and catalyzing the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate [45]. In our research, the RNAi experiment proved that the expression of *fbpase* was significantly reduced by irisin. Another significant way to control glucose metabolism was glycolysis. A study on a single muscle cell found that miR-142-3p could inhibit the expression of *FNDC5*, thereby inhibiting glycolysis [46]. In human adipocytes, it was found that irisin promoted glycolysis in visceral adipocytes through UCP-1-independent pathway [47]. Studies have also shown that, after 8 days of continuous treatment with 50 nM irisin, the level of glycolysis was significantly increased in human adipocytes [48]. In our study, we found the vital enzyme activity of glycolysis and the expression of its mRNA levels, such as *gk* (glucokinase), *hk* (hexokinase), and *pfk*, was inhibited after siRNA injection. In this study, serum glucose content was significantly increased after treatment with siRNA. Similar studies have shown that irisin treatment of human hepatocytes glucose output was significantly reduced [49]. Through the study of its function, we screened out the corresponding downstream gene targets, laying the foundation for perfecting the regulation path of irisin on common carp glucose metabolism.

Studies have confirmed that irisin could inhibit gluconeogenesis, cholesterol, and glycogen synthesis [9, 50]; improve glucose uptake; and inhibit glycogen breakdown



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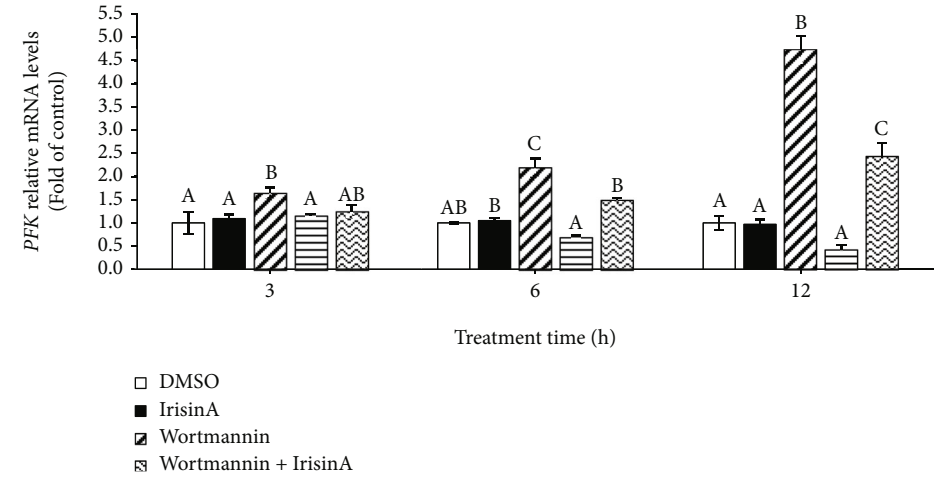

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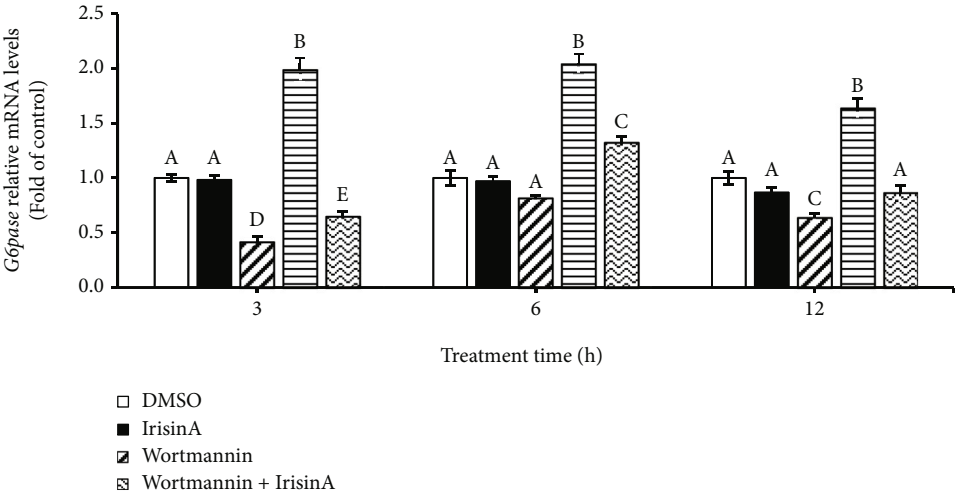

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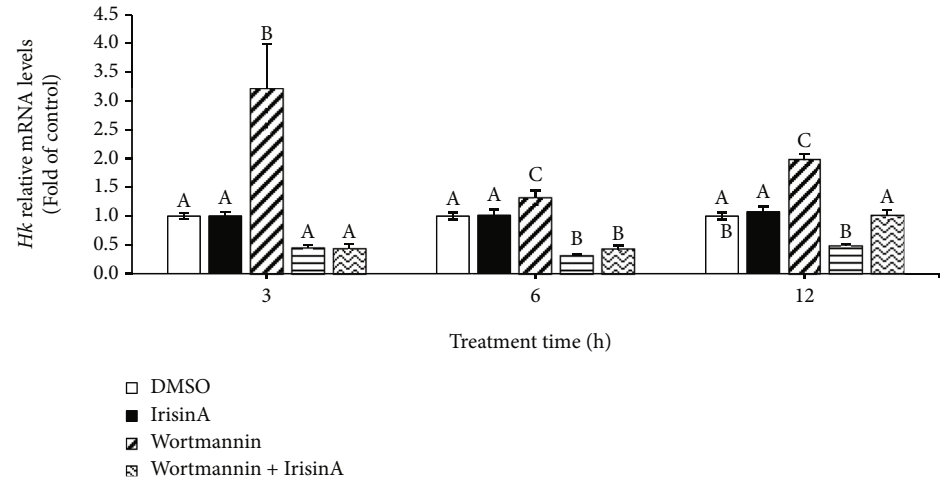

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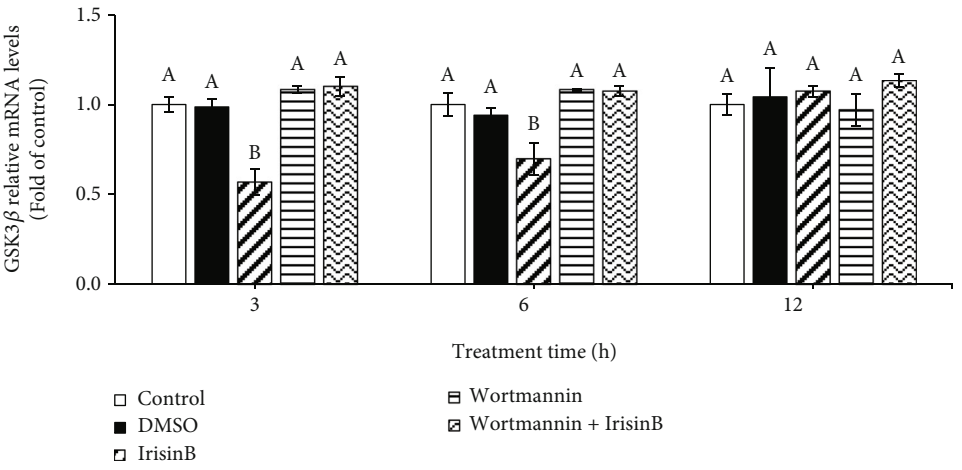

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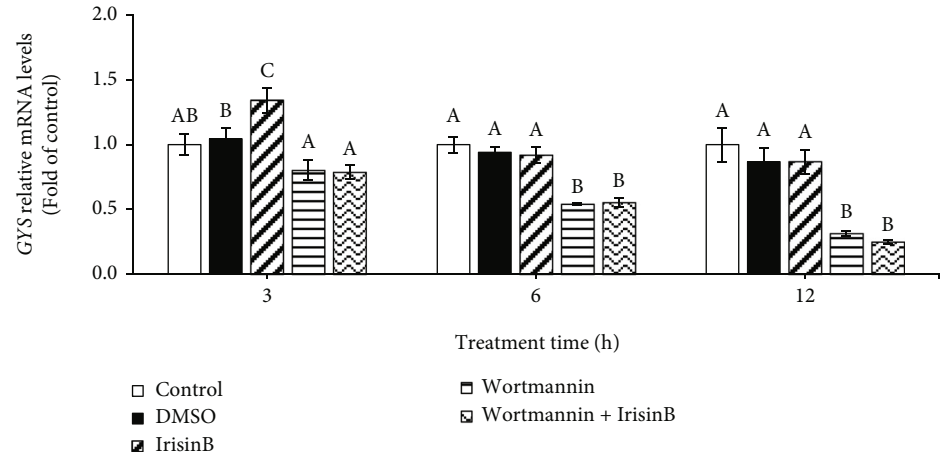

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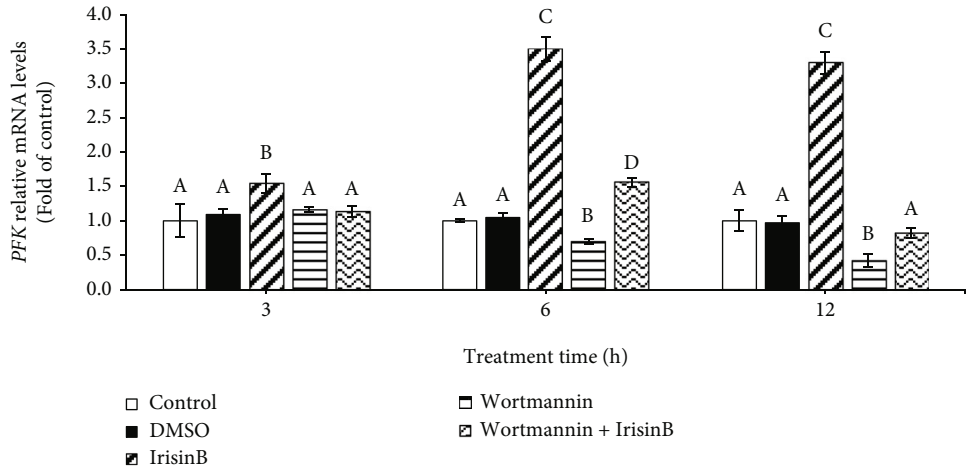
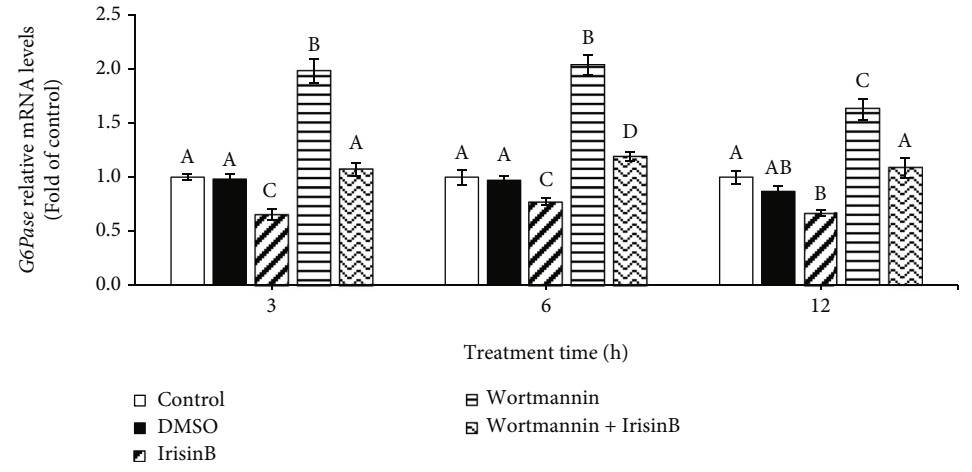
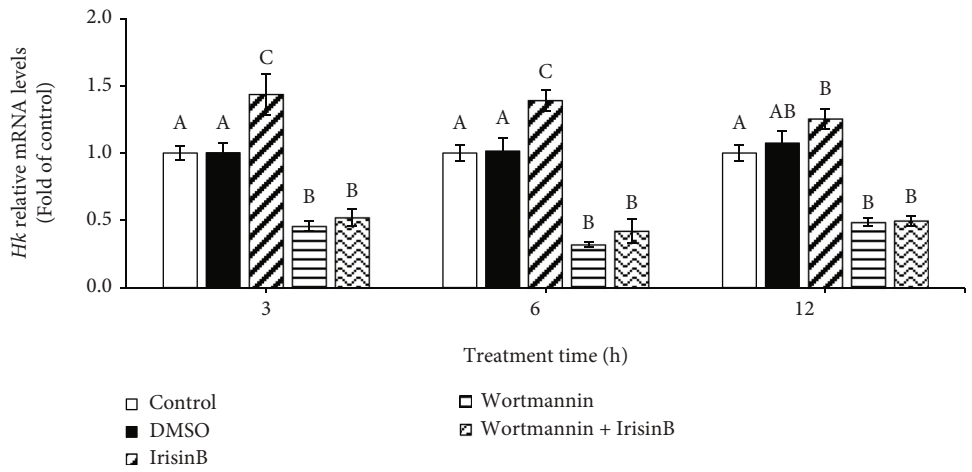


(g)



(h)



(i)

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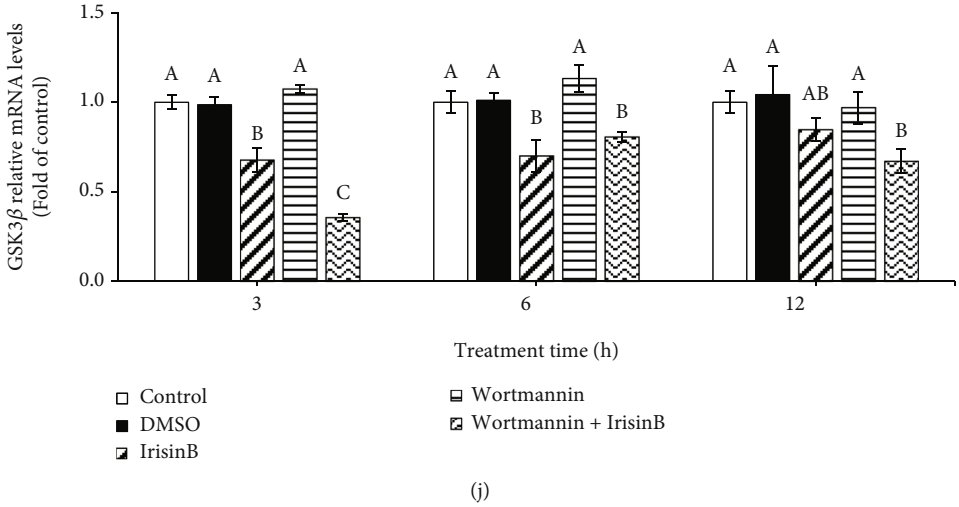
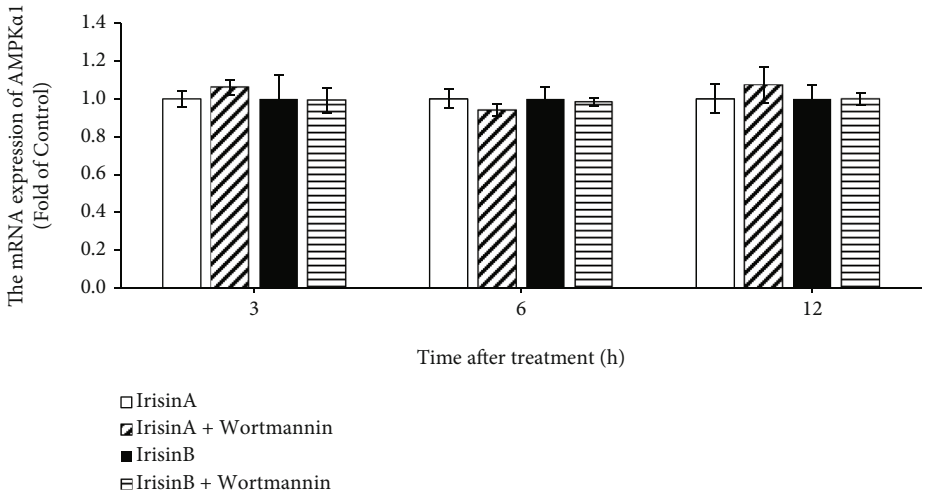


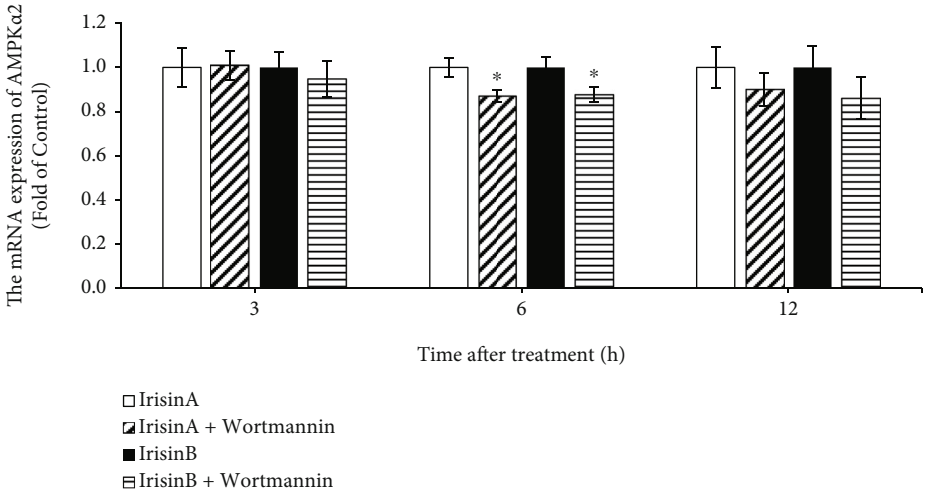
Figure 8: Irisin regulates downstream genes through PI3K/Akt to improve common carp glucose metabolism. The common carp hepatocytes were incubated with irisinA (1000 nM), irisinB (1000 nM), and compound C (10 nM). The mRNA expression of (a) *gs*, (b) *pfk*, (c) *g6pase*, (d) *hk*, and (e) *gsk3b* after incubated with irisinA and wortmannin. The mRNA expression of (f) *gs*, (g) *pfk*, (h) *g6pase*, (i) *hk*, and (j) *gsk3b* after incubated with irisinB and wortmannin. The gene expression levels were represented as the fold of control. All data were shown as the mean ± S.E.M. ($n = 6$). The difference of the one-way ANOVA analysis was represented by different superscript letters.

[17]. The activation of AMPK seems to be a key signal of irisin's action in the liver and skeletal muscle [17]. As a negative regulator, AMPK activation reduces liver glucose production by stimulating glycolytic enzymes and inhibiting gluconeogenic enzymes in the liver [51]. This study showed that the AMPK was activated by irisinA or irisinB. One study has shown that using AMPK inhibitor compound C or silencing AMPK could block the regulatory effect of irisin on *FNDC5* expression [52]. In the hepatocytes of humans and diabetic mice, irisin could downregulate *pepck* and *g6pase* by activating AMPK to affect gluconeogenesis [9]. In addition, the AMPK signaling pathway was activated by irisin in skeletal muscle cells to regulate glucose absorption [53]. According to reports, the PI3K/Akt was considered a crucial signal pathway to moderate glucose metabolism [9]. Its activation mediates insulin receptor signal transduction by further regulating downstream target proteins GSK3, GLUT4, and GYS [54]. In this study, the PI3K-Akt signaling pathway was activated by irisinA or irisinB. Similar results have shown that irisin could inhibit hepatic glucose production and increase glycogen synthesis through the PI3K/Akt pathway [9]. In tilapia, irisin through the PI3K/Akt pathways inhibits the secretion of growth hormone [24], stimulating the synthesis and secretion of insulin in pancreatic islet cells [55]. In general, the AMPK and PI3K/Akt signaling pathways could be activated by irisin and play a positive role in glucose metabolism.

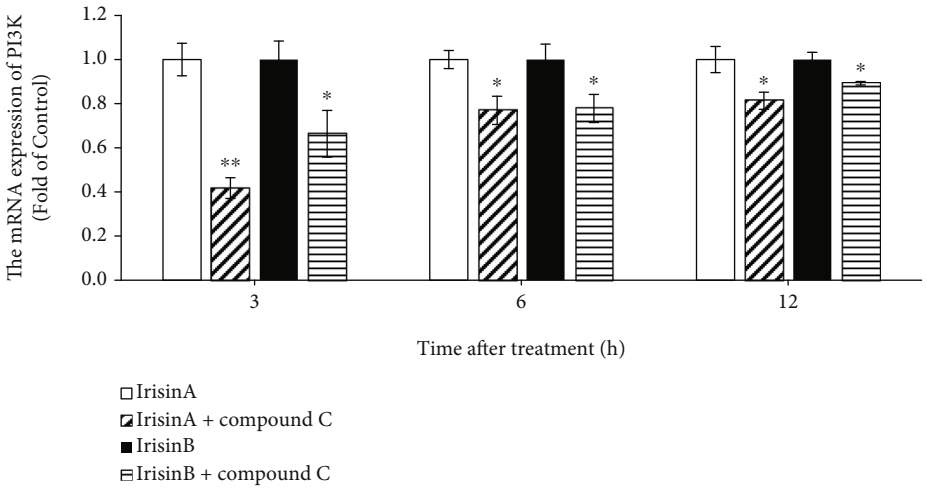
To explore the mechanism of irisin regulating common carp glucose metabolism, we tested *ampka1* and *ampka2*. This study found that the mRNA expression of *ampka1* and *ampka2* were significantly upregulated by irisin at different times or concentrations. Then the hepatocytes were coincubated with the inhibitor of AMPK signaling pathway compound C. When compound C was added, the downregulation of mRNA expression of *fbpase*, *g6pase*, and *pepck* and the upregulation effect of *gk* were eliminated, which means that irisin could regulate *fbpase*, *g6pase*, *pepck*, and *gk* through AMPK signaling pathway, thereby regulating glucose metabolism. After irisin treatment, the *pi3k* and *akt* was markedly increased. After adding wortmannin, an inhibitor of PI3K, the mRNA upregulation effects of *gs*, *pfk*, *hk*, and the downregulation effect of *g6pase* and *gsk3b* were eliminated. Therefore, irisin can regulate *gs*, *pfk*, *hk*, *gsk3b*, and *g6pase* through PI3K/Akt signaling pathway to regulate glucose metabolism. However, we found that *g6pase* was regulated in AMPK and PI3K/Akt. We speculate whether there was a relationship between AMPK and PI3K/Akt in the regulation of common carp by irisin. We further studied that after using compound C, we further studied that the *pi3k* and *akt* were markedly lower than that of hepatocytes incubated with irisin alone. After incubated with wortmannin, the *ampka1* was not changed significantly, but the *ampka2* was significantly decreased at 6 h. According to some research, AMPK can also impact the expression of PI3K/Akt, and it interacts with the PI3K/Akt. In the villous trophoblast cells of the mouse PCOS model, the scientists discovered that when LY294002 or MK2206 HCl were given, the phosphorylation of AMPK levels decreased, as did the phosphorylation of Akt and FOXO3a downstream of AMPK [56]. Some studies showed that the activation of AMPK could promote the phosphorylation of IRS1 at Ser794, therefore, blocking the PI3K/Akt pathways [54]. Above these studies, we know that irisin activates the AMPK and PI3K/Akt pathways in common carp hepatocytes, thereby regulating the common carp glucose metabolism.



(a)

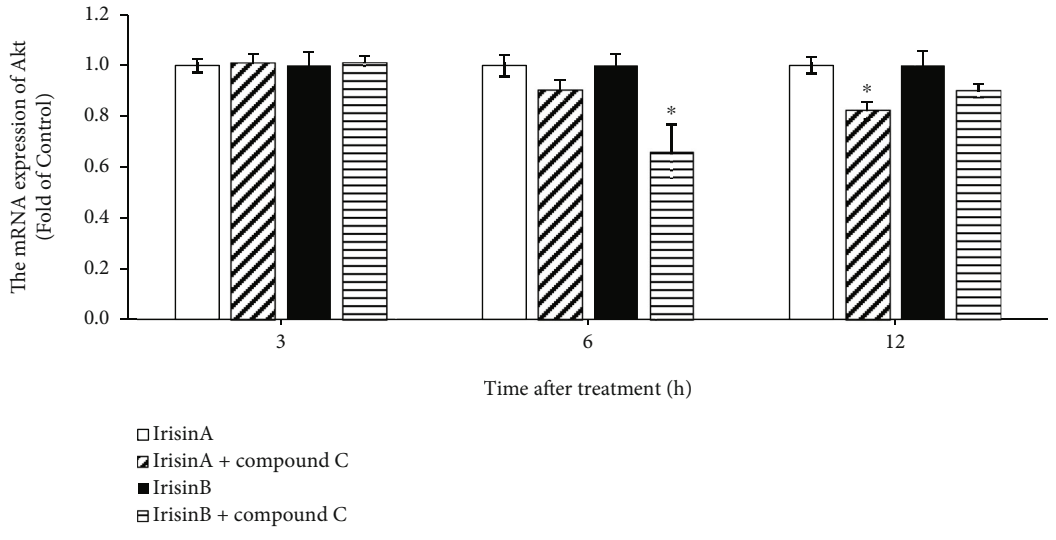


(b)



(c)

Figure 9: Continued.



(d)

Figure 9: Irisin affects the expression of PI3K/Akt by regulating AMPK. The common carp hepatocytes were incubated with irisinA (1000 nM), irisinB (1000 nM), compound C (10 nM), and wortmannin (10 nM). The mRNA expression of (a) *ampka1* and (b) *ampka2* after incubated with irisinA, irisinB, and wortmannin. The mRNA expression of (c) *pi3k* and (d) *akt* after incubated with irisinA, irisinB, and compound C. The gene expression levels were represented as the fold of control. All data were shown as the mean ± S.E.M. ($n = 6$). The probability less than 0.05 indicated a significant difference in T-test. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

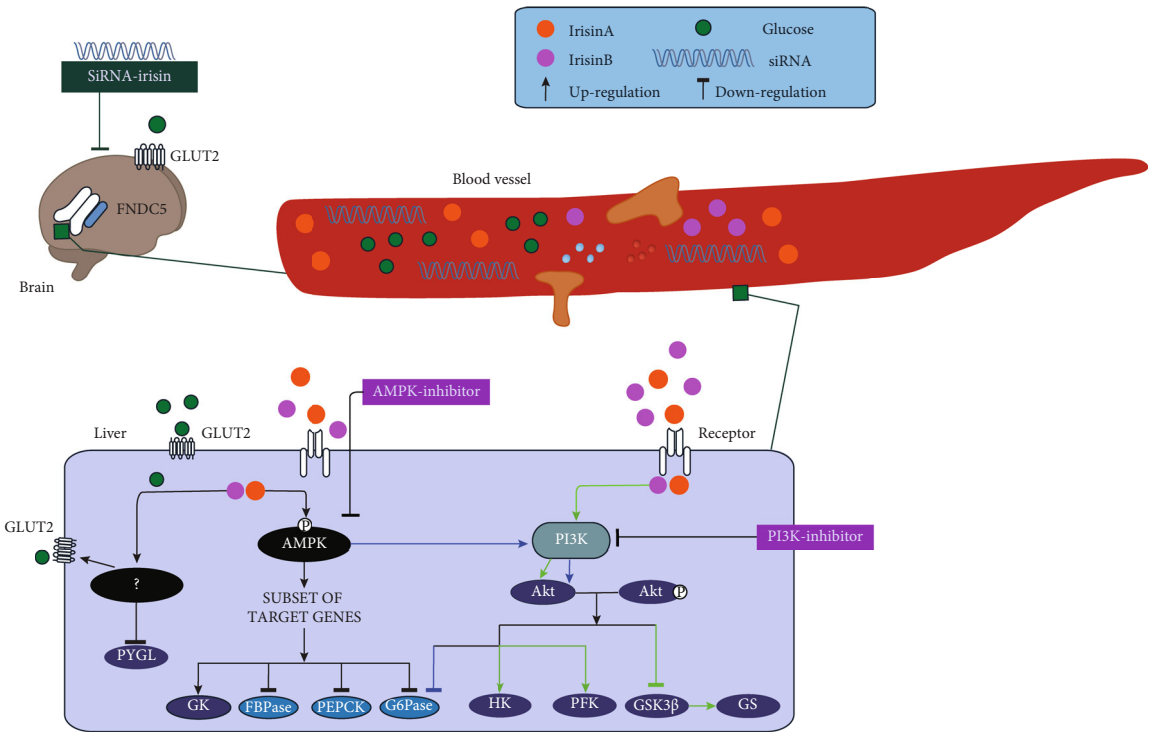


Figure 10: Schematic diagram showing the roles of irisin in the regulation of glucose metabolism in the hepatopancreas of common carp. Irisin upregulated the expression of enzyme genes related to glycolysis (*gk*, *hk*, and *pfk*) and glycogen synthesis (*gs*) and downregulated the expression of enzyme genes related to glycogen phosphorylation (*gsk3b*) and gluconeogenesis (*fbpase*, *pepck*, and *g6pase*) via AMPK orPI3K/Akt. Finally, irisin caused the increase in hepatopancreas glycogen content.

5. Conclusion

In the present study, after a long-term high-glucose and high-fat feeding trial, the *FNDC5a* and *FNDC5b* were markedly increased. Insulin inhibits the *FNDC5a* mRNA expression in the liver and gut, and glucagon promotes the *FNDC5a* in the liver. *FNDC5b* was not sensitive to insulin and glucagon in the liver and gut. Intraperitoneal injection experiments, cell experiments, and intraventricular injection experiments showed that irisin could promote glucose transport, glycogen synthesis, and glycolysis and inhibit glycogen decomposition and gluconeogenesis. It could regulate the mRNA levels of essential genes of hepatic glucose metabolism through the AMPK and PI3K/Akt pathways, thereby regulating the entire common carp glucose metabolism (Figure 10).

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplementary Figure 1: the irisinA siRNA screening and condition optimization. The siRNAs (10 ng/g B.W.) were intraperitoneal injected at 12, 24, and 36 h. (A) In the brain, the mRNA expression levels of FNDC5a. (B) In the liver, the mRNA expression levels of FNDC5a. The probability less than 0.05 indicated a significant difference in T-test.$*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ (n =9). Supplementary Figure 2: the irisinB siRNA screening and condition optimization. The siRNAs (10 ng/g B.W.) were intraperitoneal injected at 12, 24, and 36 h. (A) In the brain, the mRNA expression levels of FNDC5b. (B) In the liver, the mRNA expression levels of FNDC5b. The probability less than 0.05 indicated a significant difference in T-test. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ (n =9). Supplementary Figure 3: the operational procedure of i.c.v injection in common carp. The 60-65 g common carp with i.c.v injection was achieved using a 50-μL Hamilton syringe with 26 G 1/2-inch needles inserted through the top of the skull (A, B). The depth of penetration was limited at 5 mm by a collar fitted on the needle (C). The tangent line of the back edge of the eye was drawn lightly on the skin (D), the injection site was on this line and about 1-2 mm vertical distance from the dorsal line (E). 5 min after injection with 10 μL methylene blue, postmortem examination indicated that the cranial cavity was dyed blue (F, G). *(Supplementary Materials)*

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