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Research paper

FoxO1 in *Micropterus salmoides*: Molecular characterization and its roles in glucose metabolism by glucose or insulin-glucose loading

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ABSTRACT

Forkhead box O1 (*FoxO1*), a nuclear transcription factor, plays an important role in insulin-mediated glucose metabolism. In this study, *FoxO1* gene from largemouth bass (*Micropterus salmoides*) was cloned and characterized, and its effects on hepatic glucose metabolism regulated by insulin-AKT pathway were investigated in response to glucose or insulin-glucose injection. The full-length cDNA of *FoxO1* consisted of 2541 bp and encoded 680 amino acids. Sequence alignments and phylogenetic analysis revealed that *FoxO1* exhibited a high degree of conservation among teleost, retaining one forkhead domain, one transactivation domain, and three phosphorylation sites. *FoxO1* mRNA was expressed in a wide range of tissues, and high in the brain and liver. Glucose loading resulted in persistent hyperglycemia, and plasma insulin levels remained unchanged except at 1 h. After the insulin-glucose injection, insulin levels were significantly elevated and glucose levels recovered to the basal value after 6 h, which indicated insufficient insulin secretion caused persistent hyperglycemia in this species. Compared with the glucose injection group, transcript levels and enzyme activities of hepatic glycolysis-related genes (GK and PK) were significantly activated, and gluconeogenesis-related genes (PEPCK and G6Pase) were significantly depressed at 3 h after the insulin-glucose injection. Besides, phosphorylation of AKT-FoxO1 pathway was significantly activated. Therefore, insulin improved glucose metabolism by activating the AKT-FoxO1 phosphorylation to decrease hyperglycemia stress after the meal, which indicated insufficient insulin secretion was the reason for glucose intolerance in largemouth bass. Meanwhile, conserved S267 and S329 phosphorylation sites of FoxO1 were confirmed to be regulated by AKT and mediated the glucose metabolism. In conclusion, activation of insulin-AKT-FoxO1 pathway improved glucose tolerance through mediating glucose metabolism in largemouth bass.

1. Introduction

The teleost, especially the carnivorous fish, are considered to be glucose intolerant with persistent hyperglycemia, which is often observed after oral or glucose injection or intake of high carbohydrate diets (Mazur et al., 1992; Garcia-riera and Hemre, 1996; Peres et al., 1999; Legate et al., 2001; Enes et al., 2011; Li et al., 2016; Chen et al., 2018). At present, several valid hypotheses have been proposed to explain the sluggish glucose clearance of carnivorous fish, including low glucose phosphorylation capacity, insulin receptors and glucose transporters, unbalanced regulation of glycolysis and gluconeogenesis, poor capacity for peripheral tissues utilization of glucose, low secretion of insulin and low blood levels of insulin response to glucose loading, etc. (Hemre et al., 2002; Kirchner et al., 2008; Polakof et al., 2012).

The glucose tolerance test (GTT) has been used in many fish species to study the glucose tolerance capability (Moon, 2001; Legate et al., 2001; Enes et al., 2011, 2012; Chen et al., 2018). In general, it is well known that glucose stimulates insulin secretion, which stimulates glucose uptake by peripheral tissues (e.g. the liver, skeletal muscle, and the adipose tissue), and then promotes glycolysis and inhibits gluconeogenesis for clearing a glucose load to maintain glucose homeostasis (Hemre et al., 2002). However, the insulin activation and related metabolic responses in peripheral tissues after a GTT are highly variable among fish species and strongly associated with glucose origin and dose, season, and nutritional status (Moon, 2001; Polakof et al., 2012). Previous studies showed that plasma insulin levels not change in response to glucose loading in carnivorous fish, such as chinook salmon (*Oncorhynchus tshawytscha*) (Mazur et al., 1992), rainbow trout (*Oncorhynchus*

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mykiss), American eel (*Anguilla rostrata*) (Legate et al., 2001), and European sea bass (*Dicentrarchus labrax*) (Enes et al., 2011). Many studies also suggested that insufficient insulin secretion was the main reason for glucose intolerance in carnivorous fish (Moon, 2001; Hemre et al., 2002; Enes et al, 2011; Polakof et al., 2012). In addition, another reason to elucidate the poor glucose utilization in carnivorous fish is postprandial uncontrolled gluconeogenesis, which is drawing more attention during the latest decade (Polakof et al., 2012; Gong et al., 2015; Li et al., 2019). Some researchers also proposed that glucose is not probably an important insulin secretagogue (Mommsen et al., 2001) and the sensitivity of pancreatic somatostatin-secreting cells to glucose is higher than the insulin-secreting cells for fish (Enes et al., 2011), resulting in insufficient plasma insulin levels. But little information is available on the relationship between a GTT and the regulation mechanism of insulin and glucose metabolism in teleost.

Forkhead box O1 (*FoxO1*), a nuclear transcription factor, is a central regulator in the insulin signaling pathway and has been implicated in hepatic gluconeogenesis and glycolysis pathways to regulate glucose metabolism in mammals (Zhang et al., 2006; Dong, 2017). In the mice, liver-specific inactivation or inhibition of FoxO1 significantly reduced the postprandial plasma glucose level, which was beneficial to improve glucose tolerance and increase the utilization ability of glucose (Altomonte et al., 2003; Samuel et al., 2006; Matsumoto et al., 2007). So far, the full-length cDNA of *FoxO1* sequences have been cloned in medaka (*Oryzias latipes*) (Shen et al., 2012), grass carp (*Ctenopharyngodon idella*) (Sun et al., 2017), hybrid grouper (*Epinephelus fuscoguttatus* ♀×*E.lanceolatus* ♂) (Li et al., 2018), turbot (*Scophthalmus maximus*) (Pan et al., 2019), and spotted sea bass (*Lateolabrax maculatus*) (Li et al., 2020). Until now, only a few studies in fish illustrated the functions of *FoxO1* at the transcript level (Lin et al., 2018; Li et al., 2019; Pan et al., 2019; Yu et al., 2019).

The largemouth bass (*Micropterus salmoides*), a carnivorous fish, has been used as a typical model to investigate glucose-induced liver disease due to its low utilization capability of starch. Several studies have suggested that no more than 10% of starch was contained in the commercial diet of this species (Ma et al., 2019; Zhang et al., 2020). Insulin-AKT-FoxO1 pathway was demonstrated to play an important role in regulating glucose metabolism in mammals (Zhang et al., 2006; O-sullivan et al., 2015), whereas little information is available on the regulation of glucose metabolism mediated by this pathway in fish. Yu et al. (2019) had found that activating AKT and reducing *FoxO1* gene transcript level could alleviate starch-induced liver fibrosis via inhibiting gluconeogenesis-related genes expression in largemouth bass. However, no more data are available for this species on the regulation of glucose metabolism by insulin-AKT-FoxO1 pathway. Therefore, this study aims to illustrate the role of FoxO1 in mediating hepatic glucose metabolism via AKT-FoxO1 pathway after glucose and insulin-glucose load in largemouth bass, which may help to improve understanding of glucose intolerance in carnivorous fish.

2. Materials and methods

During the whole experimental period, the fish were maintained in compliance with the Laboratory Animal Welfare Guidelines of China (General Administration of Quality Supervision, Inspection, and Quarantine of the People's Republic of China, Standardization Administration of China, GB/T 35892–2018).

2.1. Animals and injection trials

Largemouth bass was obtained from the commercial Aquafarm (Tangshan, Hebei, China). All fish were acclimated and fed the based diet (crude protein 52%, crude lipid 11.5%, starch 10.2%) before the commencement of the trial. During the period, the water temperature was maintained at 24–27 °C, pH = 7.2–8.0, dissolved oxygen > 6.0 mg/L, and ammonia-N < 0.4 mg/L.

After a two-month acclimation with feed intake of 2% body weight in the facility, 96 fish (65.83 ± 5.52 g) divided into 16 separate aquariums (160 L each) at a rate of 6 fish per tank. After 24 h starvation, 6 fish from one random tank were rapidly anesthetized with MS-222 (200 mg/L), and then blood and liver tissues were collected as the baseline samples (0 h). The remaining fish were immediately weighted, and injected intraperitoneally with exact 10 mL 0.75% saline solution/kg body weight (BW), 1 g glucose/kg BW, the mixture of 0.05 mg bovine insulin (approximate 1.35 U)/kg BW and 1 g glucose/kg BW, respectively with 5 tanks per treatment for various sampling time of 1, 3, 6, 9, and 12 h after injection. A saline solution (0.75%) containing 200 mg glucose per mL was used for glucose injection. The control group was injected with equal volumes of 0.75% saline solution. The appropriate physiological dose of glucose and insulin used in this study referred to the previous studies (Jin et al., 2014; Xu et al., 2018; Lu et al., 2018). Blood was rapidly sampled from the caudal vein, centrifuged (4000 g, 10 min, 4 °C) to obtain plasma and then stored at −80 °C for analysis. Liver samples were dissected, and then immediately frozen in liquid nitrogen, and kept at −80 °C for mRNA isolation, tissue homogenate, and western blot analysis.

Tissues for the determination of the *FoxO1* gene expression were dissected from the anterior, middle, and posterior intestines as well as from the visceral adipose tissues, kidneys, livers, muscles, brains, gills, and skins of six normal healthy largemouth bass. All tissues used were frozen at −80 °C before use.

2.2. Plasma biochemical and hepatic enzymatic analysis

The level of plasma glucose was detected using commercial assay kits (Shanghai Rongsheng Biotech Co., Ltd., China) following the protocols. The plasma insulin and hepatic glucokinase (GK), pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase) enzymes were determined by the commercial ELISA kits (Jiangsu Meimian industrial Co. Ltd., China). The insulin ELISA kits were coated with salmon (*Oncorhynchus kisutch*) insulin antibody (Sink and Lochmann, 2006). During the determination, we used a blank control and a negative IgG control (X0396, DAKO, USA), only target antibody showed specialized reaction.

2.3. RNA extraction, cDNA synthesis, and qPCR analysis

Total RNA extraction and cDNA synthesis were carried out as described previously (Yu et al., 2019) using the RNAiso Plus reagent and PrimeScript RT reagent Kit (TaKaRa, Japan). A housekeeping gene, *EF1α* (GenBank accession no. KT827794), was used as an endogenous reference to normalize the template amount. The gene-specific primers used for mRNA quantification by qPCR were shown in Table 1. The qPCR analysis was performed using a CFX96™ Real-Time System (Bio-Rad, USA) in a 20 μL reaction volume containing iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA). The qPCR program was 95 °C for 30 s followed by 35 cycles of 10 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. To ensure that only one PCR product was amplified and detected, the melting curve analysis was included and the fluorescence was collected from 72 °C to 95 °C at the end of each cycling protocol. Each sample was run in triplicate and qPCR data was analysed using the $2^{-\Delta\Delta Ct}$. The mRNA levels of target genes are shown as the n-fold difference relative to the calibrator.

2.4. Cloning and identification of FoxO1 gene

Gene-specific primers (Table 1) were designed to obtain the 5′ and 3′ ends of each cDNA based on liver transcriptome data of largemouth bass, using the SMART™ RACE cDNA Amplification Kits (Clontech, USA), according to the manufacturer's instructions. The PCR product was purified using a gel extraction kit (Tiangen, China) and ligated with the pMD-19 T vector (TaKaRa, Japan). After their transformation into

Table 1
Primers used in the present study.

Primer Name	Sequence (from5′ to 3′)	*E*-Values (%)	Purpose
FoxO1-5′GSP	TCAGAACTGTGTCCCAGAGAGCCCGTCA		5′RACE
FoxO1-5′NGSP	TCTTCCCGCCCTCTGGGTTTAGCATCCA		5′RACE
FoxO1-3′GSP	TGGGAGGAGGTAACGGTCACCCGCATAG		3′RACE
FoxO1-3′NGSP	GTATGCCTGGTGGCTTCTGCCCTCTCA		3′RACE
GK-F	ACAGAGTGGTGGACGAGACC	102.6	qPCR
GK-R	TCGTTCACCAGCTTCATCAG		qPCR
PK-F	CCTATCGGAATTGCACTGGA	99.0	qPCR
PK-R	TTCTTGTAGTCGAGCCAGAG		qPCR
PEPCK-F	TGCTTGACTGGATGTTCAGG	94.5	qPCR
PEPCK-R	TTCCTCACCTCATCCACCTC		qPCR
G6Pase-F	GGGAGTCCAGGTGTGTGTCT	90.9	qPCR
G6Pase-R	CAGCGAAGGAGGTCAAGAAG		qPCR
EF1α-F	TGCTGCTGGTGTTGGTGAGTT	102.8	qPCR
EF1α-R	TTCTGGCTGTAAGGGGGCTC		qPCR
FoxO1-F	CTATGAATGGCCGCTTGCTCA	97.3	qPCR
FoxO1-R	TCGTCCATATCCGTTGGTGTTG		qPCR

Trans1-T1 Phage Resistant Chemically Competent cells (TransGen Biotech, China), the recombinants were identified through blue-white screening, then the positive clones were sequenced in both directions by Rui Biotech (Beijing, China).

2.5. Sequence analysis of FoxO1 gene

The full-length of *FoxO1* cDNA sequence was analyzed using the BLAST algorithm from the NCBI website (http://www.ncbi.nlm.nih.gov/blast), and the putative amino acid sequence was analyzed using the Expert Protein Analysis System (http://www.expasy.org). Multiple alignments and phylogenetic analysis were performed with MEGA 7 software (http://www.megasoftware.net).

2.6. Western blots

Protein extraction of the liver samples was carried out as described previously (Yu et al., 2019). The protein concentration was measured using a BCA Protein Quantification Kit (Bio-Rad, USA). The primary antibodies used were β-tubulin (#2146, CST, USA), anti-AKT (#9272, CST, USA), anti-phospho AKT (S473) (#4060, CST, USA), anti-FoxO1 (ET1608-25, Huaan, China), anti-phospho FoxO1(S256) (#9461, CST, USA), anti-phospho FoxO1(S319) (AP0176, Abclonal, China). Automated western blots were performed on a Jess™ system (Protein Simple) using a 12–230 kDa Pre-filled Plates according to the manufacturer's standard instructions.

2.7. Statistical analysis

All statistical procedures were performed with the aid of the SPSS software version 26.0 for Windows. All results were presented as the mean ± standard error (SEM). After the homogeneity of the variances was tested, all data means were analyzed. One-way ANOVA followed by Duncan's multiple range test was used to analyze the tissue distribution of FoxO1. Two-way ANOVA was used to analyze the significant differences among treatment means based on sampling times, injection treatments, and their interactions for plasma parameters, hepatic enzymes, gene, and protein expression. $P < 0.05$ was considered statistically significant. The graphics were drawn by GraphPad Prism 7.0 (GraphPad, USA).

3. Results

3.1. Identification and sequence analysis of FoxO1 gene

The full-length cDNA of *FoxO1* was identified with 2541 bp (GenBank Accession No: MT534172). The open reading frame of *FoxO1* was 2043 bp, encoding peptides of 680 amino acids with a predicted molecular weight of 73.69 kDa and a theoretical isoelectric point of 6.32 (Fig. 1). The forkhead domain, transactivation domain, and phosphorylated sites of the largemouth bass were highly conserved among teleost and mammals (Fig. 2). Based on human FoxO1, three evolutionarily conserved threonine and serine residues (T27, S267, S329) phosphorylated upon AKT were found in FoxO1 of largemouth bass (Fig. 2).

The results of sequence similarity showed that largemouth bass FoxO1 exhibited high identities with bony fish orthologs. Largemouth bass Foxo1 shared 94.0%, 91.8%, and 91.4% sequence similarity with *Epinephelus fuscoguttatu*, *Amphiprion percula*, and *Seriola dumerili*, respectively (Table 2). Phylogenetic analysis revealed that largemouth bass FoxO1 remained largely conserved compared with other vertebrates and clustered with bony fish. The FoxO1 of largemouth bass was clustered closest to Perciformes, with 88% bootstrap support related to *E. fuscoguttatus* (Fig. 3). As a result, the phylogenetic analysis further confirmed the annotation of the largemouth bass *FoxO1* gene.

3.2. Tissue distribution analysis of FoxO1 mRNA

It was shown that *FoxO1* gene was widely expressed in various tissues, and the abundance of mRNA varied among tissues of largemouth bass (Fig. 4). The mRNA level of *FoxO1* was highest in the brain, followed by liver, skin, midgut, gill, kidney, foregut, hindgut, visceral adipose, and lowest in muscle. The expressions of *FoxO1* in the gill, intestine, and kidney were similar.

3.3. Plasma glucose and insulin levels after the glucose or insulin-glucose injection

As shown in Fig. 5, plasma glucose and insulin levels were significantly ($P < 0.01$) affected by sampling times, injection treatments, and their interactions. No significant differences ($P > 0.05$) were observed in plasma glucose and insulin levels after the saline injection within 12 h. In contrast, the glucose injection resulted in significantly elevated plasma glucose levels with a maximum level at 1 h of 15.39 mM/L ($P < 0.05$), and then a decreased significantly ($P < 0.05$) to basal value at 12 h. Plasma insulin levels significantly increased ($P < 0.05$) only at 1 h after the glucose injection, however, showed no significant differences ($P > 0.05$) with the saline group at each sampling time. After the insulin-glucose injection, the plasma glucose levels were significantly decreased ($P < 0.05$) at each sampling time compared with glucose injection treatment, and the time returning to basal glucose levels was at 6 h, which was earlier than the glucose group. These results showed that exogenous bovine insulin could perform its physiological function to decrease plasma glucose levels in largemouth bass.

3.4. Hepatic glucose metabolism after the glucose or insulin-glucose injection

After the glucose or insulin-glucose injections, the transcript levels and enzyme activities of glucose metabolism-related factors were significantly ($P < 0.01$) affected by sampling times, injection treatments, and their interactions (Fig. 6 and Fig. 7). In the saline group, no statistical differences ($P > 0.05$) were observed in the mRNA levels of *GK*, *PK*, *PEPCK*, and *G6Pase* during 0–12 h. Compared with the saline group, the glucose loading resulted in a significant ($P < 0.05$) increase of *GK* and *PK* gene expressions with the highest value being obtained at 6 h and 3 h, respectively. Thereafter, the mRNA levels of *GK* and *PK* decreased significantly ($P < 0.05$), only the value of *PK* returned to the

A

Gene Name	5'UTR (bp)	3'UTR (bp)	ORF (bp)	Encoding aa	MW (KDa)	Theoretical pI	GenBank No.
FoxO1	321	179	2043	680	73.69	6.32	MT534172

B

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1     ACATGGGGGCAGACGGAACATCACTACGACTGGATAACAAAAACAAACTAGAGGGTCAACACACTGTGGACACTTAGACAACTGTTTTTCACATGAGAGGCCTTTTTAGAAACATACTTT 120
121   TTATTATTCTTAACACAGATTTTTACCGGGTTGGGATGTGACTTTCAGTGTAGTTTTTGCGCCCAGGCGGAGCACTATTTTCCCGGACAGATTCATCTTGTGTTCGCTCACATTTCAAAG 240
241   GCGAAGTTGCTACTTTTGAGGGGCGACAGGTTCGTCACATTTACATGACATTGTAACACTTGGCACCGTCACTTT                                              315
316   TCG ACC atg GCG GAA GCG GCC CAG CAG CCA CAC CTG GTG GAA ATC GAC CCG GAA TTT GAG CCC CTG TCG CGG CCC CGG TCG TGC ACT TGG  405
1           M   A   E   A   A   Q   Q   P   H   L   V   E   I   D   P   E   F   E   P   L   S   R   P   R   S   C   T   W   28
406   CCC TTG CCC CGG CCG GAG TTC ATC AAC CCC GGT GAC TCC AAC ACG TCC TCG CCG GTA CCG TCT GTG AAG CAG GAG CCC TCC GGC AAC GCC  495
29    P   L   P   R   P   E   F   I   N   P   G   D   S   N   T   S   S   P   V   P   S   V   K   Q   E   P   S   G   N   A   58
496   GAG TTC ATC AAC AAC CTT AGT CTG CTA GAG GAG ACC GAG GAC TTT ACG GAG CAG AAG CCT GTC ATC CTG TGC ACT GAT TTC CAG TGT CAG  585
59    E   F   I   N   N   L   S   L   L   E   E   T   E   D   F   T   E   Q   K   P   V   I   L   C   T   D   F   Q   C   Q   88
586   GAG AGT TGC GTC CAC CAG TCA CCC ACA CCG CAG CAG CAG CAG CAA CAT CAG CAG CAT CAG CAG CAA CAT CAG CAG CAC CCG AAC CCG CAA  675
89    E   S   C   V   H   Q   S   P   T   P   Q   Q   Q   Q   Q   H   Q   Q   H   Q   Q   Q   H   Q   Q   H   P   N   P   Q   118
676   CTC CCG CAT CAT CAT CAT CAT CAT CAT CAC CAG CAG CCG CAG CAG CAG CAG CAA CAG CAG GTG CCT CTG CTG TCC TCC CCG GTC GGC TCG  765
119   L   P   H   H   H   H   H   H   H   Q   Q   P   Q   Q   Q   Q   Q   Q   Q   V   P   L   L   S   S   P   V   G   S   148
766   TCG TCC ACG GCG GCC GCA GCT GCC GCC GCT GCC CAG AGG AAG AGC AGC TCC TCC CGA CGA AAC GCA TGG GGG AAT ATG TCT TAT GCA GAC  855
149   S   S   T   A   A   A   A   A   A   A   Q   R   K   S   S   S   S   R   R   N   A   W   G   N   M   S   Y   A   D   178
856   CTC ATA ACC AAA GCT ATC GAA AGT TCT CCT GAG AAC CGA CTA ACT CTG TCT CAG ATT TAT GAC TGG ATG GTG AAG AGT GTG CCT TAT TTT  945
179   L   I   T   K   A   I   E   S   S   P   E   N   R   L   T   L   S   Q   I   Y   D   W   M   V   K   S   V   P   Y   F   208
946   AAG GAC AAA GGA GAC AGC AAT AGC TCT GCA GGC TGG AAG AAC TCC ATT AGG CAC AAC CTG TCG CTG CAT AGC CGC TTT ATT CGC GTC CAG 1035
209   K   D   K   G   D   S   N   S   S   A   G   W   K   N   S   I   R   H   N   L   S   L   H   S   R   F   I   R   V   Q   238
1036  AAT GAG GGG ACT GGG AAG AGC TCC TGG TGG ATG CTA AAC CCA GAG GGC GGG AAG AGT GGC AAG TCC CCA CGA CGC AGG GCA GTC TCC ATG 1125
239   N   E   G   T   G   K   S   S   W   W   M   L   N   P   E   G   G   K   S   G   K   S   P   R   R   R   A   V   S   M   268
1126  GAC AAC AAC AGC AAG TTC ACC AAG AGC AGA GGA AGA GCA GCT AAG AAA AAG CTG TCC CTG CAG GGT GGC CCT GAG GGA GGC GCA GAC AGT 1215
269   D   N   N   S   K   F   T   K   S   R   G   R   A   A   K   K   K   L   S   L   Q   G   G   P   E   G   G   A   D   S   298
1216  CCA GGA TCA CAC TCC AAG TGG CCT GGC AGC CCC AAC TCC CAC AGC AAC GAC GAC TTT GAT GCG TGG AAC AGC TTC AGG CCT CGG ACC AGC 1305
299   P   G   S   H   S   K   W   P   G   S   P   N   S   H   S   N   D   D   F   D   A   W   N   S   F   R   P   R   T   S   328
1306  TCC AAT GCC AGC ACT CTG AGT GGC CGC CTC TCA CCC TTC ATG CCC GAG GAT GAC CTC GGT GAG GCA GAC GTG CAT ATG GGC TAC CCA GGA 1395
329   S   N   A   S   T   L   S   G   R   L   S   P   F   M   P   E   D   D   L   G   E   A   D   V   H   M   G   Y   P   G   358
1396  GCC CCC GGG ACC AAG ATG ACA GCC ACG CTG CCC AGT CTC ACA GAG ATG ACG GGC TCT CTG GGA CAC AGT TCT GAG AAT GTC ATG GAG AAC 1485
359   A   P   G   T   K   M   T   A   T   L   P   S   L   T   E   M   T   G   S   L   G   H   S   S   E   N   V   M   E   N   388
1486  CTT CTG GAC AAC CTG AAC TTG CTC TCA CCC AAC AAC TCT CAG GTT GGA GGA GGA GCC ACG ACC CCA GGC TCC TCC CAG TCC TCC CCC TCT 1575
389   L   L   D   N   L   N   L   L   S   P   N   N   S   Q   V   G   G   G   A   T   T   P   G   S   S   Q   S   S   P   S   418
1576  CCT ATG ATG CAG CCA AGC CCT GGG TAT CCT GCT TAC AGC TCC CCT AGC ATG GCC TCA CAG TCC CAG CAG GAG TAC CGC AAG TGT GTG TAT 1665
419   P   M   M   Q   P   S   P   G   Y   P   A   Y   S   S   P   S   M   A   S   Q   S   Q   Q   E   Y   R   K   C   V   Y   448
1666  GGC CAA GCA GGG ATG AAC ACC CTG TCT CCC ATG TCT CTG CAG ACA CTG CCA GAG AGT AAG CCA AGC TTT GGG CCC AGT ATG GGC CAG TAC 1755
449   G   Q   A   G   M   N   T   L   S   P   M   S   L   Q   T   L   P   E   S   K   P   S   F   G   P   S   M   G   Q   Y   478
1756  AGC TGC CCT GCG GGG CTT TTG AAG GAG CTG CTG ACA TCA GAT ACA GAC CAG CAT GGA GAG CTC ATG CCG TCA GTG GAC ACT GTG GTT TCT 1845
479   S   C   P   A   G   L   L   K   E   L   L   T   S   D   T   D   Q   H   G   E   L   M   P   S   V   D   T   V   V   S   508
1846  CAG TCT AGC GGA GGC TGC ATG CTG CCG CCA TAC AGC AGC AGC CAG CAT ACA GCT AAA CTC ATG GGA GGA GGT AAC GGT CAC CCG CAT AGT 1935
509   Q   S   S   G   G   C   M   L   P   P   Y   S   S   S   Q   H   T   A   K   L   M   G   G   G   N   G   H   P   H   S   538
1936  CTC TCT CAC ACT CAC CCC CAC AAT GTG CAC AGC CAA GCC CCG GCC CCG TCA CCA GCT ATG AAT GGC CGC TTG CTC ATG TCC CTG AGC CAC 2025
539   L   S   H   T   H   P   H   N   V   H   S   Q   A   P   A   P   S   P   A   M   N   G   R   L   L   M   S   L   S   H   568
2026  ACC GGG GGC ACC ACG CGT TTG CCT ACA GCC AAA AGC CCT ATG CAG ATG CCT TAT GGT TTC TCC AGC CAC CTC AGC GGG GGA GGT ATG CCT 2115
569   T   G   G   T   T   R   L   P   T   A   K   S   P   M   Q   M   P   Y   G   F   S   S   H   L   S   G   G   G   M   P   598
2116  GGT GGC TTC TGC CCT CTC AAC ACC AAC GGA TAT GGA CGA CCG GGC CTG CCA CAG CCC TTG CAC CCA GAG AAG CTG CCC AGT GAC CTG GAC 2205
599   G   G   F   C   P   L   N   T   N   G   Y   G   R   P   G   L   P   Q   P   L   H   P   E   K   L   P   S   D   L   D   628
2206  GAC ATG TCC ATC GAG AAG TTT GAG TTT GAC ATG GAG ACA GTC CTC CAC GAA ACT CTG ATG GAC GGG GAC TCG CTG GAC TTC AAC TTT GAG 2295
629   D   M   S   I   E   K   F   E   F   D   M   E   T   V   L   H   E   T   L   M   D   G   D   S   L   D   F   N   F   E   658
2296  CCA ATG GTG ACC CAA CAA GGT TTC TCC CAT GGA GTG AAG ACC ACC ACG CAC AGC TGG GTG TCA GGG tag AAA CTG TAG AGT GGA AAA AGT 2385
659   P   M   V   T   Q   Q   G   F   S   H   G   V   K   T   T   T   H   S   W   V   S   G   *                           680
2386  TCTTGTTAACTGACTTCTTGTTGTTGTTGCTGTTTGGTAAAAGACATGATATGTATCTTAGGATGTCTTAGGATCACAAAATATGTATCTTAGGATGTAATTCTCATCCCAACTTCACCT 2505
2506  GTAGTTTCTGTACACAAAAAAAAAAAAAAAAAAAAA                                                                                      2541
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Fig. 1. (A) The cDNA and predicted protein information of FoxO1 from largemouth bass. (B) Complete nucleotide and deduced amino acid sequence of largemouth bass *FoxO1* cDNA. Start codon (ATG) and stop codon (TAG) were marked with lowercase "*atg*" and "*", respectively.

normal levels. The transcript levels of *PEPCK* and *G6Pase* decreased significantly ($P < 0.05$) to the minimum value at 3 h and 1 h post-injection, respectively. As time went on *PEPCK* and *G6Pase* gene expressions gradually increased to the basal value at 9 h. The insulin-glucose administration led to a significant increase ($P < 0.05$) mRNA levels of *GK* and *PK* during 3 h. Thereafter, the mRNA levels of *GK* and *PK* decreased. The mRNA levels of *PEPCK* and *G6Pase* significantly ($P < 0.05$) down-regulated with the lowest at 1 h post-injection and increased to normal value at 9 h.

In addition, after the glucose or insulin-glucose injection, both hepatic GK and PK enzyme activities were significantly higher ($P < 0.05$) at 3 h than those of 0 and 12 h, whereas the opposite was true for PEPCK and G6Pase activities (Fig. 7). In terms of injection types, the transcript levels and enzymatic activities of GK and PK in the insulin-glucose group were significantly higher ($P < 0.05$) than those of the other groups, whereas the opposite was true for that of the PEPCK and G6Pase.

3.5. Activated phosphorylation of AKT-FoxO1 pathway after the glucose or insulin-glucose injection

After the glucose or insulin-glucose administration, the hepatic p-AKT/AKT, p-FoxO1(S267)/FoxO1, and p-FoxO1(S329)/FoxO1 ratio were significantly ($P < 0.05$) affected by sampling times, injection treatments, and their interaction (Fig. 8). Western blot analysis showed that the ratio of p-AKT/AKT, p-FoxO1(S267)/FoxO1, and p-FoxO1 (S329)/FoxO1 was significantly ($P < 0.05$) increased at 3 h, then decreased after glucose or insulin-glucose injection. In terms of injection types, the ratio of p-AKT/AKT, p-FoxO1(S267)/FoxO1, and p-FoxO1 (S329)/FoxO1 in the insulin-glucose group were significantly higher than those of the other groups ($P < 0.05$).

4. Discussion

In the present study, a complete cDNA sequence of *FoxO1*, which encoded 680 amino acid residues, was isolated from largemouth bass liver. The largemouth bass FoxO1 was clustered closest to Perciformes among teleost in the phylogenetic tree and exhibited a highly conversed forkhead box domain and a transactivation domain. From the invertebrate to the vertebrate, the forkhead box domain in the N-terminal region and the transactivation domain in the C-terminal region were highly conserved (Brunet et al., 1999; Gross et al., 2008). Until now, studies involved in cloning *FoxO1* sequence have been reported in medaka (Shen et al., 2012), grass carp (Sun et al., 2017), hybrid grouper (Li et al., 2018), turbot (Pan et al., 2019), and spotted sea bass (Li et al., 2020), all of which indicated that FoxO1 contained a conserved forkhead and transactivation domain. These domains were related to regulating the function of FoxO1 proteins by protein–protein or protein-DNA interactions (Calnan and Brunet, 2008). For example, PGC-1α (peroxisome proliferator-activated receptor γ coactivator 1α) interacted with elements in this region and functioned together with FoxO1 in the liver to stimulate gluconeogenesis (Yoon et al., 2001). Given that these domains are very conserved, we can infer that FoxO1 of largemouth bass has a similar regulation function as that of mammal.

The brain-liver axis played a key role in the regulation of numerous

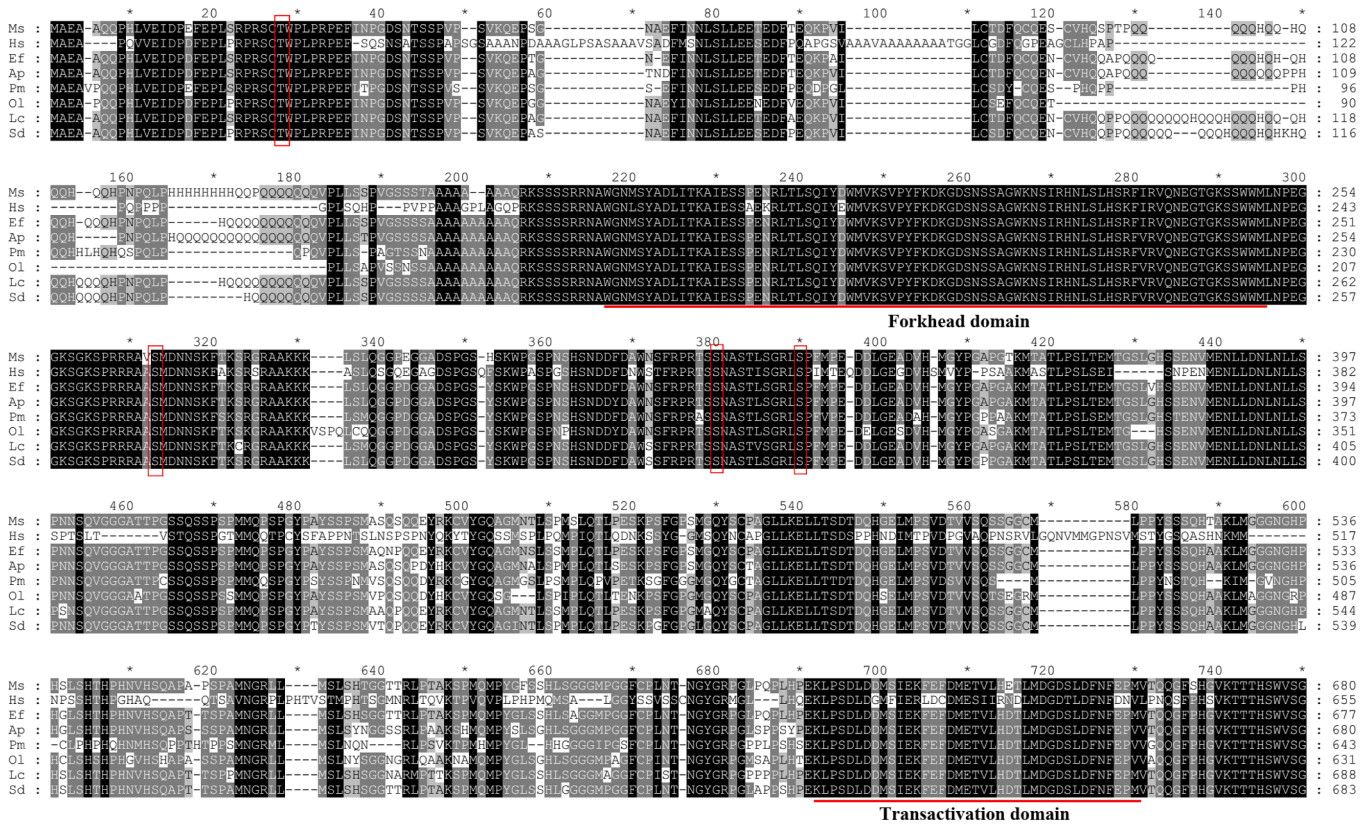


Fig. 2. Comparison of the deduced amino acid sequences of FoxO1 from largemouth bass with that from human and bony fish. Identical residues were shaded black, and similar residues were shaded gray. The forkhead domain and transactivation domain were noted by red lines. Three amino acids (T27, S267, S329) representing the conserved AKT phosphorylated sites were marked by red rectangle boxes. Protein IDs are as follows: Ms: *Micropterus salmoides*; Ef: *Epinephelus fuscoguttatus* (tr|A0A384X150); Lc: *Lates calcarifer* (XP018520954.1); Sd: *Seriola dumerili* (XP|022604638.1); Ap: *Amphiprion percula* (tr|A0A3P8SVQ7); Ol: *Oryzias latipes* (tr|C1K302); Pm: *Periophthalmus magnuspinnatus* (tr|A0A3B3ZF53); Hs: *Homo sapiens* (sp|Q12778).

Table 2
Alignment of FoxO1 amino acid sequence identity (%) from the largemouth bass and other bony fish.

	Dr FoxO1	Ef FoxO1	Ap FoxO1	Ca FoxO1	Pm FoxO1	Ip FoxO1	Ci FoxO1	Ol FoxO1	Lc FoxO1	Tf FoxO1	Sd FoxO1
Ms FoxO1	70.4	**94.0**	91.8	66.8	80.7	67.6	70.6	82.6	91.7	66.7	91.4
Dr FoxO1		71.5	70.7	88.3	71.2	75.7	94.3	71.9	69.8	76.4	71.4
Ef FoxO1			93.6	68.2	82.3	69.4	72.1	84.0	94.4	68.4	94.1
Ap FoxO1				67.2	81.9	69.4	71.4	84.6	91.3	67.8	91.4
Ca FoxO1					67.4	71.6	89.6	68.0	66.1	72.3	67.8
Pm FoxO1						67.2	71.5	79.4	80.7	68.0	82.0
Ip FoxO1							75.7	66.3	68.2	93.4	67.8
Ci FoxO1								72.1	70.3	76.3	71.9
Ol FoxO1									82.4	67.3	83.5
Lc FoxO1										66.9	94.8
Tf FoxO1											67.2

The closest homologs of largemouth bass FoxO1 proteins are shown as bold figures.
Protein IDs are as follows: Ms: *Micropterus salmoides*; Ef: *Epinephelus fuscoguttatus* tr|A0A384X150; Lc: *Lates calcarifer* XP_018520954.1; Sd: *Seriola dumerili* XP|022604638.1; Ap: *Amphiprion percula* tr|A0A3P8SVQ7; Ol: *Oryzias latipes* tr|C1K302; Pm: *Periophthalmus magnuspinnatus* tr|A0A3B3ZF53; Ip: *Ictalurus punctatus* tr|W5UE46; Tf: *Tachysurus fulvidraco* XP |027003659.1;Ca: *Carassius auratus* XP|026119056.1; Dr: *Danio rerio* sp|A3RK74; Ci: *Ctenopharyngodon idella* tr|A0A0U2ILB4.

endocrine events, especially contributing to glycemic homeostasis in the control of hepatic glucose (Ribeiro and Antunes, 2018; Stanley et al., 2019). In mammals, *FoxO1* gene was highly expressed in several insulin-responsive tissues such as the brain, liver, muscle, and pancreatic β cells (Carter and Brunet, 2007; Huang and Tindall, 2007). The high expression level of *FoxO1* mRNA in the brain (central nervous system) was related with food intake and energy balance (Kim et al., 2006). In response to starvation, up-regulated *FoxO1* mRNA enhancement of orexigenic genes expression (AgRP, NPFF, and NPY), and inhibition of anorectic genes expression (POMC and CART) (Kitamura et al., 2006; Otero-Rodiño et al., 2018; Li et al., 2020). After the feeding, the hepatic glycolysis and gluconeogenesis would be activated and inhibited, respectively. Meanwhile, the hepatic FoxO1 also down-regulated in response to exogenous glucose loading (Calnan and Brunet, 2008). Therefore, FoxO1 in the brain was closely related with hepatic glycolysis or gluconeogenesis. Fish, including grass carp (*FoxO1b*) (Sun et al., 2017), hybrid grouper (Li et al., 2018), turbot (Pan et al., 2019), and largemouth bass, observed in the present study, the *FoxO1* gene was abundant in the liver and brain, but very low in the muscle. In mammals, the FoxO1 acted as a key regulator to maintain the delicate balance between muscle protein synthesis and degradation, which mediated the muscle growth and atrophy (Gross et al., 2008; Schiaffino et al., 2013).

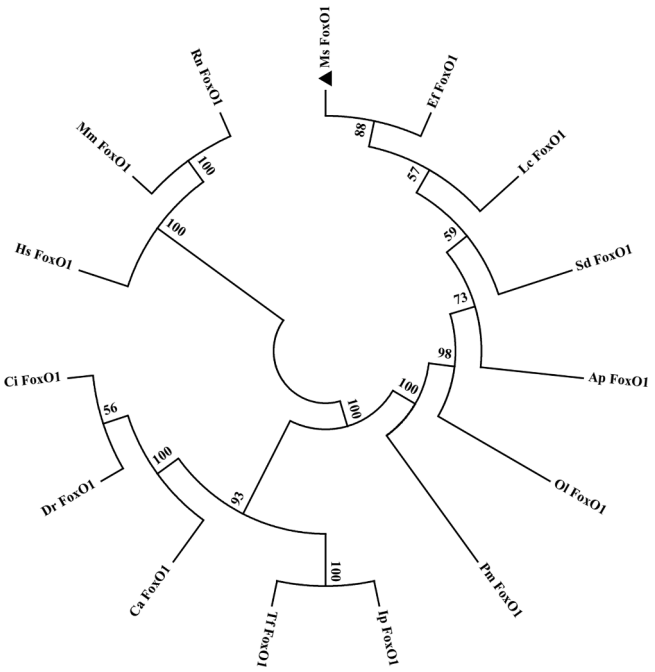


Fig. 3. Phylogenetic analysis of largemouth bass *FoxO1* gene. The phylogenetic tree was constructed by the amino acid sequences from several representative mammals and teleost with the neighbor-joining method. The numbers represented the frequencies (%) with which the tree topology presented was replicated after 1000 iterations. The largemouth bass *FoxO1* gene was labeled with a black triangle. Protein IDs are as follows: Ms: *Micropterus salmoides*; Ef: *Epinephelus fuscoguttatus* (tr|A0A384X150); Lc: *Lates calcarifer* (XP018520954.1); Sd: *Seriola dumerili* (XP|022604638.1); Ap: *Amphiprion percula* (tr|A0A3P8SVQ7); Ol: *Oryzias latipes* (tr|C1K302); Pm: *Periophthalmus magnuspinnatus* (tr|A0A3B3ZF53); Ip: *Ictalurus punctatus* (tr|W5UE46); Tf: *Tachysurus fulvidraco* (XP |027003659.1); Ca: *Carassius auratus* (XP|026119056.1); Dr: *Danio rerio* (sp|A3RK74); Ci: *Ctenopharyngodon idella* (tr|A0A0U2ILB4); Hs: *Homo sapiens* (sp|Q12778); Mm: *Mus musculus* (sp|Q9R1E0); Rn: *Rattus norvegicus* (sp|G3V7R4).

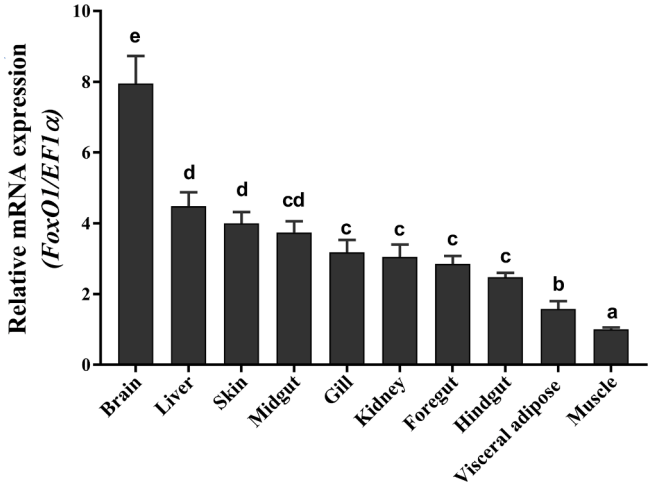
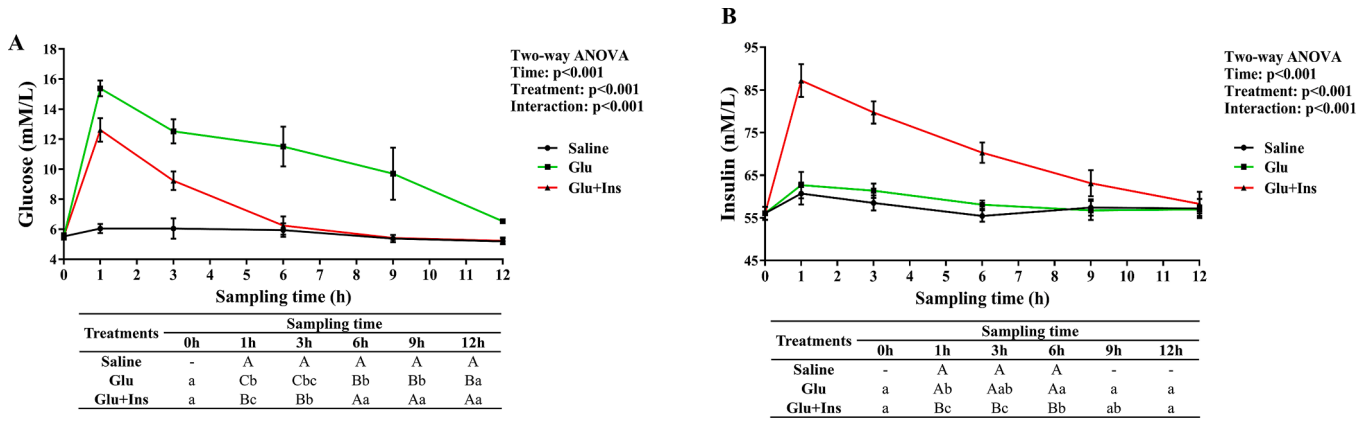


Fig. 4. Tissue distribution of *FoxO1* mRNA in the largemouth bass by qPCR. The results were expressed as the mean ± SEM (n = 6). Significant differences ($P < 0.05$) were indicated by the lower-case letters.

Overexpression of FoxO1 led to muscle atrophy and a decrease in muscle mass in mice (Sandri et al., 2004; Kamei et al., 2004). Whereas, most the fish possessed the extraordinary ability to augment their skeletal musculature throughout life (Johnston et al. 2011). So, we hypothesized that the low expression of FoxO1 gene in the piscine muscle may be related with whole-life muscle growth. Based on the sequence conservation and high expressions in the brain and liver, the FoxO1 could play a vitally important role in mediating glucose metabolism in largemouth bass, but might not same with mammals in the muscle.

After an intraperitoneal injection of 1 g glucose/kg BW in largemouth bass, the levels of plasma glucose were significantly elevated, with a peak value at 1 h, and then decreased significantly to basal value at 12 h. The short time to reach a plasma glucose peak was observed in largemouth bass. Similar results also were reported in turbot (Garcia-riera and Hemre, 1996), gilthead seabream (*Sparus aurata*) (Peres et al., 1999), Australian snapper (*Pagrus auratus*) (Booth et al., 2006), silver perch (*Bydianus bydianus*) (Stone et al., 2003), white seabream (*Diplodus sargus*) (Enes et al., 2012) and tilapia (*Oreochromis niloticus*) (Chen et., 2018) injected with the same glucose dose. However, time to re-establish a basal level of plasma glucose in largemouth bass (within 12) or other carnivorous fish (12–24 h) (Garcia-Riera and Hemre, 1996; Peres et al., 1999; Booth et al., 2006; Enes et al., 2011) was much longer than that of the omnivorous and herbivorous fish species (4–6 h) (Stone et al., 2003; Enes et al., 2012; Li et al., 2016; Chen et., 2018), which suggested that the largemouth bass owned the poor ability to utilize dietary carbohydrate. Generally, plasma glucose peak is correlated with maximal insulin levels in the teleost, which could be observed at 1–3 h after a glucose load or feeding (Moon, 2001; Hemre et al., 2002). In this study, plasma insulin kept stable at 1–6 h after the glucose loading when compared to the saline-injected largemouth bass. The similarity results were also reported in rainbow trout, American eel, Chinook salmon, and European sea bass, and researchers further investigated that glucose tolerance curves were poorly correlated with plasma insulin levels (Mazur et al., 1992; Legate et al., 2001; Enes et al., 2011). In contrast, in omnivorous and herbivorous fish, such as common carp (*Cyprinus carpio*), zebrafish (*Danio rerio*), blunt snout bream (*Megalobrama amblycephala*), and hybrid or GIFT tilapia, a remarkable increase in plasma insulin levels were observed after glucose injections (Stone et al., 2003; Lu et al., 2018; Li et al., 2016; Chen et., 2018). Thus, the reason for glucose intolerance of largemouth bass was closely related to insufficient insulin secretion. Some researchers proposed that glucose was not probably an important insulin secretagogue, which led to insufficient insulin secretion (Mommsen et al., 2001). Previous studies had indicated that amino acids, especially arginine and lysine, were more potent insulin secretagogues than glucose in the teleost (Mommsen et al., 2001; Andoh, 2007). In addition, the sensitivity of pancreatic somatostatin-secreting cells to glucose was higher than the insulin-secreting cells for fish (Enes et al., 2011), so the increased somatostatin levels also inhibited insulin secretion (Sheridan and Kittilson 2004).

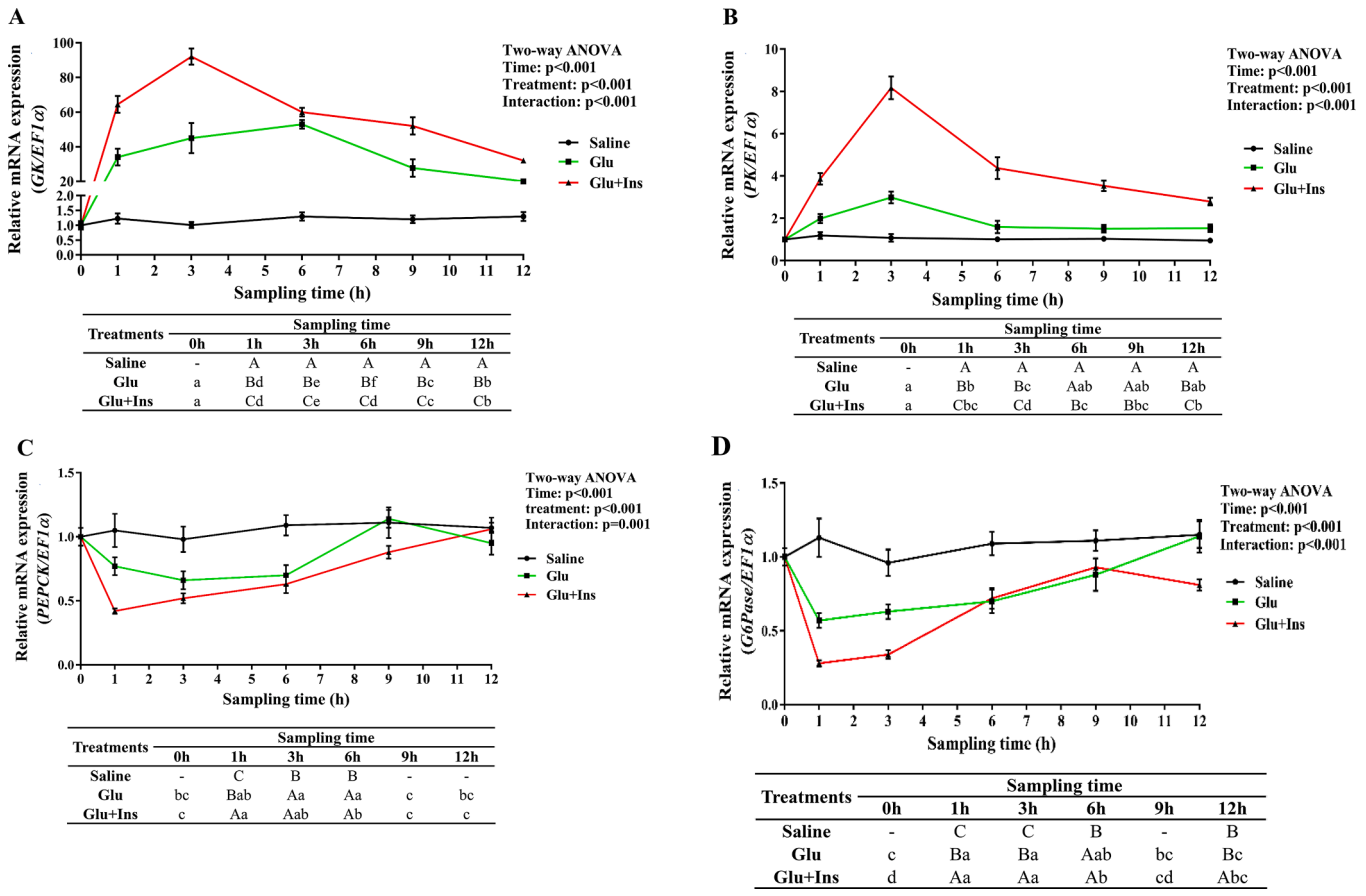
Many studies had indicated that exogenous insulin, which originated from fish, swine, and bovine, could profoundly affect the plasma glucose levels to mediate glucose metabolism in fish (Moon, 2001; Hemre et al., 2002; Polakof et al., 2010). Either fasted or fed a high carbohydrate diet or a glucose load, bovine insulin is known to induce hypoglycemia in rainbow trout (Polakof et al., 2010; Jin et al., 2014) and in other fish (Moon, 2001; Hemre et al., 2002; Caruso and Sheridan, 2011). In zebrafish, blood glucose could be effectively decreased without impairing the function of tissue glucose metabolism after insulin-glucose injection (0.046 mg bovine insulin and 1 g glucose/kg BW), and the time returning to basal glucose level was shortened within 2 h (Lu et al., 2018). Similarly, after insulin-glucose injection, the plasma glucose levels of largemouth bass significantly decreased, then restored to the basic values in 6 h. Therefore, exogenous insulin was effective to promote the clearance of plasma glucose in largemouth bass, which further confirmed that insufficient secretion of insulin was the main reason for glucose intolerance. According to multiple alignment analysis, three highly conserved AKT phosphorylation sites in largemouth bass FoxO1 including T27, S267, and S329, equivalent to T24, S256, and S319 in human FoxO1 respectively were critical for regulating FoxO1 nuclear export and transactivation activity (Carter and Brunet, 2007; Calnan and



Treatments	Sampling time					
	0h	1h	3h	6h	9h	12h
Saline	-	A	A	A	A	A
Glu	a	Cb	Cbc	Bb	Bb	Ba
Glu+Ins	a	Bc	Bb	Aa	Aa	Aa

Treatments	Sampling time					
	0h	1h	3h	6h	9h	12h
Saline	-	A	A	A	-	-
Glu	a	Ab	Aab	Aa	a	a
Glu+Ins	a	Bc	Bc	Bb	ab	a

Fig. 5. Plasma glucose (A) and insulin (B) levels of largemouth bass after the glucose or insulin-glucose injection. The results were expressed as the mean ± SEM (n = 6). Significant differences ($P < 0.05$) among sampling times within each treatment were indicated by the lower-case letters. Upper-case letters indicated a significant difference ($P < 0.05$) among three treatments at each sampling point.



Treatments	Sampling time					
	0h	1h	3h	6h	9h	12h
Saline	-	A	A	A	A	A
Glu	a	Bd	Be	Bf	Bc	Bb
Glu+Ins	a	Cd	Ce	Cd	Cc	Cb

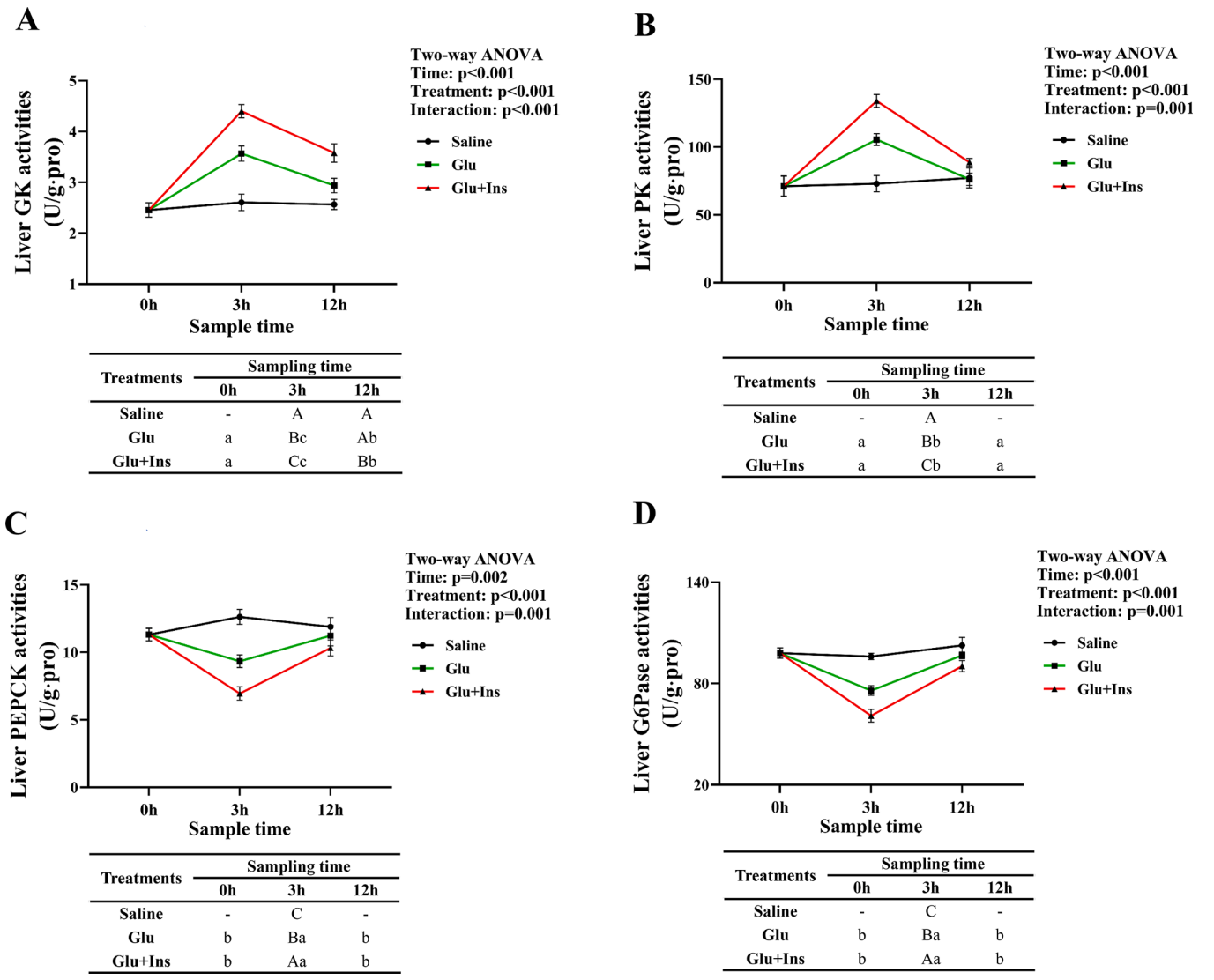
Treatments	Sampling time					
	0h	1h	3h	6h	9h	12h
Saline	-	A	A	A	A	A
Glu	a	Bb	Bc	Aab	Aab	Bab
Glu+Ins	a	Cbc	Cd	Bc	Bbc	Cb

Treatments	Sampling time					
	0h	1h	3h	6h	9h	12h
Saline	-	C	B	B	-	-
Glu	bc	Bab	Aa	Aa	c	bc
Glu+Ins	c	Aa	Aab	Ab	c	c

Treatments	Sampling time					
	0h	1h	3h	6h	9h	12h
Saline	-	C	C	B	-	B
Glu	c	Ba	Ba	Aab	bc	Bc
Glu+Ins	d	Aa	Aa	Ab	cd	Abc

Fig. 6. Relative mRNA levels of hepatic glycolytic and gluconeogenic genes in largemouth bass after the glucose or insulin-glucose injection. The results were expressed as the mean ± SEM (n = 6). Significant differences ($P < 0.05$) among sampling times within each treatment are indicated by the lower-case letters. The upper-case letters indicated a significant difference ($P < 0.05$) among three treatments at each sampling point. (A) *GK*; (B) *PK*; (C) *PEPCK*; (D) *G6Pase*.

Brunet, 2008). Previous studies revealed that mutation of T24 and/or S319 with alanine had no significant effect on the utilization of insulin, but mutation of S256 with an aspartate residue was sufficient to impair insulin-dependent transactivation by FoxO1, which indicated phosphorylation of S256 was essential for insulin to regulate glucose metabolism in human (Rena et al., 1999). In addition, the phosphorylated ability of S256 site was more rapid than the other two sites (Rena et al., 2001). Subsequently, further studies indicated that phosphorylation of S256 may act as a gatekeeper in a process of hierarchical phosphorylation and nuclear/cytoplasmic trafficking, and was also required for subsequent phosphorylation of T24 and S319 (Guo et al., 1999; Zhang et al., 2002). Phosphorylated S319, in turn, was followed by phosphorylation of S322 and S325 by casein kinase 1 and S329 by the dual-specificity kinase 1, respectively, which contributed to form a nuclear export proteins complex (Ran and Crm-1) to promote nuclear exclusion (Woods et al., 2001; Rena et al., 2002). Furthermore, phosphorylation of T24 associated with 14–3-3 proteins promoted cytoplasmic sequestration of FoxO1 (Rena et al., 2001). Thus, each of three



Treatments	Sampling time		
	0h	3h	12h
Saline	-	A	A
Glu	a	Bc	Ab
Glu+Ins	a	Cc	Bb

Treatments	Sampling time		
	0h	3h	12h
Saline	-	A	-
Glu	a	Bb	a
Glu+Ins	a	Cb	a

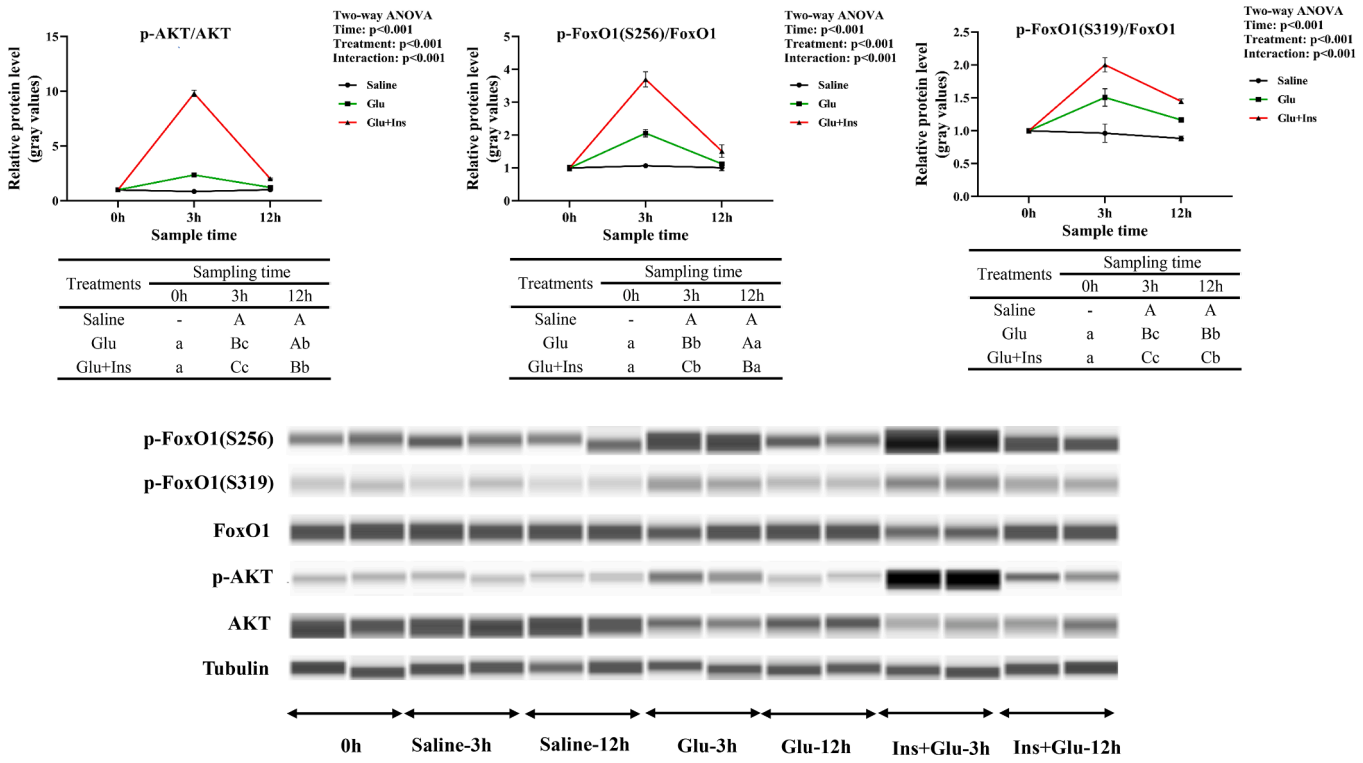
Treatments	Sampling time		
	0h	3h	12h
Saline	-	C	-
Glu	b	Ba	b
Glu+Ins	b	Aa	b

Treatments	Sampling time		
	0h	3h	12h
Saline	-	C	-
Glu	b	Ba	b
Glu+Ins	b	Aa	b

Fig. 7. The enzymatic activities of hepatic glycolysis and gluconeogenesis in largemouth bass after the glucose or insulin-glucose injection. The results were expressed as the mean ± SEM (n = 6). Significant differences ($P < 0.05$) among sampling times within each treatment were indicated by the lower-case letters. The upper-case letters indicated a significant difference ($P < 0.05$) among three treatments at each sampling point. (A) GK; (B) PK; (C) PEPCK; (D) G6Pase.

AKT phosphorylation sites on FoxO1 participated in insulin-mediated FoxO1 action, and S256 phosphorylation may be more critical for FoxO1 because of mediating subsequent phosphorylation of T24/S319 or inhibiting the function of FoxO1 independent of T24/S319 phosphorylation (Rena et al., 1999 and 2001; Zhang et al., 2002). Except for the phosphorylation sites of T24, S256, and S319, many new target sites, such as S229 (Woods et al., 2001), S322 and S355 (Rena et al., 2002), S276 (Wu et al., 2018), and S22, S383, T649 (Saline et al., 2019) were identified in the control of FoxO1 bioactivity, nuclear exclusion, and glucose homeostasis in human. Although the AKT/FoxOs pathway has been widely studied in mammals, it was scattered in teleost (Lansard et al., 2009; Aedo et al., 2015; Otero-Rodiño et al., 2017; Velasco et al., 2018). In the present study, both of S267 and S329 phosphorylation sites of FoxO1 were significantly activated by AKT phosphorylation after glucose or insulin-glucose injection in largemouth bass, which confirmed that S267 and S329 phosphorylation sites of FoxO1 mediated the glucose metabolism. Unfortunately, we do not find a specific phosphorylated FoxO1(T24) antibody to detect the FoxO1(T27) phosphorylation levels of largemouth bass. Hence, more research on phosphorylation sites of largemouth bass FoxO1 regulated by AKT is needed to reveal their roles in regulating glucose metabolism.

In general, after intake of a high carbohydrate diet or glucose injection, plasma insulin level increases, and concomitantly stimulation of hepatic glycolysis, glycogen storage as well as lipogenesis and inhibition of gluconeogenesis were provoked to maintain the glucose homeostasis (Moon, 2001; Hemre et al., 2002). The FoxO1 was demonstrated as the downstream target of insulin signaling to be involved in the regulation of hepatic glycolysis and gluconeogenesis pathways in animals (Dong, 2017). Following the binding of insulin and insulin receptors, AKT was activated by phosphorylation, which phosphorylated FoxO1 proteins to regulate protein or transcript levels of downstream genes (Carter and Brunet, 2007; Gross et al., 2008). In response to starvation, the increased levels of FoxO1, which was mediated by dephosphorylated AKT, could activate the hepatic gluconeogenic-related genes (PEPCK and G6Pase) expression and inhibit glycolysis-related genes (GK and PK) expression to maintain normal plasma glucose levels in mouse (Qu et al., 2006; Zhang et al., 2006). Besides, FoxO1 had been shown to interact directly with DNA binding sites in the promoter region of several genes involved in PEPCK and G6Pase (Schmoll et al., 2000; Hall et al., 2000), and promoted glucose production in mouse hepatocytes (Yoon et al., 2001). Inactivation or inhibition of hepatic FoxO1 resulted in the reduction of glucose production and blood glucose in mouse, moreover specifically



Treatments	Sampling time		
	0h	3h	12h
Saline	-	A	A
Glu	a	Bc	Ab
Glu+Ins	a	Cc	Bb

Treatments	Sampling time		
	0h	3h	12h
Saline	-	A	A
Glu	a	Bb	Aa
Glu+Ins	a	Cb	Ba

Treatments	Sampling time		
	0h	3h	12h
Saline	-	A	A
Glu	a	Bc	Bb
Glu+Ins	a	Cc	Cb

Fig. 8. The activation of AKT/FoxO1 pathway after the glucose or insulin-glucose injection. The results were expressed as the mean ± SEM. Significant differences ($P < 0.05$) among sampling times within each treatment were indicated by the lower-case letters. The upper-case letters indicated a significant difference ($P < 0.05$) among three treatments at each sampling point.

knockout of hepatic FoxO1 led to lower blood glucose levels neither fasting nor non-fasting conditions (Matsumoto et al., 2007; Xiong et al., 2013; O-sullivan et al., 2015). So, the suppression of liver FoxO1 activity was beneficial to improve the capability of glucose tolerance and starch utilization in fish (Altomonte et al., 2003; Matsumoto et al., 2007). In contrast to the mammals, the research focused on the insulin-AKT-FoxO1 pathway mediating glucose metabolism in teleost was relatively little. Li et al. (2018) showed that the mRNA levels of hepatic *FoxO1* significantly decreased in hybrid grouper after the insulin loading or high dietary carbohydrate fed. Up-regulated phosphorylation levels of FoxO1(T24) induced by the insulin, oleate, or octanoate were observed in the hepatocytes of rainbow trout (Lansard et al., 2009; Velasco et al., 2018). The AKT-FoxO1 (T24) pathway was transiently inhibited by dephosphorylated induced by lipopolysaccharide in rainbow trout myotubes (Aedo et al., 2015). Besides, the levels of FoxO1 protein were increased significantly in the liver of zebrafish after insulin receptor a (InsRa) knockout (Gong et al., 2018). However, Li et al. (2019) indicated activation of the insulin pathway could effectively regulate glycolysis, but not the gluconeogenesis pathway in largemouth bass. Both in *vitro* (*FoxO1*-siRNA in primary hepatocytes) and in *vivo* (fed high carbohydrate diets) trials showed that down-regulated *FoxO1* expression in turbot could not result in significant depression of gluconeogenesis (*G6Pase2*) and activation of glycolysis (*GK*) (Pan et al., 2019). Thus, the insulin-AKT-FoxO1 pathway mediating hepatic glucose metabolism needs to further be clarified in teleost. In the present study, compared with the glucose injection group, transcript levels and enzyme activities of hepatic glycolysis (GK and PK) were significantly activated, and gluconeogenesis (PEPCK and G6Pase) were significantly depressed. AKT-FoxO1 pathway was significantly activated by phosphorylation at 3 h after the insulin-glucose injection. Taken together, insulin significantly improved glucose metabolism by activating the phosphorylation AKT-FoxO1 pathway to decrease hyperglycemia stress after the meal.

In summary, the *FoxO1* gene was firstly cloned from the largemouth bass liver. Glucose and insulin-glucose injection trials indicated that insufficient insulin secretion was the main reason for glucose intolerance in largemouth bass. Activation of insulin-AKT-FoxO1 pathway improved glucose tolerance by depressed the hepatic gluconeogenesis and activated glycolysis in largemouth bass after meals. Meanwhile, both S267 and S329 phosphorylation sites of FoxO1 regulated by AKT were confirmed to mediate the glucose metabolism. Further studies, namely inactivation or inhibition of hepatic FoxO1 level and regulation of FoxO1 phosphorylation sites, would help to further clarify the relation among FoxO1, glucose intolerance, and poor ability to utilize the dietary carbohydrate in carnivorous fish.

CRediT authorship contribution statement

Pei Chen: Conceptualization, Methodology, Formal analysis, Writing - original draft. **Xiufeng Wu:** Resources. **Xu Gu:** Formal analysis. **Juan Han:** Methodology. **Min Xue:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing. **Xiaofang Liang:** Formal analysis, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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