Effects of glucose on biochemical immune responses and hepatic gene expression in common carp, *Cyprinus carpio* L

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**ABSTRACT**

Glucose metabolism is a key biological process in animals, as it is related to multiple activities including immune response and diseases. In this study, we focused on serum parameter changes and hepatic gene expression to explore the immune response to glucose injection. We performed serum analysis and high-throughput sequencing analysis in the liver of common carp (*Cyprinus carpio* L). The serum GLU and albumin were up-regulated by glucose injection. The transcriptome analysis showed that the categories of response to external stimulus in GO category, PPAR-signalling pathway, complement and coagulation cascades pathways in KEGG analysis were enriched in genes that were differently expressed between the glucose injection group and the control group. Among these pathways, redox-regulatory protein FAM213A-like, extracellular superoxide dismutase [Cu–Zn]-like and putative natural resistance-associated macrophage protein (NRAMP) were up-regulated by glucose stimulation. The 25-hydroxyvitamin D-1 alpha hydroxylase, vitamin D 25-hydroxylase and natterin-3-like were significantly down-regulated by glucose injection. The present study is the first report of serum and hepatic transcriptome changes in common carp in response to glucose increase in serum. The obtained data provide valuable clues for future efforts into understanding the connection between the energy metabolism and the immune system.

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**Introduction**

The nutrition and metabolism determine the feeding strategy, feed formula and physiological status in fish. Thus, understanding the mechanisms of nutrient metabolism is a crucial objective in aquaculture research [1–4]. It is well known that certain nutrients play a regulatory role in anabolism and catabolism [5,6]. However, few studies have demonstrated the correlation between nutrition regulation and immune response. A previous study reported that lack of adequate nutrients can cause immune deficiency in mammals [7]. Food intake and its components affect the response to stress, inflammation and microflora in organisms [8]. On the other hand, the immune pathways participate in the metabolic control. MAPK (mitogen-activated protein kinase) and TOR (target of rapamycin) pathways mediate nutrient signals to regulate organismal growth and homeostasis [9,10]. The nutrient components, such as sugar, protein, fatty acids and microelements, regulate the immune response mechanisms as well [7].

In fish, carbohydrates are ingested, absorbed and utilized in anabolism [3,11]. Carbohydrates are the main energy source for fish [12]. However, unlike mammals, fish have low ability to utilize carbohydrates. In addition, glucose-sensing mechanisms may be relevant in different feeding habits [6]. Glucose is the key intermediate in both carbohydrate catabolism and anabolism. Low activity of lipid syntheses by glucose stimulation was found in Atlantic cod (*Gadus morhua*) [13]. Moreover, in teleosts, the liver is the key tissue responsible for the regulation of glucose storage [14]. These findings support the idea that the liver plays a crucial role both in nutrient metabolism and in immunomodulation. Impaired glucose tolerance is associated with increased serum levels of interleukin 6 [15]. Injection with a glucose analogue,
2-deoxy-D-glucose, provoked hyperglycaemia and affected the immune activity of leucocytes in seabream (*Sparus aurata* L.) [16]. High levels of serum glucose in fish are related to constant metabolic stress and lead to suppressed immune functions [1]. However, glycogen deposition affected the immune function in Atlantic salmon (*Salmo salar*) only to a minor extent [17].

Based on the previous results, we presumed that glucose regulates the immune activities in fish blood and liver. Thus, the aim of the present study was to investigate the integrated effects of glucose injection on main immune parameters in the blood and the transcriptome in the liver of common carp (*Cyprinus carpio*), which is a popular farmed fish around the world. The present study highlights the response of common carp to the high glucose levels from immune scenarios.

**Materials and methods**

**Fish and sampling**

Fourteen common carp of similar size (75.29 ± 9.92 g) obtained from Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, were kept in acrylic tanks at 26 °C and 12-h light:12-h dark photoperiod. The individuals were randomly divided into two groups (control and glucose injection group) and acclimatized for 7 days prior to the experiment. The control group and the glucose injection group were injected with saline and 1 g/kg D-glucose (Sigma), respectively. After 6 h, blood samples were collected from the fish for biochemical analyses. The serum was obtained after centrifugation (3000 ×g, 5 min, Eppendorf 5810-R, Germany). Liver tissue was collected and stored at −80 °C for RNA analyses.

**Measurement of blood biochemical indicators**

The serum total protein (TP), albumin (ALB), globulin (GLB), total cholesterol (TC), triglyceride (TG) and glucose (GLU) concentration were assayed by A6 semiautomatic biochemical analyzer (Runlian, China). The leukocyte counts were counted under a light microscope. The C3, C4, IgA, IgG and IgM were determined by an ELISA (enzyme-linked immunosorbent assay) kit from Beijing Sino-Uk Institute of Biological Technology, Beijing, China.

**High-throughput sequencing and analysis**

Liver RNA was isolated by RNA Trizol (Invitrogen) according to the manufacturer’s instructions. The RNA was analyzed by agarose gel, ultraviolet (UV) spectrophotometer (DU800, Beckman) and Agilent 2100 bioanalyzer (Agilent). Two cDNA libraries were constructed by mixing the RNA pool of each group. The mRNA from the RNA pool was isolated by oligo(dT) magnetic beads (Life Technologies). After obtaining the cDNA libraries, Hiseq™ 4000 platform was used to sequence these cDNA libraries.

The raw data were filtered and *de novo* assembly was done using Trinity software (http://trinityrnaseq.sourceforge.net/) as previously described [18]. Differentially expressed genes (DEGs) were identified using the fragments per kilobase million (FPKM) method. The genes with a *p*-value lower than 0.05 and fold changes with log2*fold_change* ≥ 1 were identified as DEGs. To illustrate the function of DEGs, GO and KEGG analyses were performed. The Blast2Go software [19] assigned the DEGs to GO and KEGG annotation by NCBI (National Center of Biotechnology Information) blastx to the KEGG database.

**Quantitative polymerase chain reaction (qPCR)**

According to the high-throughput sequencing result, six genes were analyzed by qPCR: redox-regulatory protein FAM213A-like, extracellular superoxide dismutase [Cu–Zn]-like, putative natural resistance-associated macrophage protein (NRAMP), 25-hydroxyvitamin D-1 alpha hydroxylase, vitamin D 25-hydroxylase and natterin-3-like genes. The total RNAs isolated from liver samples were reverse transcribed by PrimeScript™ reagent Kit with gDNA Eraser (Takara) according to the manufacture’s instructions. The mRNA expression levels were analyzed by Pikoreal Real-time PCR System (Thermo). The PCR primers were designed by Primer

| Table 1. Primer sequences used in this study. | Forward (5’ to 3’) | Reverse (5’ to 3’) |
|---------------------------------------------|-------------------|-------------------|
| Redox-regulatory protein FAM213A-like | CATTAGTGCTCCCCCGCTTCT | CTAAGAGAGACGTGCTGTCTG |
| Extracellular superoxide dismutase [Cu–Zn]-like | GATCTTTCGGCAGGCTCTTCTT | GGGCCTGGTGATCAT |
| Putative natural resistance-associated macrophage protein (NRAMP) | CTTCCAGGGCTTTCTATGG | TTTGATTGGGCTGGGAAGG |
| 25-Hydroxyvitamin D-1 alpha hydroxylase | AGCCAAACGCTAGATCATCACA | ACGGAGCTGGGCTGAG |
| Vitamin D 25-hydroxylase | CCTGGGAGGAATTCCGAACGTTG | AAGGGGAGGAGCCTGATC |
| Natterin-3-like | CAGGGAACCGGTGTCGAAGG | GTGGAATTCTCCCAAATGAA |
| β-Actin | GCTATGTCCTTCGTCCTG | CCGTCAGGCAGCTCATAGCT |
Express V3.0 (Table 1). Reaction mixtures (10 μL volumes) for qPCR were prepared containing 5 μL SYBR Premix Ex Taq II (Tli RNaseH Plus 2×) (Takara), 0.4 μL forward primer, 0.4 μL reverse primer, 0.8 μL DNA and 3.4 μL sterile distilled water. The PCR was started with denaturation at 95°C for 5 min and 40 cycles of 95°C for 5 s and 60°C for 30 s. β-Actin was used as an internal reference. The relative expression levels were calculated according to the 2−ΔΔCT method [20].

Statistical analysis

The statistical analyses were performed using SPSS 17.0 software and the data are shown as mean values with standard deviation (±SD). Paired two-tailed t-test was used to identify significant differences between the glucose injection group and the control group. When p < 0.05, the differences between the two groups were considered statistically significant.

Results and discussion

Haematological and serum analyses

The results from the biochemical analyses of GLU, TC and TG are presented in Figure 1. Glucose injection significantly increased the serum GLU levels (p < 0.05). However, the glucose injection had no significant effects on the serum TC and TG levels in common carp (p > 0.05). These results are in agreement with previous reports that, in gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax) glucose injection increased the glucose levels in plasma [21]. In our study, the serum total protein and globulin levels were not significantly changed by glucose injection (p > 0.05), whereas the albumin level significantly increased (p < 0.05) (Figure 1), suggesting stimulation of immune activities.

Serum immune activities

The serum C3 and C4 levels are presented in Figure 2. There were no statistically significant differences between the glucose injection group and the control (p > 0.05). The IgA levels in the glucose injection group were significantly decreased compared to the control (p < 0.05). The IgG and IgM levels in serum (Figure 2) did not show statistically significant differences between the two groups (p > 0.05). The leucocyte counts were increased in the glucose injection group (Figure 2) but this was not statistically significant.
according to the two-tailed t-test ($p > 0.05$). Although C3, C4, IgG and IgM showed no significant difference comparing the two groups, IgA was decreased by glucose injection. The leucocyte counts were also increased by glucose injection. As reported before, blood glucose is related to insulin secretion [22]. Meanwhile, insulin regulates the immune activities [18,23]. Thus, it is reasonable to understand that the glucose injection triggered an immune reaction in the serum. These clues indicated that the increased level of glucose may selectively stimulate some serum immune parameters but not others.

### Changes of hepatic transcriptome

The high-throughput sequencing generated 61,635,965 reads and 50,517,328 clean reads after sequence trimming. De novo assembly obtained 45,191 unigenes with N50 of 1686 bp. Using FPKM analysis, 499 unigenes were identified as DEGs, including 249 down-regulated and 250 up-regulated genes in the glucose injection group (Figure 3(a)). The GO enrichment analysis indicated that several GO categories associated with glucose/glycogen process, fat process and energy metabolism process were enriched comparing the two transcriptomes in DEGs (Figure 3(b)). Response to external stimulus as a GO category was found enriched in DEGs (Figure 3(b)). The KEGG enrichment analysis showed that PPAR-signalling pathway, complement and coagulation cascades pathways were enriched in DEGs (Figure 3(c)).

In order to assess the results of DEGs from high-throughput sequencing, the mRNA relative expression of selected genes were analyzed by qPCR (Figure 4). The mRNA levels of redox-regulatory protein FAM213A-like, extracellular superoxide dismutase [Cu–Zn]-like and putative natural resistance-associated macrophage protein (NRAMP) were up-regulated in the glucose injection group, while 25-hydroxyvitamin D-1 alpha hydroxylase, vitamin D 25-hydroxylase and natterin-3-like were down-regulated by glucose injection.

The function of immune cells depends on glucose metabolism in organisms. Glucose transporter 1 is a key gene in glucose metabolism which plays a key role in glucose uptake in T cells [24]. This suggests that glucose metabolism is associated with immune activities. On the other hand, the liver mediates the immune activities and may suffer injury due to glycogen accumulation. The present study sequenced the transcriptome in the liver to reflect the RNA expression changes by glucose injection. It was not surprising that the glucose increase in the serum regulates glucose/glycogen processes, fat processes and energy metabolism.
metabolism processes. In addition, the analysis of the response to external stimulus from GO terms revealed the enriched DEGs. These results indicated that the acute glucose increase in the blood induced a response from the liver. The KEGG pathway analysis revealed that the PPAR-signalling pathway, complement and coagulation cascades pathways were enriched in DEGs. In these pathways, complement C3-S, complement C3-H1, complement C3-H2 and complement component C7-like were found stimulated in the glucose injection group. The up-regulated complement genes expression indicated that these activities were activated on the transcriptome level in the liver upon glucose injection. We suppose that this may due to the active glycogen synthesis in the liver. However, the acute increasing of glucose in serum could not affect the serum complement levels in such a short time.

FAM213A was reported down-regulated in pig white adipose tissue by lipopolysaccharide injection [25]. Therefore, the antioxidant FAM213A showed evidence of immune activation function. Extracellular superoxide dismutase [Cu–Zn]-like is a member of the SOD family, which has important anti-disease activity. NRAMP was involved in iron metabolism and resistance to pathogens [26]. The up-regulated expression of these immune genes determined by qPCR indicated immune activation after glucose injection stress. On the contrary, 25-hydroxyvitamin D-1 alpha hydroxylase, vitamin D 25-hydroxylase and natterin-3-like were down-regulated in the glucose injection group as determined by qPCR. Vitamin D is a modulator of the figure 3.

**Figure 3.** RNA-Seq analysis of differentially expressed genes in fish subjected to different treatment. (a) Differentially expressed genes between the glucose injection group and control. (b) Gene Ontology enrichment analysis of differentially expressed genes. (c) KEGG enrichment analysis of differentially expressed genes.
immune system [27, 28] and 25-hydroxyvitamin D-1 alpha hydroxylase functions in murine macrophages [29]. Vitamin D 25-hydroxylase in liver transforms vitamin D into 25-hydroxyvitamin D, which participates in vitamin D metabolism [30]. Based on these clues we speculate that glucose injection regulated the immune activities via vitamin D. The natterin family induces edema and cleaves kininogen-derived synthetic peptides [31]. Thus, the glucose injection might also participate in the control of kininogen-derived synthetic peptides and may regulate the immune response in the liver.

Conclusions
The present results gave clues about the response of common carp to glucose injection. Short acute stimulation by glucose injection increased the serum GLU and albumin levels. These stimulating effects implied that the immune system was regulated by blood glucose. Besides the serum changes, the hepatic gene expression was regulated by glucose injection. Several genes associated with the response to external stimuli, the PPAR-signalling pathway, the complement and coagulation cascades pathways were identified by KEGG enrichment analysis. The qPCR results also confirmed the gene expression changes indicated by the transcriptome analysis. The present study adds information about the mechanisms underlying the response to increased glucose in the blood regulating the immune activities.

Disclosure statement
No potential conflict of interest was reported by the authors.

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