Chronic stress of high dietary carbohydrate level causes inflammation and influences glucose transport through SOCS3 in Japanese flounder Paralichthys olivaceus

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Carnivorous fish is thought to be high-glucose intolerance. But the reasons were still unclear. The aim of the present study is to investigate the effects of high level of dietary carbohydrate on the survival, growth and immune responses of Paralichthys olivaceus, and the underlying molecular mechanism related to the immune and glucose metabolism. P. olivaceus were fed with 8%, 16% and 24% of dietary carbohydrate for 10 weeks, respectively. After that, a glucose tolerance test (GTT) was conducted. Results showed that excessive (24%) dietary carbohydrate significantly decreased the growth and glucose tolerance ability according to the GTT. It significantly increased hepatic NADPH oxidase activity and malondialdehyde content and serum contents of IL-6 and advanced glycation end products. The expressions of glucose transport-relevant genes in liver and the content of related hormones in serum were analyzed. In conclusion, it was confirmed that IL-6 increased the expression of suppressor of cytokine signaling 3 (SOCS3) and regulated the downstream targets of PI3K-AKT mediated signal transduction, and then downregulated the glucose transporter 2 activity in liver of P. olivaceus fed diet with excessive carbohydrate level. It was suggested that SOCS3 served as a bridge between immune response and glucose metabolism in P. olivaceus.

Carbohydrate is an excellent energy source for vertebrates, but cannot be fully and efficiently used by fish, especially for carnivorous fish species. Most carnivorous fish have been considered as glucose intolerant and often display a prolonged postprandial hyperglycemia after oral or injected glucose loading and intake of carbohydrate-enriched diets1-3. In addition, fish showed high mortality, reduced growth performance, low nutrient utilization efficiencies and poor physiological functions, when administrated with excessive carbohydrate levels4-8. Many reasons were speculated to explain high-glucose intolerance in carnivorous fish, including a higher sensitivity of insulin to amino acids rather than glucose, inefficiencies in peripheral glucose utilization and absorption, inadequacies in homeostatic glucose regulation and imbalances of endogenous versus exogenous glucose sources9-13. However, there was no clearly conclusion at present.

Pronounced changes in glucose metabolism in fish could be induced by dietary carbohydrate levels. These changes included activities of the key enzymes involved in glucose metabolism, such as pyruvate kinase and glucokinase14,15. In previous study, genes of most of these enzymes have been cloned and characterized in fish. Meanwhile, the regulation of these enzymes at the molecular level by dietary carbohydrate manipulation has been
reported\(^{16-20}\). However, most of the previous studies focused on the glycolysis and gluconeogenesis. Little research was reported on the molecular regulation of the upstream pathways.

Previous studies showed that the carnivorous fed high level of dietary carbohydrate could have suppressed immunity\(^{21,22}\). However, only some apparent parameters were analyzed, such as growth performance, mortality and activity of the immune related enzymes\(^{23,24}\). In mammals, it was confirmed that glucose metabolism was related to the immune response of animals, such as anti-oxidative capacity and inflammation\(^{25-28}\). Production of H\(_2\)O\(_2\) has been shown to regulate insulin release to modulate proximal and distal insulin signaling\(^{25,29}\). At the same time, impaired immunity including ROS and inflammation could also affect insulin signal pathway through activating numerous intracellular serine kinases and negative regulators of cytokine signaling, such as c-Jun N-terminal kinase (JNK) and suppressor of cytokine signaling (SOCS)\(^{30-32}\). Recent years, human diabetes was even regarded as a chronic inflammatory response\(^{33}\). So, it is worth to investigate if the high-glucose intolerance in fish is linked to the impaired immunity.

Japanese flounder (\textit{Paralichthys olivaceus}) is a typical marine carnivorous fish species. After a 45-day feeding trial with different dietary carbohydrate sources, the previous study found that the Japanese flounder utilized dextrin more efficiently than glucose, and dextrin was a better source of energy than lipid. In that study, however, only the apparent parameters including growth performance, feed utilization and body compositions were analyzed\(^{34}\). The aim of the present study is to investigate the effects of high level of dietary carbohydrate on the survival, growth and immune responses of \textit{P. olivaceus}, and the underlying molecular mechanism related to the immune and glucose metabolism.

**Results**

**Survival and growth performance.** There were no significant differences in survival rate (SR, about 95\%) among all the treatments (Fig. 1). With the increase of dietary carbohydrate levels, specific growth rate (SGR) significantly increased in the C16 group, and then decreased in the C24 group (\(p\)-value = 0.002). The lowest value of SGR was observed in the C8 group.

**Serum parameters after feeding trial.** Data of the serum parameters are shown in Table 1. Compared with the C8 and C16 group, contents of total protein (TP), albumin (Alb), globulin (Glo), and advanced glycation end products (AGEs) in serum reached the significant highest values in the group of C24 (TP, \(p\)-value < 0.001; Alb, \(p\)-value = 0.024; Glo, \(p\)-value < 0.001; AGEs, \(p\)-value < 0.001). There were no significant differences in the content of tumor necrosis factor (TNF-\(\alpha\)) and fasting serum insulin (FINS), activities of aspartate transaminase (AST) in serum among all the groups. The highest concentration of interleukin 6 (IL-6) in serum was found in C24 group, which was significantly higher than that in C16 group (\(p\)-value = 0.015). The activities of glutamic-pyruvic transaminase (ALT) and alkaline phosphatase (ALP) in serum significantly increased with dietary carbohydrate levels (ALT, \(p\)-value = 0.025; ALP, \(p\)-value = 0.045). There was no significant difference in the content of leptin between the group of C16 and C24. Both of them were significantly higher than that in C8 group (\(P < 0.001\)). There were no significant differences in concentration of adiponectin in serum among all the three groups.

**Analysis of GTT.** The fasting serum glucose (FSG) of the three groups were 1.15 ± 0.17, 1.25 ± 0.21 and 1.35 ± 0.12 mmol/L, respectively. In addition, there were no significant differences among all the three groups (Table 2). At the beginning, glucose injection induced a notable increase of the serum glucose level in all the groups, and then resulted in a treatment (C8, C16 and C24) × time (sampling time points after injection) interaction (Fig. 2a). During the test, serum glucose concentrations in all groups were significant higher than the FSG. At the 48th hour after injection, the serum glucose concentration in all the groups returned to the basal level.

The parameters of fitting equation (equation (3)) are shown in Table 3. Coefficients of all parameters were with 95% confidence bounds. The values of R square in all equations were over 0.84. According to the fitted equation,
the max serum glucose concentration (Cmax) of serum peaked at 7.35 ± 0.90, 7.65 ± 0.40, 3.21 ± 0.20 hour with the Cmax at 10.67 ± 0.35, 11.85 ± 0.32, 14.02 ± 0.13. No significant differences were found in areas under the curve of glucose during the 48 h of GTT (AUCg) among all the groups (Table 2). The highest value of clearance rate during the 48 h of GTT (CR48) was found in C16 group (2.38 ± 0.23%/h), and the lowest one was in C24 group (0.27 ± 0.25%/h). Compared with that in C24 group, the time return to basal serum glucose (Tbasal) was significantly shorter in C16 group (33.32 ± 0.35%).

In the regard to the three groups, there were no significant differences in the serum insulin concentrations among all the sampling time points after injection of glucose. And there was no clear trend on the serum insulin concentrations changing with the sampling times (Fig. 2b).

**Histological structure and oxidation resistance of liver.** Hepatocytes from group C8 and C16 showed normal histology (Fig. 3). In C24 group, some hepatic cell outlines were indistinguishable and some nuclei were dissolved. Obvious hepatocytes swelling, nucleus polarization and lipid vacuolization were observed in C24 group. The value of total anti-oxidative capacities (T-AOC) in liver in C24 group was significantly lower than those in C8 and C16 groups (Table 2) (p-value = 0.004). The C8 group had a significant higher superoxide dismutase (SOD) activity in liver than C24 group (p-value = 0.007). The C24 group had a significant higher malondialdehyde (MDA) in liver than C8 and C16 groups (p-value = 0.002). C16 group had a significant lower NADPH oxidase (NOX) activity in liver than C24 (p-value = 0.040), and there was no significant difference between C8 and C16.

**Gene and protein expression in liver.** The gene expressions of glucose utilization-relevant genes are shown in Fig. 4a. The mRNA levels of insulin receptor substrate 2 (IRS2), c-Jun N-terminal kinase 1 (JNK1) and protein kinase B 1 (AKT1) in liver had no significant differences among all the three groups. Gene expressions of the insulin receptor substrate 1 (IRS1) and glucokinase (GK) in C24 group was significantly lower than that in C16 group (IRS1, p-value = 0.039; GK, p-value = 0.003). Compared with C8, the mRNA level of SOSC3 had the significant highest value in C24 group (p-value = 0.014), and the significant lowest value in C16 group (p-value = 0.001). The mRNA level of phosphoinositide 3-kinase (PI3K), protein kinase B 2 (AKT2), glucose transporter 2 (GLUT2) and phosphofructokinase (PFK) had the significant highest value in C16 group (PI3K, p-value = 0.014; AKT2, p-value = 0.002; GLUT2, p-value = 0.008; PFK, p-value = 0.001), but no differences were found between C8 and C24. The mRNA levels of pyruvate kinase (PK) had the significantly higher and lower value in C16 and C24, respectively (p-value < 0.001). At the same time, phosphorylation of AKT (P-AKT) level in

| Parameters                  | Diets       | C8  | C16 | C24 |
|-----------------------------|-------------|-----|-----|-----|
| TNF-α (pg/mL)               |             | 49.22 ± 7.9 | 51.33 ± 9.31 | 50.81 ± 1.71 |
| ALT (U/L)                   |             | 5.40 ± 0.36b | 5.80 ± 0.42ab | 6.40 ± 0.36a |
| AST (U/L)                   |             | 12.90 ± 1.36 | 12.13 ± 1.69 | 12.55 ± 0.62 |
| ALP (U/L)                   |             | 61.65 ± 3.10b | 64.68 ± 2.31ab | 68.47 ± 3.41a |
| AGEs (mg/ml)                |             | 4.26 ± 0.11b | 4.63 ± 0.23b | 5.24 ± 0.23a |
| FINS (mIU/L)                |             | 21.33 ± 2.72 | 21.37 ± 2.04 | 21.89 ± 3.34 |
| Leptin (ng/ml)              |             | 8.15 ± 0.50b | 11.76 ± 0.74a | 11.57 ± 0.80a |
| Adiponectin (µg/ml)         |             | 6.82 ± 1.29 | 7.09 ± 1.69 | 7.70 ± 1.39 |

Table 1. Serum parameters of Japanese flounder after the 10-week feeding trial. Values are presented as mean ± S.D., n = 3 (8 fish/replicate). Values followed by different letters in the same row are significantly different (P < 0.05).

| Parameters                  | Diets       | C8  | C16 | C24 |
|-----------------------------|-------------|-----|-----|-----|
| FSG (mmol/L)                |             | 1.15 ± 0.17 | 1.25 ± 0.21 | 1.35 ± 0.12 |
| AUCg (mmol/L × h)           |             | 219.27 ± 1.46 | 229.52 ± 4.19 | 222.82 ± 7.90 |
| Cmax glucose (mmol/L)       |             | 10.67 ± 0.35c | 11.85 ± 0.32b | 14.02 ± 0.13a |
| Tmax (h)                    |             | 7.35 ± 0.90a | 7.65 ± 0.40a | 3.21 ± 0.20b |
| CR48 (%/h)                  |             | 1.29 ± 0.33b | 2.38 ± 0.23a | 0.27 ± 0.25c |
| Tbasal (h)                  |             | 37.22 ± 1.17b | 33.32 ± 1.61b | 45.72 ± 2.13a |

Table 2. Analyzed parameters in the glucose tolerance test. Values of fasting serum glucose are presented as mean ± S.D., n = 3 (8 fish/replicate) and other values are presented as mean ± S.D., n = 3 (3 fish/replicate). Values followed by different letter in the same row are significantly different (P < 0.05).
Figure 2. Response to glucose tolerance test of Japanese flounder after the 10-week feeding trial. (a) Serum glucose concentrations (b) Serum insulin concentrations. Values at 0 h are presented as mean ± S.D., n = 3 (8 fish/replicate) and the other values represented the means ± S.D., n = 3 (3 fish/replicate). * represents significant difference between C24 and C8 (P < 0.05); ** represents significant difference among all groups, (P < 0.05).

| Diets | Parameters | a    | b            | c   | d   | R²   |
|-------|------------|------|--------------|-----|-----|------|
| C8    | 18.02      | 0.05038 | 15.36        | 0.2876 | 0.8523 |
| C16   | 20.60      | 0.05443 | 17.97        | 0.2546 | 0.8973 |
| C24   | 32.01      | 0.06802 | 29.32        | 0.1586 | 0.8489 |

| Diets | Parameters | a    | b            | c   | d   | R²   |
|-------|------------|------|--------------|-----|-----|------|
| C8    | 34.65      | 0.07830 | 31.80        | 0.1876 | 0.8801 |
| C16   | 39.42      | 0.07570 | 36.49        | 0.1689 | 0.8966 |
| C24   | 67.51      | 0.09075 | 65.05        | 0.1434 | 0.8990 |

Table 3. Parameters in the regression equations after the glucose tolerance test. Coefficients of all parameter are with 95% confidence bounds.
C16 was significantly higher than that in C24 (p-value = 0.009) and the total of AKT (T-AKT) in C16 was significantly higher than other two groups (T-AKT, p-value = 0.032) (Fig. 4b–d).

Discussion

Based on the growth rate, the optimal levels of dietary carbohydrate for the carnivorous species, such as gilthead sea bream, giant croaker, Chinese long snout catfish, were determined less than 20% 15,35,36. Instead, compared to that in the carnivorous species, the optimal levels for herbivorous species and omnivore species, such as gibel carp, white sea bream and grass carp, can be higher than 30%, even to 50%37–39. Excessive level of dietary carbohydrate could result in negative effects. In the present study, dietary carbohydrate supplementation at 24% led to reduced growth compared with 16% of dietary carbohydrate. Liver is a main metabolic organ. According to the histological analysis, the liver cell of Japanese flounder fed with 24% of dietary carbohydrate potentially indicated the impaired function of liver. In addition, more oxidative damage in the liver of Japanese flounder was found in group with 24% of dietary carbohydrate compared with the other two groups. Based on these data, it was suggested 24% of dietary glucose concentration was overdose for Japanese flounder.

Impaired immunity in fish by overdose of dietary carbohydrate was observed in previous studies. It was reported that high level of dietary carbohydrate reduced plasma IgM and lysozyme levels in European whitefish40. The blood haemoglobin was negatively correlated with dietary carbohydrate levels in Atlantic salmon by long-term feeding of a high carbohydrate diet (20–30%) 24. After the 10-week feeding trial, in the present study, the concentration of TP, Alb, Gln, AGEs and IL-6, the activities of ALP and ALT in serum and the content of MDA in liver significantly increased, while the activity of T-AOC and SOD in liver of Japanese flounder significantly decreased by the excessive dietary carbohydrate inclusion at 24%. This suggested that excessive dietary carbohydrate level could increase the oxidative stress and inflammation in Japanese flounder.

A fact was found that the dietary carbohydrate intake affected the glucose tolerance in fish, while it seemed to have a poor ability to take care of excess glucose41,42. In mammals, fasting glucose concentration and fasting insulin concentration were used in calculating insulin sensitivity or resistance, in order to evaluate the glucose utilization ability. The relationship between blood glucose concentration in fish and dietary carbohydrate level was inconclusive. Hemre et al. found that blood glucose level in Atlantic salmon was correlated with dietary carbohydrate level43, while some studies reported serum glucose did not differ between the groups fed different dietary carbohydrate levels44,45. Insulin is a major pancreatic endocrine hormone, which regulates blood glucose levels and the underlying metabolic pathways in higher vertebrates. In the present study, FSG was not significantly

Table 4. Anti-oxidative parameters in liver of Japanese flounder after the 10-week feeding trial. Values are presented as mean ± S.D., n = 3 (8 fish/replicate). Values followed by different letters in the same row are significantly different (P < 0.05).

| Parameters       | Diets     | C8         | C16         | C24         |
|------------------|-----------|------------|-------------|-------------|
| T-AOC (U/mg protein) | 0.66 ± 0.07a | 0.82 ± 0.16a | 0.41 ± 0.07b |
| SOD (U/mg protein)  | 1.45 ± 0.10a | 1.30 ± 0.05ab | 1.19 ± 0.04b |
| MDA (nmol/mg protein) | 0.96 ± 0.09b | 0.90 ± 0.07b    | 1.22 ± 0.08a |
| NOX (U/mg protein)  | 0.98 ± 0.05ab | 0.90 ± 0.25b    | 1.65 ± 0.45a |

C16 was significantly higher than that in C24 (p-value = 0.009) and the total of AKT (T-AKT) in C16 was significantly higher than other two groups (T-AKT, p-value = 0.032) (Fig. 4b–d).
affected by dietary levels. Meanwhile, the levels of circulating insulin kept the same level in Japanese flounder fed different levels of dietary carbohydrate in this study. This result was in accordance with an earlier study in rainbow trout, in which it was found that the insulin content in plasma of trout fed with the carbohydrate-enriched diet was not significantly increased when compared with that with carbohydrate-free diet45.

In fact, apart from FSG and in FINS, GTT is a more accurate way to evaluate the glucose utilization ability, where AUC of glucose was used to estimate the content of individual loaded glucose, CR and Tbasal were used to judge the speed of glucose disappearance. A lower CR and longer Tbasal with a large AUC were regarded as impaired glucose tolerance46–48. GTT is required when humans have high fasting glucose levels but do not meet the diagnostic criteria for diabetes. GTT have been conducted in many fish species and is useful for evaluating glucose tolerance, such as rainbow trout46, channel catfish49 and white sturgeon50. In the present study, the mathematical modeling was used to evaluate the ability of glucose utilization during GTT in Japanese flounder after a 10-week feeding trial with different dietary carbohydrate levels. Consistent with the increased SGR, although no significant differences in AUCs were found, the fish fed with 16% of dietary carbohydrate had a significant higher CR and lower Tbasal. According to the data on growth and GTT, it was suggested that the ability of glucose utilization of Japanese flounder was improved when fed with 16% of dietary carbohydrate. And the feeding adapting was helpful for glucose tolerance.

Ability of glucose utilization is related to glucosesensing. Rainbow trout fed a diet with 20% of carbohydrate showed a higher postprandial glucose than that fed carbohydrate-free diet. This showed a positive response to the dietary carbohydrate based on the major components of glucosesensing system including glucose transporters (GLUTs)51. Glucose metabolism depends on the intake of glucose. Insulin-induced PI3K-AKT signaling contributes to the regulation of GLUT2-mediated glucose uptake in liver. PI3K and AKT, which were two major nodes downstream of insulin receptor substrate (IRS), have been implicated in many of the metabolic actions of insulin, such as glucose transport. The rate-limiting enzymes in the glycolysis pathways, such as GK, PFK and PK, played key roles in glucose metabolism. Results in the present study showed that the upregulation of PI3K,
AKT2, GLUT2, PK and PFK transcription and activities of the total AKT and AKT phosphorylation were found in C16 group. These could be helpful to explain why the Japanese flounder fed with 16% of dietary carbohydrate had better glucose utilization ability as shown by the data on growth and GTT.

Moreover, many studies in mammals have found that leptin and adiponectin could improve the glucose transport12-24, even though the detail was not clear. Fish definitely do have these hormones with some functional similarities and many other distinct features45. In the present study, there were no significant differences in concentration of adiponectin in serum among all the three groups. However, the concentrations of leptin in groups of C16 and C24 were significantly higher than that in C8 group. It was suggested that an increasing level of leptin could improve glucose utilization in Japanese flounder. In the present study in zebrafish, it was also found that leptin signaling regulates glucose homeostasis56. In the present study, however, Japanese flounder fed with higher dietary carbohydrate (24%) had a longer Tbasal with lower CR, a decreased activity of PI3K-AKT and glycolysis pathways, a downregulated GLUT2 transcription than those fed with 16% of dietary carbohydrate. This result implied that excessive dietary carbohydrate inhibited the ability of glucose transportation and glucose catabolism, which was not associated with the content of serum insulin, leptin and adiponectin.

Previous studies suggested many reasons to explain the high-glucose intolerance in fish, and many issues remained under debate. A growing number of studies indicated that the oxidative stress influences the insulin signal pathway in mammals55,56. Although these findings were reported in muscle cell, it is expected that they also occur in hepatocytes because the primary metabolic abnormalities in these cells also induce ROS production, thus affecting glucose utilization. AGEs accumulate under high-glucose conditions and affect ROS producing by NADPH oxidase59-62. In the present study, it was implied that ROS level increased in organism with a significant up-regulation in AGEs and NADPH oxidase in Japanese flounder fed diet with excessive carbohydrate level (24%). Houstis et al. pointed out that increases in ROS levels precede the onset of insulin resistance (IR) and might be causally linked to it44. It is still not clear exactly how oxidative stress causes insulin resistance or metabolic dysfunction in mammals. The possible mechanism suggested is that ROS could inhibit IRS to regulate the insulin signal pathway by c-Jun N-terminal kinase (JNK)31,32. JNK family members are encoded by three genetic loci, JNK1-3. JNK1 isoform is the major contributor to insulin resistance64. Activation of JNK1 has been shown to directly phosphorylate IRS-1 at inhibitory sites that prevent recruitment of this protein to the activated insulin receptor56. In the present study, no significant difference in JNK1 gene expression was found although it was suggested that Japanese flounder fed with 24% of dietary carbohydrate was under oxidative stress. Another fact was found that inflammation can be induced by ROS through the induction of pro-inflammatory cytokines (e.g., TNF-α) in C24 group compared with C16 group. These results indicated that TNF-α could inhibit IRS to regulate glucose utilization. In conclusion, dietary carbohydrate is necessary for the growth and immune response of Japanese flounder. Diet formulation and its proximate composition are given in Table 5. Three isonitrogenous and isolipidic diets are formulated with graded levels of carbohydrate (8%, 16% and 24%, respectively). They were named as C8, C16 and C24, respectively. All ingredients were finely ground, well mixed, and dry extruded in a laboratory pellet mill (EL220, Shangdong Haiyang, China). The diameters of the diet particles were 3 mm and 5 mm die. The particles were dried in a forced air oven at 50 °C for 8 h and stored in a refrigerator (−20 °C) until used.

Materials and Methods

Experimental diet. Diet formulation and its proximate composition are given in Table 5. Three isonitrogenous and isolipidic diets are formulated with graded levels of carbohydrate (8%, 16% and 24%, respectively). They were named as C8, C16 and C24, respectively. All ingredients were finely ground, well mixed, and dry extruded in a laboratory pellet mill (EL220, Shangdong Haiyang, China). The diameters of the diet particles were 3 mm and 5 mm die. The particles were dried in a forced air oven at 50 °C for 8 h and stored in a refrigerator (−20 °C) until used.
Animals and feeding trial. Japanese flounders were obtained from Haiyang, Shandong Province, China. They (initial body weight: 7.14 ± 0.10 g) were randomly distributed into 9 tanks (3,000 L) in a flow-through water system supplied with sand filtered seawater at a flow rate of 2.5 L/min. Three tanks were used for one treatment (150 fish/tank). The fish were fed to apparent satiation twice daily (8:00 and 18:00) for 10 weeks. During the feeding trial, the dissolved oxygen content was approximately 7.4 mg/L. Water temperature naturally ranged from 21 to 24 °C. The photoperiod was maintained at intervals of 13-h light: 11-h dark. All procedures performed in study were in strict accordance with the recommendations in the Guide for the Use of Experimental Animals of Ocean University of China. The protocols for animal care and handling used in this study were approved by the Institutional Animal Care and Use Committee of Ocean University of China.

Sampling. At the end of the 10 weeks feeding trial, all the fish were fasted for 24 h after the last feeding and anaesthetized with MS-222 (Sigma, 50 mg L⁻¹ water) before sampling. Fish in each tank were weighed and counted to determine SGR and SR. Eight fish per tank were sampled to collect blood from coccygeal vertebra vein. The serum was separated and stored at −80°C until analysis. After that, these fish were dissected to collect liver. And the livers were quick frozen using liquid nitrogen and stored at −80°C for subsequently analysis.
Glucose tolerance test. At the end of the feeding trial, the serum of fish starved for 24 h was used to measure FSG and FINS concentration, which were set as a baseline for an AUC of glucose calculation and for comparison with insulin content after GTT, respectively. Another 30 anaesthetized fish per tank were used for GTT according to the method of López-Olmeda82. Anaesthetized fish per tank were intraperitoneal injected with glucose at a concentration of 600 mg per kg fish. After that, the blood was taken at 1, 3, 6, 9, 12, 18, 24 and 48 h after injection, respectively, to analyze serum glucose and insulin concentrations. Three fish per tank were used for collecting the serum at each sampling time point. The AUC of glucose, CR, Cmax, Tbasal and the time reach the max serum glucose concentration (Tmax) were calculated.

| Primers | Forward (5′-3′) | Reverse (5′-3′) |
|---------|----------------|----------------|
| SOCS3   | TTCTCTACCCCTGTCGCTG | CCAACCGTTCCTCCAATGAT |
| IRRK    | TGGTGGGTTGTAGGTTTGGT | TTCCTGCTGCTTGGGCTTTT |
| IRS1    | CCCACATTAGAAGGAGAAGAAG | AGTACAGGAGACGGAGGATC |
| IRS2    | GAGGGATAGGCAAAGTGAAT | AAGAAGAAGCTGTCCGAGAT |
| AKT1    | GAGGGAAGAGGAGGACGAGAG | TTCACGGAGTGGTGAAGAT |
| AKT2    | CATCCTCTTCACAAACACTA | CTGTAACCAATGCAGTTCG |
| GLUT1   | GAAAGCAGACAGAGAGAGG | ACAAGCAGAACATTGACCAT |
| PK      | GCTGTCGAGACTAAGAATC | CTCGTTGACTGCGCATGGT |
| G2       | GGGATGATTGTTGGCACT | ATGGATTGCTGATGGTG |
| PFK     | TTGTTGAGGAGGTTCG | ATTTGTGCTGATGGTG |
| β-ACTIN | GGAATGCTGCTGAGCATTAAG | CCTCTGAGACAGGGACCCT |

Table 6. List of PCR primer pairs used for the real-time PCR analysis.

Serum and liver parameters. The concentrations of glucose, TP, Alb and Glo, and the activities of ALP, ALT and AST in serum were analyzed by the automatic biochemical analyzer (AU5400, Beckman). The concentration of insulin, leptin, adiponectin, IL-6, AGEs and TNF-α in serum were determined using a double antibody sandwich enzyme-linked immunosorbent assay. Kits for adiponectin and AGEs were purchased from Cusabio, China. Kits for insulin, leptin, IL-6 and TNF-α were purchased from Mybiosource, USA. All these kits were developed using antigenic regions completely conserved in fish83–86. The protein concentration, T-AOC, MDA, and SOD activity in liver were measured by commercial kits (Nanjing Jiancheng Bioengineering Institute, China)87–89. And the activity of NOX in liver was detected according to Li et al.89.

Histological analysis. Liver samples were fixed in 10% neutral buffered formalin for 24 h and then transferred to 70% ethanol. Samples were then dehydrated through graded levels of ethanol (70%, 80%, 85%, 90%, 95%, 100%) using a tissue processor, cleared and embedded in paraffin wax (PDDT-12C1, Ceike, China). Transverse sections (~7 μm) were prepared using a rotary microtome (RM2235, Leica, China), mounted onto slides and processed for staining (Thermo Gemini A2, USA) with hematoxylin and eosin (H&E, Nanjing Jiancheng Bioengineering Institute, China). Slides were viewed under light microscopy and photographed with a digital camera (Olympus, DP72, Nikon, Japan).

Real time PCR. Total RNA was extracted from 0.1 g of liver tissue using Trizol Reagent (Invitrogen, USA). It was then quantified and the purity was assessed by spectrophotometry. The 260:280 ratios were 1.8 ~ 2.0. Complementary DNA (cDNA) was synthesized from 1 mg of total RNA using PrimeScript® RT reagent Kit with gDNA Eraser (Takara, Japan). Expressions of the selected genes were analyzed by the real-time PCR, which was performed with an ABI 7500 instrument (Applied Biosystems) using SYBR Green PCR (Takara). The Reaction mixture contained 2.0 μl of cDNA template, 10.0 μl of SYBR® Premix Ex Taq™ II, 0.8 μl of Forward Primer, 0.8 μl of Reverse Primer, 4.0 μl of ddH2O and 0.8 μl of DNA template. The reaction consisted of 10 cycles at 95 °C for 15 s and 60 °C for 60 s. The PCR amplification was performed with 30 cycles at 95 °C for 5 s, 58 °C for 5 s and 72 °C for 30 s. The resulting product was separated on a 1% agarose gel (3 μl of each), stained with gel red (0.5 μg/μl) and visualized under UV illumination. The gel was photographed using a gel documentation system (SimpleDoc™ 1500, UVP, UK). The bands were analyzed with Quantity one software (BioRad). The density of the bands was corrected by β-actin and the quantification was calculated using the ΔΔCt method, and the primers used are shown in Table 6.

Western blot analysis. Tissues were homogenized with glass Tenbroeck tissue grinders (Kimble Chase, USA) on ice and lysed with 50 mM Tris-HCl, 150 mM NaCl, 0.5% NP-40, 0.1% SDS, and 1 mM EDTA, pH 7.5, with protease and phosphatase inhibitor cocktails (Roche, Switzerland) at 4 °C for 1 h and cleared by centrifugation at 12,000g for 20 min. Protein concentrations were determined with a BCA protein assay kit (Beyotime, Shanghai, China). The samples were separated by SDS-PAGE and transferred to a 0.45 μm PVDF membrane (Millipore, USA) for Western analysis. The membrane was blocked with 5% nonfat milk in TBST buffer (20 mM Tris-HCl, 500 mM NaCl, 0.1% Tween 20) for 1 h at room temperature and incubated with primary antibody overnight at 4 °C before horseradish peroxidase (HRP)-conjugated secondary antibodies were added and visualized using ECL reagents (Goodhere, China). The following antibodies were used: antibodies against protein kinase B (AKT, 9272), phospho-AKT (Ser473, 9271) and β-tubulin (2146) were purchased from Cell Signaling Technology. All these antibodies were developed using
antigenic regions completely conserved in fish, and could be used in scophthalmidae turbot\(^9\). The Western bands were quantified using NIH Image 1.63 software.

**Calculation and statistical analysis.** The following parameters were calculated as:

\[
\text{Survival rate (SR, %)} = 100 \times \text{final fish number/initial fish number} \tag{1}
\]

Specific growth rate (SGR, %) = 100 × \((\ln \text{final body weight} - \ln \text{initial body weight}) \times \text{days}^{-1}\) \tag{2}

The PROC NLIN was used to fit exponential curves for glucose concentration during 48 hours GTT, using the following equation performed by Metlab2017:

\[
F(t) = a \times \exp(-b \times t) + c \times \exp(-d \times t); \tag{3}
\]

where \(F(t)\) was the metabolite concentration at time \(t\) (hour); the method was modified based on Pires et al.\(^9\). CR (%/h), \(C_{\text{max}}\) (mmol/L), \(T_{\text{basal}}\) (h) and \(T_{\text{max}}\) (h) were calculated as the actual equation. CR was calculated as:

\[
\text{CR; %/h) = ([ln(ta) - ln(tb)]/(tb - ta)) \times 100}; \tag{4}
\]

where \([ta]\) was the concentration of glucose at time \(a\) (ta), and \([tb]\) was the concentration of glucose at time \(b\) (tb). The AUC was calculated using the trapezoidal method and actual concentration values, after discounting the baseline concentration (FSG, calculated by averaging value before injection)\(^9\).

All statistical evaluations were analyzed using the software SPSS 19.0. The growth parameters, immune parameters and gene expression were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple range tests. The effects of time and diets and their interactions were analyzed by two-way ANOVA. Tukey’s multiple range tests were used to examine treatment differences among the interactions. When the interaction was significant, the results were further analyzed using one-way ANOVA and Tukey’s multiple range test. In case unequal variance was determined by Levene’s test, the data were square root-transformed before statistical analysis. Differences were regarded as significant when \(P < 0.05\). All data were expressed as means ± standard deviations.

**Data availability.** The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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**Author Contributions**

K.D. completed the experiment and prepared the manuscript. M.P., J.L., M.Y., Z.G., Y.Z., G.L. and D.L. analyzed the samples. W.Z. designed the experiment and revised the manuscript. K.M. designed the experiment. All authors have read and approved the final manuscript.
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