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Transcriptomic responses to low temperature stress in the Nile tilapia, *Oreochromis niloticus*

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ABSTRACT

The Nile tilapia, *Oreochromis niloticus*, is a species of high economic value and extensively cultured. The limited stress tolerance of this species to a low temperature usually leads to mass mortality and great loss. Nevertheless, there is limited information on the molecular mechanisms underlying the susceptibility to low temperature in the tilapia. In this study, tilapia was treated at 28 °C to a lethal temperature of 8 °C by a gradual decrement. Transcriptomic response of the immune organ, kidney, in tilapia was characterized using RNA-seq. In total, 2191 genes were annotated for significant expression, which were mainly associated with metabolism and immunity. Pathway analysis showed that immune-related pathways of phagosome and cell adhesion molecules (CAMs) pathway were significantly down-regulated under low temperature. Moreover, ferroptosis, a significantly changed pathway involved in tissue damage and acute renal failure, is reported here for the first time. The levels of serum parameters associated with kidney damage such as urea and uric acid (UA) increased significantly under low temperature. The immunofluorescence staining of the kidney showed that cell apoptosis occurred at low temperature. The results of the present study indicate that exposure to low temperature can cause kidney disfunction and down-regulate the immune-related pathway in the kidney of tilapia. This study provides new insight into the mechanism of kidney damage in fish under low temperature.

1. Introduction

Water temperature is an essential extrinsic factor that influences the physiology and behavior of aquatic organism [1]. As ectotherms, fish can cope with nature temperature, such as daily or seasonal temperature fluctuations in their aquatic environments. However, fish suffer low temperature stress when the temperature decreases to exceed their thermal tolerance capability, which may result in mortality [2]. Several authors have reported that the immune functions of fish are influenced by variances in temperature [3,4]. Several species of fish have evolved several adaptive mechanisms to cope with cold stress to protect them against damage, for instance, Antarctic notothenioid [5]. Several reports have characterized the transcriptional responses elicited by cold stress in a number of fish species, and the results reveal that a large number of cold-regulated genes involved in a variety of biological processes are associated with acclimation to both daily and seasonal low temperatures [6]. Nonetheless, there are quantities of fish which are not able to survive the winter, for example, the tilapia.

Belonging to the Cichlidae family, tilapia (*Oreochromis niloticus*) is one of the most important commercial freshwater fish in aquaculture worldwide due to its fast growth rate, relatively low production cost and high tolerance to adverse conditions. Tilapia is widely cultivated in Guangdong, Guangxi and Fujian provinces in China, which belong to the torrid and subtropical zones. Tilapias can grow up between 16 and 38 °C, with an optimum range between 25 and 28 °C [7], which suggested that they are unable to thrive at low temperatures [8]. Tilapia is unable to survive at temperatures lower than 10 °C [9], however, it is inevitable of large-scale tilapia farming to suffer the low temperature in winter. The coldest temperature in winter in Guangdong province can reach to 5 °C, and the average temperature is around 10 °C.

Until now, low temperature stress still limits tilapia's cultivation expansion and cause a great loss due to a mass mortality in winter

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[10,11]. Thus, how to improve the low temperature resistance of tilapia has become an important issue in aquaculture development. Previous research on the impact of low temperatures on the performance of tilapia has mainly concentrated on the effects on appearance, physiology, and biochemistry [12,13]. However, there have been few reports revealing the mechanism of tilapia coping with lethal temperature at the molecular level. To explore the contributions of gene expression patterns in the lethal temperature, our lab used to compare the transcriptional plasticity of the gill of tilapia at the lethal temperature (8 °C) [14]. Besides, the fish kidney is involved in numerous regulatory processes including fluid and electrolyte balance (osmoregulation), control of blood pressure and volume, excretion of nitrogenous wastes, and acid-base balance. Moreover, in fish, the kidney is very important for hematopoiesis and immunity and can be compared to the bone marrow of mammals. It plays a role in the formation and maturation of erythrocytes and leukocytes, including B lymphocytes, granulocytes, macrophages, and monocytes [15–17]. Thus, as an important organ in fish, kidney response to lethal temperature in tilapia is suitable to elucidate the mechanism of this species' adaption to low temperature.

High-throughput sequencing technology of transcriptome provides a new approach to identify fish gene expression variance under low temperature. In the present study, we examined the transcriptome responses of the *O. niloticus* kidney, after a low temperature challenge. RNA sequencing was performed by using the Illumina sequencing technology, and the sequencing reads were mapped to the *O. niloticus* reference genome. Subsequently differentially expressed genes (DEGs) were studied using heatmap analyses of hierarchical clustering, gene ontology (GO) functional enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and EuKaryotic Orthologous Groups (KOG) classification. The objective of this study was to identify the kidney response to low temperature in the tilapia and to investigate the molecular mechanisms involved in its susceptibility to this type of stress, to extend our understanding of its adaptation to environmental challenges.

2. Materials and methods

2.1. Experiment design and sampling

Tilapia (*O. niloticus*) were bought from Weiye tilapia factory (Maolin city, Guangdong province), and reared at 28 °C for at least 5 generations under laboratory conditions. They were fed 4% of their body weight on the same commercial diet daily. For cold treatment, six-month-old tilapias were subjected to a low temperature in a temperature-adjustable cultivation chamber (Ningbo Jiangnan Instrument Factory, Ningbo, China). The temperature gradually decreased from 28 °C to 8 °C in 36 h at a rate of ∼0.56 °C/h and was then maintained at 8 °C for 6 h (Fig. 1). Fish groups were named as C, A, and B according to the three time points chosen (28 °C, 8 °C/0 h, and 8 °C/6 h respectively) and the C group was selected as the control one. The kidney samples were collected at these three time points, flash frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction.

2.2. Hematological parameters analysis

In two groups (B and C), at least ten fish from each tank were randomly sampled and blood samples were taken from the caudal vein using a 1 ml syringe and allowed to clot at 4 °C for 4 h. Serum was obtained by centrifugation at 3000*g* for 5 min (4 °C) and then stored at −20 °C until use. A BS-200 automatic biochemical analyzer (Mindray, Shenzhen, China) was used to measure the serum biochemical parameters using the corresponding Mindray Kit, based on methods used by Ibrahim et al. (2008) [18]. The activity of alanine transaminase (ALT),

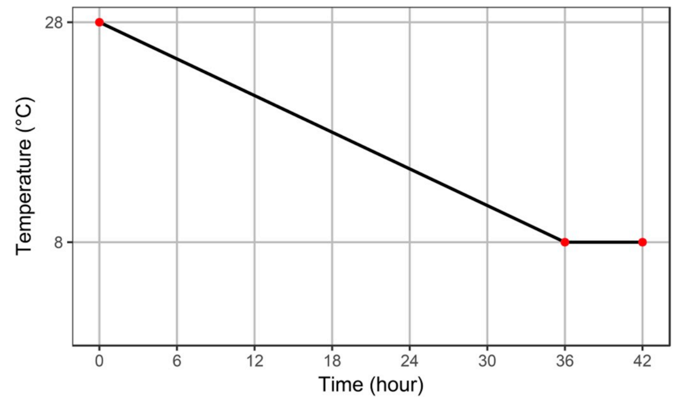

Fig. 1. Schematic diagram showing the cold treatment and the sampling regimes. The fish were subjected to the following two temperatures: 28 °C and 8 °C. The red dots indicate the sampling time points. At each point, 2 fish were sampled, and RNA from kidney was isolated and pooled for RNA-Seq. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

aspartic transaminase (AST), lactate dehydrogenase (LDH) was measured as well as the concentration of non-protein nitrogen (NPN) including blood urea nitrogen (UREA) and uric acid (UA). The concentration of blood lipids triglyceride (TG) and serum glucose (GLU) were also quantified. The biochemical data were analyzed by one-way ANOVA using SPSS for windows version 22.0 (SPSS, inc., USA) and expressed as mean ± standard error. LSD and Duncan's multiple range tests were applied to determine significant differences between all groups. A p-value of less than 0.05 was considered statistically significant and marked as "*"; those treatments less than 0.01 were marked as "**".

2.3. RNA isolation and sequencing

Total RNA was extracted from the kidney sample using TRIzol Reagent according to the manufacturer's protocol (Invitrogen, USA). The RNA quality of each sample was assessed on an Agilent Bioanalyzer Chip RNA 7500 series II and its concentration was determined by a Qubit fluorometer. We used three micrograms of RNA from each sample to prepare the mRNA-Seq library with the TruSeq RNA Sample Prep Kit (Illumina) following the manufacturer's instructions. Proper index codes were used to attribute sequences to individual samples. Briefly, poly(A)+ RNA was purified and fragmented using divalent cations at elevated temperature. RNA fragments were converted to cDNA using random primers, followed by second-strand cDNA synthesis and end repair. Illumina PE adaptors were attached to the cDNA ends. Fragments that were approximately 300 bp in length was extracted from a 2% low-range ultra-agarose sizing gel. Adaptor-tagged cDNA fragments were enriched using the manufacturer's cocktail and 10-cycle PCR. The library quality and insert length were checked using the DNA High Sensitivity DNA Kit (Bioanalyzer 2100, Agilent) to ensure the proper insert size of 300–500 bp. The libraries were diluted to 10 pM, and equal amounts of 3 distinctively indexed libraries were mixed and subjected to 109 cycles of paired-end (2 × 109 bp) sequencing on one lane of an Illumina HiSeq 2000 system. A total of 3 libraries were separately generated from the kidneys of tilapia in three groups (A, B, and C) using the same protocol.

2.4. RNA-seq reads processing and mapping

Raw reads were initially cleaned by removing the low-quality ones and adaptor sequence (Sanger base quality < 20) using Trimmomatic v0.32 [19]. Clean reads were mapped to the *Oreochromis niloticus* reference genome (http://asia.ensembl.org/info/data/ftp/index.html) using HISAT v2.0.4 with default values and the reads from each sample were mapped separately [20].

2.5. Annotation

Gene and function annotations of the Nile tilapia were performed based on the existing data of *O. niloticus* from the ensembl databse (http://.asia.ensembl.org/info/data/ftp/index.html). Protein sequences of tilapia's total genes were downloaded from it and then compared with public databases for homology annotation, including the NCBI nonredundant protein (Nr), Swiss-Prot (http://.www.ebi.ac.uk/.uniprot/), and Clusters of Orthologous Groups (COG) (http://www.ncbi.nlm.nih.gov/COG/) by using the BLASTP algorithm, with an E-value cut-off of 10^{-5}. Tilapia's cDNA sequences were also download from the ensembl database and then compared with nonredundant nucleotide (Nt) databases (http://www.ncbi.nlm.nih.gov/) by using a the BLASTX algorithm, with an E-value cut-off of 10^{-5}. GO annotation was downloaded from the ensembl database after running BioMart browser sessions. KEGG annotation were also performed by putting the genes'corresponding coding sequence (downloaded from ensembl databse) onto the KASS-KEGG Automatic Annotation Sever (http://www.genome.jp/tools/kaas/).

2.6. Different expression analysis and enrichment analysis

First, we sort the bam files of the aligned reads by read name using SAMTools v1.3.1 [21]. The HTSeq v0.6.1 [22] was then used to count the number of reads that were mapped to the genes. A differential expression analysis was performed by importing the read counts of each sample into the R package edgeR [23]. We compared the gene expression level of treated groups (A and B) with the control group (C). To assess the significance of differential gene expression, the FDR threshold was set at ≤0.05 and the absolute value of log2 ratios (fold change between treatment group (A and B) and the control group (C)) at ≥1 [24,25]. After differently expressed genes (DEGs) were obtained, the function enrichment of gene ontology terms (GO, http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp.kegg) pathway were performed. Enrichment analysis was performed via a hypergeometric test, pathways with p-values ≤ 0.05 were considered to be significantly enriched in DEGs.

2.7. Real-time RT-PCR confirmation of Illumina sequencing data

To validate our Illumina sequencing data, 16 differentially expressed genes in two comparisons (C vs A, C vs B) were selected for quantitative RT-PCR (RT-qPCR) analysis, using the A, B and C groups RNA samples as transcriptome profiling. The primers were designed with the PrimerQuest tool on the sg.idtdna.com. The β-actin gene was chosen as the internal control for the qPCR analysis. Two micrograms of RNA from each sample were reverse-transcribed to cDNA using an RT-PCR kit (PrimeScript™ RT reagent Kit, Takara). All primers used were listed in Table 1. A qPCR analysis was conducted in a CFX96 Touch™ Real-Time PCR Detection System. PCR reactions were exposed to an initial denaturation (95 °C for 4 min), followed by 39 cycles of denaturing at 95 °C for 30 s, annealing at 60 °C for 20 s and extending at 72 °C for 30s in a 25 μL reaction mixture containing 1 μL of each primer (10 μM), 2 μL first-strand cDNA as a template, 12.5 μL Roche FastStart Universal SYBR Green Master (Rox) and 8.5 μL water. The relative transcript abundance was obtained by normalizing with the *O. niloticus* β-actin gene expression based on the $2^{-\Delta\Delta CT}$ method [26] and all data were given in terms of relative mRNA expression.

2.8. Detection of apoptosis

Paraffin sections were used for immunofluorescence staining of tilapia kidney from three groups (A, B and C). The kidney sections were deparaffinized in xylene and subsequently treated with sodium citrate buffer (pH 6.5) at 95 °C for 5 min. TUNEL staining was performed using a commercially kit (TUNEL FITC Apoptosis Detection kit, Vazyme, A11103). Then, the kidney sections were counterstained with DAPI (500 ng/ml) for 5 min and analyzed under a laser confocal microscope (LSM 710; Carl Zeiss, Jena, Germany).

3. Results

3.1. Serum parameters in tilapia at different temperatures

The plasma ALT activity of fish from group B was significantly higher than in the control group (C) (P < 0.01). The same happened with plasma AST activity. The plasma UREA and UA concentration were significantly higher (P < 0.01) in group B compared with the control group (Table 3). Similarly, the concentration of GLU increased significantly in B group. On the contrary, there were no significant differences in the activities of LDH and TG between the B and C groups.

3.2. Transcriptome profiles and annotation

A total of three cDNA libraries were sequenced by Illumina HiSeq2000. The sequencing totally generated 80,799,151 raw reads, which were deposited to the National Center for Biotechnology Information (NCBI) with the accession number of SRP147966. Raw reads were trimmed by removing adaptor sequences and low-quality sequences. Approximately 8.4–11.3 M clean reads were obtained from each library. The uniquely mapped percentages of these transcripts

Table 1
List of primers used for quantitative RT-PCR validation.

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon length (bp)
pnp4a	TGTGGACGCTGTTGGTATG	CTGTGTCCTCGTAACTCTTCAC	119
pnp5a	AATCCTCTGAATGGACCCAAC	GAAATCTCCATAGCCCAGATCC	126
nme2b.2	CTACGCTGGACTCTGCAAATA	GGTCTCACCCAGCATCATC	103
atic	GCTTCAGATGGATCCTGACTAC	CCTTATCGATACGTCCTCCATTC	101
gucyla3	AACTGAGACGGCGGATTTAC	TTGGCTCTGCTTGTCTTCTT	90
nt5c2a	CTCCTGAGTCGGATGAATGAAG	GACCATGAGGAAGGTCAAACA	106
adar	GGGATGGTGCTCTGTTTGATA	CCCTCACCATTCTCCACTTTAG	121
xdh	GACTCATGGAGGGACTGAAATG	GTTGGTGCTGGTCTCTGATATG	115
entpd1	CTGTCAACTTCCGACTCTATGG	GCACCTGACTGAGTTTGATTTG	115
ENPP3	CTCATCCCTGTCCTGGATAAAC	GAGACCCGTAACAATGGAGTAG	111
β-actin	GATCTGGCATCACACCTTCTAC	TCTTCTCCCTGTTGGCTTTG	104

Table 2
The raw, total clean, total mapped, unique mapped and multiple mapped reads obtained by RNA-seq analysis of tilapia in different groups.

Index	Group I		Group II		Group III	
	C-1	C-2	A-1	A-2	B-1	B-2
Raw reads	16141697	14312728	13432598	14628707	10770909	11512512
Total clean reads	13911595	12654527	11681001	12801003	9569352	10137803
Total mapped reads	11349265 (81.58%)	10220790 (80.77%)	9645871 (82.58%)	10527242 (82.24%)	7903482 (82.59%)	8250762 (81.38%)
Uniquely mapped	9756833 (70.13%)	8722548 (68.93%)	8338995 (71.39%)	9111512 (71.18%)	6823981 (71.31%)	7120269 (70.23%)
Multiple mapped	1592432 (11.45%)	1498242 (11.84%)	1306876 (11.19%)	1415730 (11.06%)	1079501 (11.28%)	1130493 (11.15%)

Table 3
Hematological parameters of tilapia under different temperature.

Parameters	C	B
UREA (mmol/L)	0.567 ± 0.28386	1.0571 ± 0.30177**
UA (μmol/L)	11.64 ± 4.42799	44.3429 ± 36.47574**
AST (U/L)	38.69 ± 28.8596	124.8571 ± 78.0826**
ALT (U/L)	10.93 ± 4.77797	54.2857 ± 29.55958**
GLU (mmol/L)	2.663 ± 0.48908	8.6743 ± 1.64269**
LDH (U/L)	470.37 ± 365.9242	625.7857 ± 476.64721
TG (mmol/L)	2.131 ± 1.62924	2.3479 ± 1.39199

Values (mean ± S.E., n ≥ 10), "*" indicates significant difference ($P < 0.05$), "**" indicates significant difference ($P < 0.01$). UREA: blood urea nitrogen; UA: uric acid; AST: aspartic transaminase; ALT: alanine transaminase; GLU: Glucose; LDH: lactate dehydrogenase; TG: triglyceride.

ranged from 68.93 to 71.39%, while the percentage of multiple mapped transcripts ranged from 11.06 to 11.45% (Table 2). Total of 27088 tilapia genes were annotated by matching them against the Nr, Nt, swissport and COG database; 26705 genes were matched to the Nr database, 26709 genes were matched to the Nt database, 26132 genes were matched to swissport database and 20966 genes were matched to the COG database.

3.3. Differently expressed genes

During the RNA mapping, at least 80.77% of the reads were aligned to the tilapia reference genome and the relative abundance of each gene from the three related libraries was estimated using HTSeq. According to the gene data, statistical analysis performed with edgeR package, the differentially expressed genes between the control group (C) and treatment groups (A, B) were identified. Compared with the control group, there were 1344 DEGs, of which 529 were upregulated and 815 were downregulated in A group. In the B group, 1748 DEGs were identified, 620 of them were upregulated and 1128 were downregulated. A substantial overlap was observed between DEGs in different comparisons (Fig. 2), 901 transcripts were significantly differentially expressed along the entire experiment. Of these 901 DEGs, 900 DEGs were expressed similarly (up- or down-regulated) in different comparison. The response to low temperature was strong not only in terms of total number of DEGs but also in the degree of transcriptional changes. A total of 532 DEGs in A group (179 over- and 353 under-expressed) and 778 DEGs in B group (315 over- and 463 under-expressed) showed large FCs ($\log2FC \geq 2$). The volcano-plot and Venn diagrams results of the DEGs are shown in Fig. 1. Hierarchical clustering based on the gene expression patterns was performed by selecting the union set DEGs (2191) (Fig. 3). The global gene expression profile of tilapia in the treatment group (A & B) showed large differences when compared with the control group (C) under low temperature stress conditions.

3.4. Gene Ontology(GO) and KEGG pathway enrichment

To better understand the functional relevance of the DEGs, GO enrichment and KEGG pathway enrichment analysis were performed by using the corresponding annotations of genes. GO enrichment was conducted for the two comparisons. In the comparison group of A/C, 1032 of 1344 DEGs could be assigned by GO classification, and the equivalent numbers for other comparison groups were 1328 of 1749 DEGs (B/C). The top two significant annotated GO biological process pathways in C vs A was oxidation-reduction process (GO:0016787) represented by 60 DEGs and proteolysis (GO: 0006508) represented by 59 DEGs, whereas in C vs B was oxidation-reduction process (GO:0055114) represented by 64 DEGs and metabolic process (GO:0008152) represented by 49 DEGs. The classification of DEGs is shown in Fig. 4. A portion of immune-related GO terms such as immune response, innate immune response and antigen processing and presentation of endogenous peptide antigen via MHC class I was significantly enriched in the biological processes in C vs A or C vs B and the majority of DEGs in these terms were downregulated. The classified genes (C, A and B group) resulted in 337 different pathways, including metabolism, genetic information processing, environmental information processing, cellular process, organism systems and human diseases. In the comparison of A/C, 554 of 1344 DEGs were assigned to 149 pathways. In the comparison of B/C, 698 of 1749 DEGs were assigned to 148 pathways. The top two significantly enriched KEGG pathways in the comparison C vs A were fatty acid elongation signaling pathway (ko 00062) and phagosome signaling pathway (ko 04145). The top two significantly enriched pathways in the comparisons C vs B were cell adhesion molecules (CAMs) pathway (ko 04514) and phagosome signaling pathway (ko 04145) (Table S1). According to the KEGG function annotations, a total of 18 and 18 significantly enriched pathways (P-value < 0.05) were identified at C vs A and C vs B (Fig. 5). The 'Biosynthesis of unsaturated fatty acids', 'Fatty acid biosynthesis', 'Fatty acid elongation', 'Fatty acid metabolism' pathways that involved in lipid metabolism were predominant in both two pairwise comparisons. Immune-related pathways including 'Cell adhesion molecules (CAMs)', 'Phagosome', 'Ferroptosis', 'Cytosolic DNA-sensing signaling', 'Apoptosis', 'necroptosis' and 'cell senescence' were significantly enriched in both comparisons. Besides, other pathways including 'Glycine, serine and threonine metabolism', 'Peroxisome', 'PPAR signaling pathway' are included in C vs A or C vs B. Here, the phagosome pathway (Fig. 6) and CAMs pathway (Fig. S1) showed an overall down-regulation in C vs A and C vs B. Genes encoding CR1(C1R), CR2(ITGAM, ITGB2), MHCI (MHC1), MHCII(MHC2) and TAP(ABCB2, ABCB3) involved this two pathways showed reduced expression in both A and B groups (Table S1).

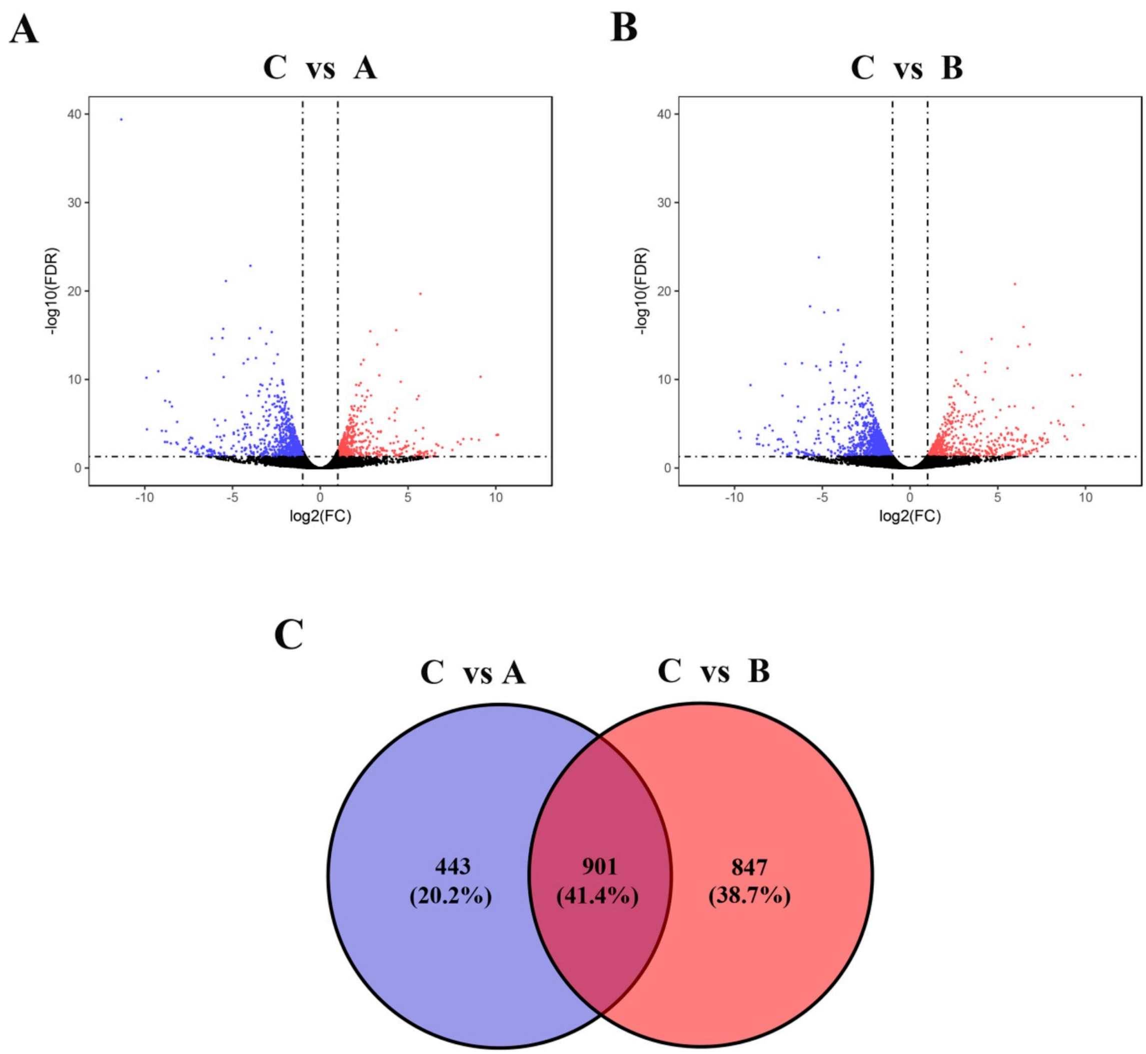

Fig. 2. Volcano Plot of differently expressed genes distribution trends among C vs A and C vs B. The $\log_2$ (A/C) indicates the mean expression level for each gene. Each dot represents one gene. Blue and red dots represent differentially expressed genes (DEGs). Black dots represent non-differentially expressed genes. (B) Volcano Plot of the distribution trends of differentially expressed genes between C and B. (C) Venn diagrams showing the overlap of DEGs between different time points comparison C vs A (blue) and C vs B (red). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. EuKaryotic Orthologous Groups (KOG) classification

A KOG analysis was performed to provide a deeper understanding of the functions of the DEGs. Total of 2191 DEGs were classified into 26 functional categories. The category 'General function prediction only' contained the largest number of DEGs (508, 20.51%) (Fig. 7), followed by the 'signal transduction mechanisms' cluster (478, 19.30%) and the 'posttranslational modification protein' cluster (223, 9.00%). The categories of greatest interest in this study were posttranslational modification, protein turnover, and chaperones (223, 9.00%), cytoskeleton (79, 3.19%) and energy production and conversion (24, 0.97%), as the genes in these categories are probably related to energy production and conversion, protein-domain-specific binding and cell signaling pathways. The majority of DEGs in energy production and conversion showed reduced expression in both A and B groups (Table S2).

3.6. Validation of RNA-Seq results with RT-qRCR

The Real-time PCR outcomes were further summarized. The 16 DEGs qPCR results, of C vs A and C vs B, are shown in Fig. 8. Grouped comparison results between qPCR and RNA-Seq were also displayed, and the results showed that all the 16 candidate genes in qPCR verification agree with the results of the RNA-Seq technology.

3.7. Apoptosis in tilapia kidney

As it is shown in Fig. 9, apoptosis occurred at two different time points, corresponding to the A and B group, and the level of apoptosis in the group B was higher than in the group A.

4. Discussion

As one of the most important environmental factors for aquatic ectotherms, the temperature has been investigated for its key role in affecting overall biological processes in various species, including zebrafish (*Danio rerio*) [27,28], common mussel (*Mytilus edulis*) [29], Manila clam (*Ruditapes philippinarum*) [6] and so on. The physiologies and lethal temperature of fishes vary a lot due to their wide distribution [30]. Cold-water and eurythermal fish tend to be relatively well adapted to lower temperture. For example, rainbow trout (*Oncorhynchus mykiss*) which thermal tolerance range between 0 and 25 °C was studied under 4 and 18 °C [31], common carp (*Cyprinus carpio*) was subjected to graded cooling regimes (23 °C, 17 °C, or 10 °C) and compared the expression profiles to control (30 °C) temperature [32].

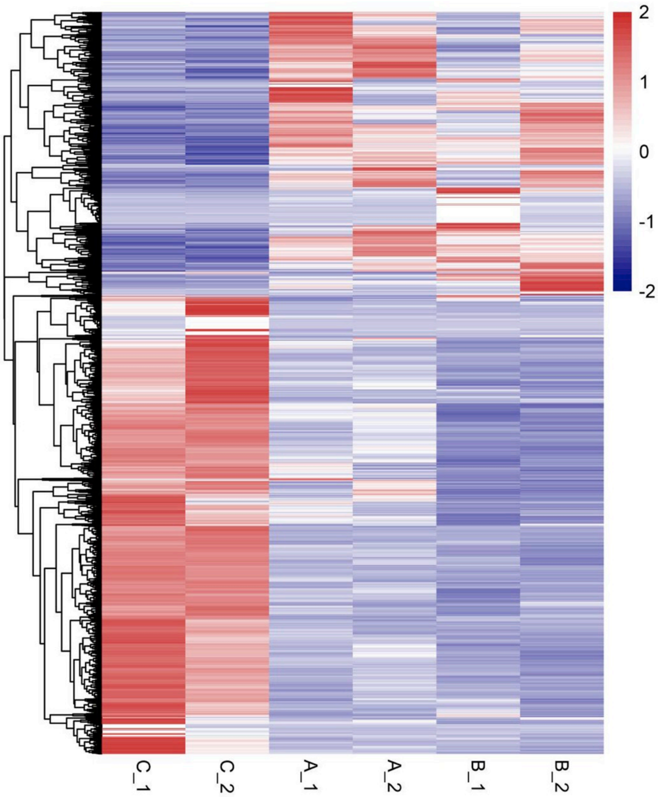

Fig. 3. Hierarchical clustering of differentially expressed (DE) mRNAs among the 6 libraries. Heatmap of the count data for DE mRNA libraries for the differentially expressed genes between C, A and B groups.

Channel catfish (*Ictalurus punctatus*) which adapt to a wide range of temperature changes (0–36 °C) was compared differential gene expression at 12 °C and 24 °C [33]. The species-specific thermal tolerance range differently in fishes. Some temperate zone fish is particularly sensitive to cold. For instance, prolonged exposure to 7.5 °C caused 100% mortality in yellow drum (*Nibea albiflora*) which was usually acclimated at 18 °C [34], and comparing to the lethal temperature of 5 °C, gilthead sea bream (*Sparus aurata*) normally acclimate at 16 °C [35]. Therefore, investigation with RNA-Seq analysis of the transcriptional response of the yellow drum exposed to two temperature regimes of 18 °C and 7.5 °C [34], and DNA microarray analysis of liver transcriptome was carried out at different time points during exposure of gilthead sea bream to 16 ± 0.3 °C and 6.8 ± 0.3 °C [35]. For the warm-water farmed fish, such as Nile tilapia which optimum temperature range between 25 and 28 °C, over-winter mortality is a main reason of economical loss in cold temperature. Therefore, our study had been focused on the gene expression patterns and regulatory mechanisms after exposed to its lethal temperture (8 °C).

In the present study, the functional classification and annotation of differential genes were performed using GO, KEGG and KOG analyses. These gene categories were mainly associated with metabolism and immunity. The metabolism in ectothermic fish is highly dependent on the environmental temperature during stressful conditions [36]. As shown in Fig. 4, under the biological processes, a higher number of GO terms were assigned to metabolic process. Besides, pathway analysis (Fig. 5) also showed that metabolic pathways such as the metabolism of amino acids, carbohydrates, and lipids represented the most abundant pathways, which is similar to the yellow drum under cold stress [34]. Purine metabolism is closely related to amino acids metabolism and correlated with the production of UA in fish. The up-regulation of purine metabolism may be due to physiological adjustments to remove excess ATP generated by the creatine kinase transgene [37]. Carbohydrates is the main source for aerobic energy production. Temperature-related changes in gene transcripts of metabolic enzymes suggest that capacity for glycolytic energy production from carbohydrates might be increased [31]. Besides, lipid metabolism involving the break down or storage of fats for energy, High levels of available energy are required for the tilapia during thermal stress, Therefore, the enriched of carbohydrates and lipid metabolism indicated that tilapia might resist cold by altering energy metabolism. The regulation of metabolic processes is an important part of temperature response in fish. However, low temperature decreases the growth rate and metabolism [38], which may accentuate mortality and lead to a variety of sublethal consequences (eg., compromised immune function, organ functions disordered) in fishes with limited energetic resources. Moreover, the result of KOG function classification showed that only 24 (0.97%) DEGs were involved in the category of "energy production and conversion", most of which (19 DEGs) were down-regulated. It might be less production and conversion of energy in the tilapias under low temperature.

Cold stress could cause immunosuppression in mice [39], fishes [35,37,40,41] and mollusks [42–44]. Proper functioning of the immune system is highly dependent on the adequate regulation of the metabolism. However, the regulation of metabolism in fish is affected by low temperatures, and cold stress causes a decline in fish immunity. It is showed that low temperature can regulate the levels of cortisol, epinephrine, catecholamine, norepinephrine, and epinephrine agonists in the blood, thereby inhibiting phagocytosis of white blood cells and lowering antibody levels [40]. In the present study (Fig. 6 and Fig. S1), the overall down-regulation of genes present in the immune-related pathways (eg., phagosome, cell adhesion molecules, ferroptosis and apoptosis) may suggest the immune suppression by low temperature.

Phagosome formation is crucial for tissue homeostasis and both innate and adaptive immune response against pathogens. The present study (Fig. 6) showed an overall down-regulation of phagosome pathway suggested the phagocytosis in kidney was suppressed under low temperature stress. This is consistent with the observation of Nile tilapia that phagocytic activity decreased significantly when transferred from 25 °C to 12 °C [40]. Certain genes (eg., MHCI, MHCII, TAP, CR1, CR3) related to the phagosome showed reduced expression in both group A and B. These genes participate in the vertebrate immune system to detect intracellular pathogens, function in phagocytosis, and to destroy the infected cells, which influence by immune-related stimulation. Such as MHCI from rainbow trout was up-regulated after infection with infectious haematopoietic necrosis virus (IHNV) [45]. It was implied that the immune function in the kidney of Nile tilapia might have been inhibited by cold stress. However, despite most of the down-regulated DEGs, two genes including TUBA and CD36 were up-regulated in B group (Fig. 6B). The alpha-tubulin (TUBA) and beta-tubulin (TUBB) share 40% amino-acid sequence identity, which are important component of microtubules and constitute the cytoskeleton of cells [46]. Evidences exist to show that cold adaptation of Antarctic fishes is based in part on expansion of TUBA gene families [47]. It is possible that in order to ensure the efficient synthesis of tubulin polypeptides during cold stress, the expression of TUBA in Nile tilapia increased. CD36 is indispensable for thermogenesis under conditions of cold stress [48]. To survive potentially life-threatening cold stress, CD36 was important to Nile tilapia and therefore up-regulated in our research.

Cell adhesion molecules (CAMs) are glycoproteins expressed on the cell surface and play a critical role in a wide array of biological processes including hemostasis, the immune response, inflammation, embryogenesis, and development of neuronal tissue. In fully developed animals, these molecules play an integral role in creating force and movement and consequently ensure that organs are able to execute their functions. Often, the aberrant expression of CAMs will result in pathologies ranging from frostbite to cancer [49]. In overall, the cell adhesion molecules (CAMs) pathway presented down-regulation in

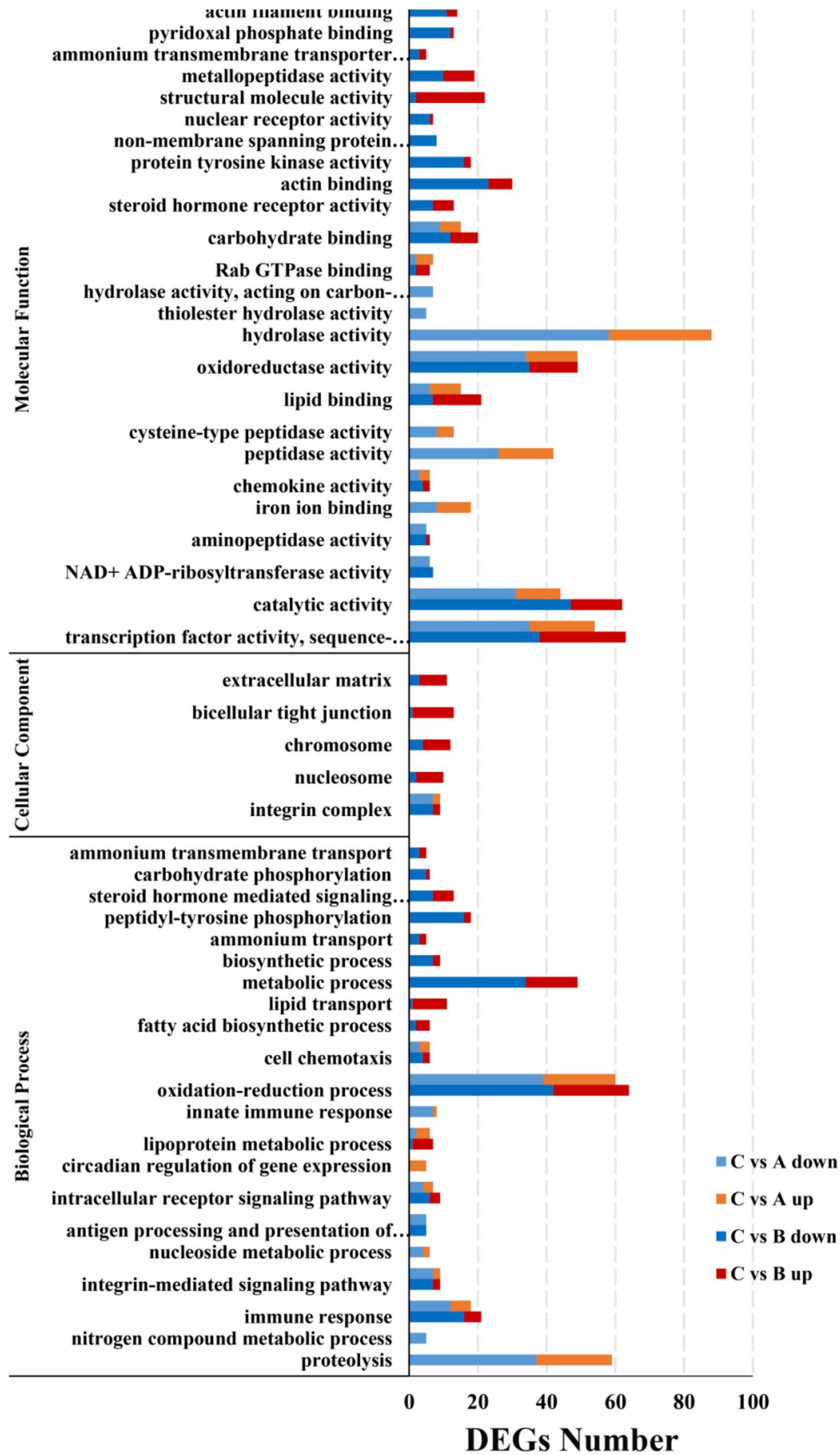

Fig. 4. Gene Ontology functional classification of DEGs. The expression level of A, B was compared with control group (C) and the numbers of significant putative DEGs (either up or down) were shown in x-axis; the Gene Ontology functional classification of putative DEGs was shown in the y-axis. Up-regulated and down-regulated genes were represented by red and blue color. C, 28 °C; A, 8 °C/0 h; B, 8 °C/6 h.

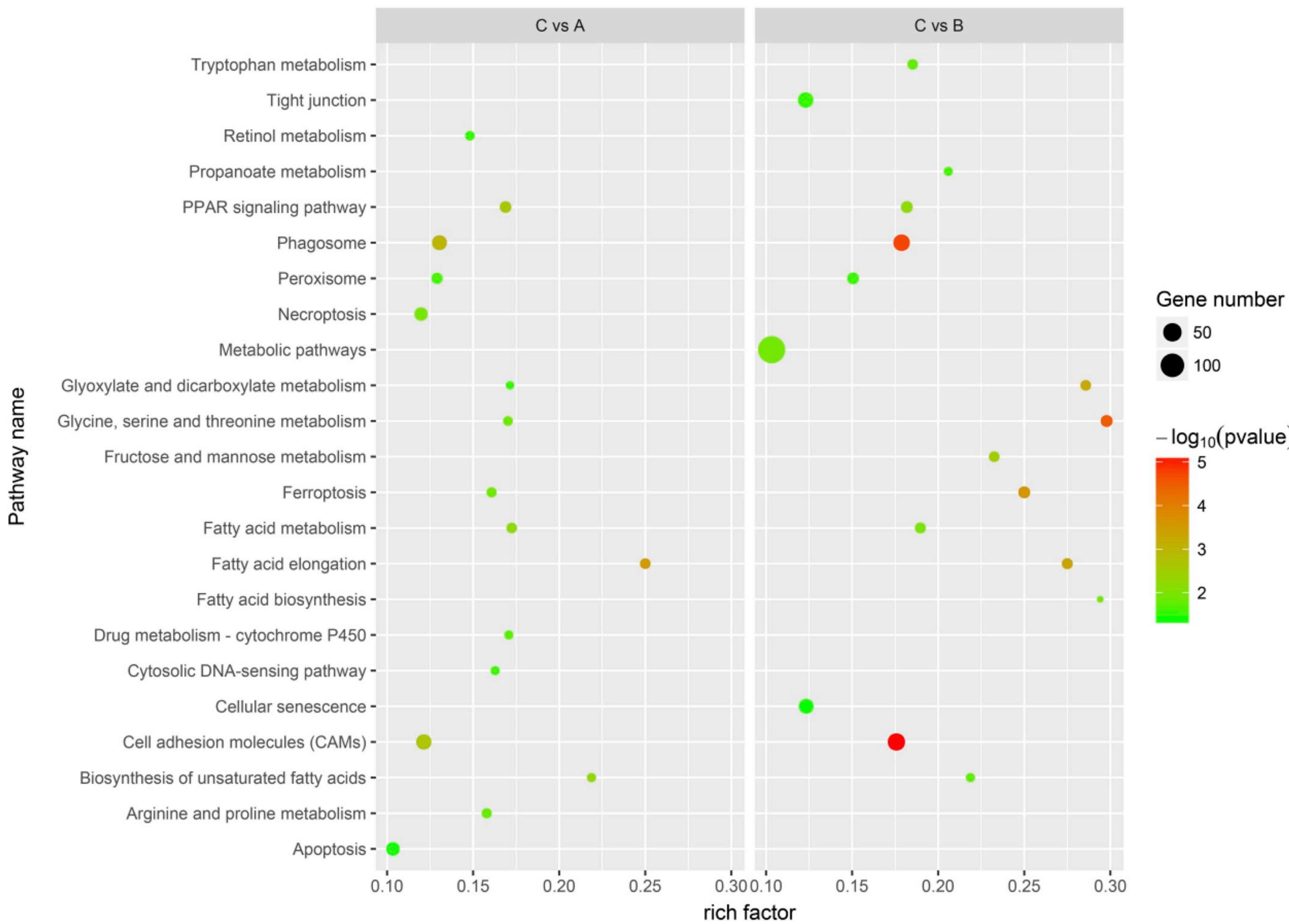

Fig. 5. KEGG enrichment analysis scatter plot representing pathways of significant DEGs in response to low temperature in A and B group. Red color indicates highly significant enrichment according to P-value range. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

both A and B groups, the DEGs from the immune system portion of this pathway were all down-regulated. The decreased expression of immune-related genes and downregulation of phagosome and CAMs pathways in this study suggested cold stress could cause immunosuppression.

Ferroptosis is a regulated form of cell death and it is characterized by a production of reactive oxygen species (ROS) from accumulated iron and lipid peroxidation, it is involved in multiple physiological and pathological processes, such as cancer cell death, neurodegenerative disease, tissue damage and acute renal failure [50–53]. The sensitivity to ferroptosis is tightly linked to numerous biological processes, including amino acid, iron, and polyunsaturated fatty acid metabolisms, and the biosynthesis of glutathione, phospholipids, NADPH, and coenzyme Q10. In our experiment, ferroptosis pathway was enriched with several genes upregulated in C vs A, such as glutamate–cysteine ligase regulatory subunit (GCLM) and acyl-CoA synthetase long-chain family member 1 a (ACSL1A). By contrast, genes encoding NADPH oxidase 2(NOX2), heme oxygenase 1(HMOX1), SLC7A11 showed reduced expression in C vs B. Quite recently, Doll et al. reported that ACSL4 (the orthologous protein of ACSL1A) drives ferroptosis via the accumulation of oxidized cellular membrane phospholipids [50]. Furthermore, transcriptional inhibition of cystine-glutamate antiporter, SLC7A11, by mutant p53 enhances ferroptosis [51]. It is likely that low temperature stress leads to damage in the kidney, which may cause a deficiency in renal functions such as filtration and excretion of nitrogenous wastes.

Blood is a pathophysiological indicator of body health, and the hematological parameters give an indication of any abnormality under environmental stress [54]. Changes in serum enzyme activities in the blood often directly reflect cell damage in specific organs [55]. ALT and AST exist mainly in the liver, and when the liver is stressed or damaged these enzymes can be released into the blood. Therefore, high activities of these enzymes may be an indicator of liver impairment [56]. In this study, the 42 h lethal timepoint (8 °C/6 h) of the cold treatment was selected (fish began to die) to examine the serum parameters of Nile tilapia. Our results indicate that the liver of this species may be affected by low temperatures and that ALT is more sensitive to these conditions when compared with AST. UREA and UA are two important components of NPN in serum, and changes in their concentrations may indicate changes in the kidney function [38]. The concentrations of UREA and UA elevated significantly in the Nile tilapia occurred at 8 °C/6 h (Table 3). The changes in the concentrations of these two components that occurred as temperature decreased may indicate the minor degree of kidney damage under low temperature. Cell damage can occur as a result of an adverse stimulus which disrupts the normal homeostasis of affected cells [57]. Among other causes, this can be due to physical, chemical, infectious, biological, nutritional or immunological factors. Apoptosis can be used as an indicator of cellular damage [58,59]. Cold stress (10 °C) caused apoptosis in cells of a warm-water fish Colossoma brachypomum [60] and zebrafish [61], which was similar to our research that apoptosis occurred (Fig. 8) and the apoptosis pathway was significantly enriched (Fig. 5) in tilapia kidney after low temperature exposure. Kidney cell death occurs when the severity of the cold stress

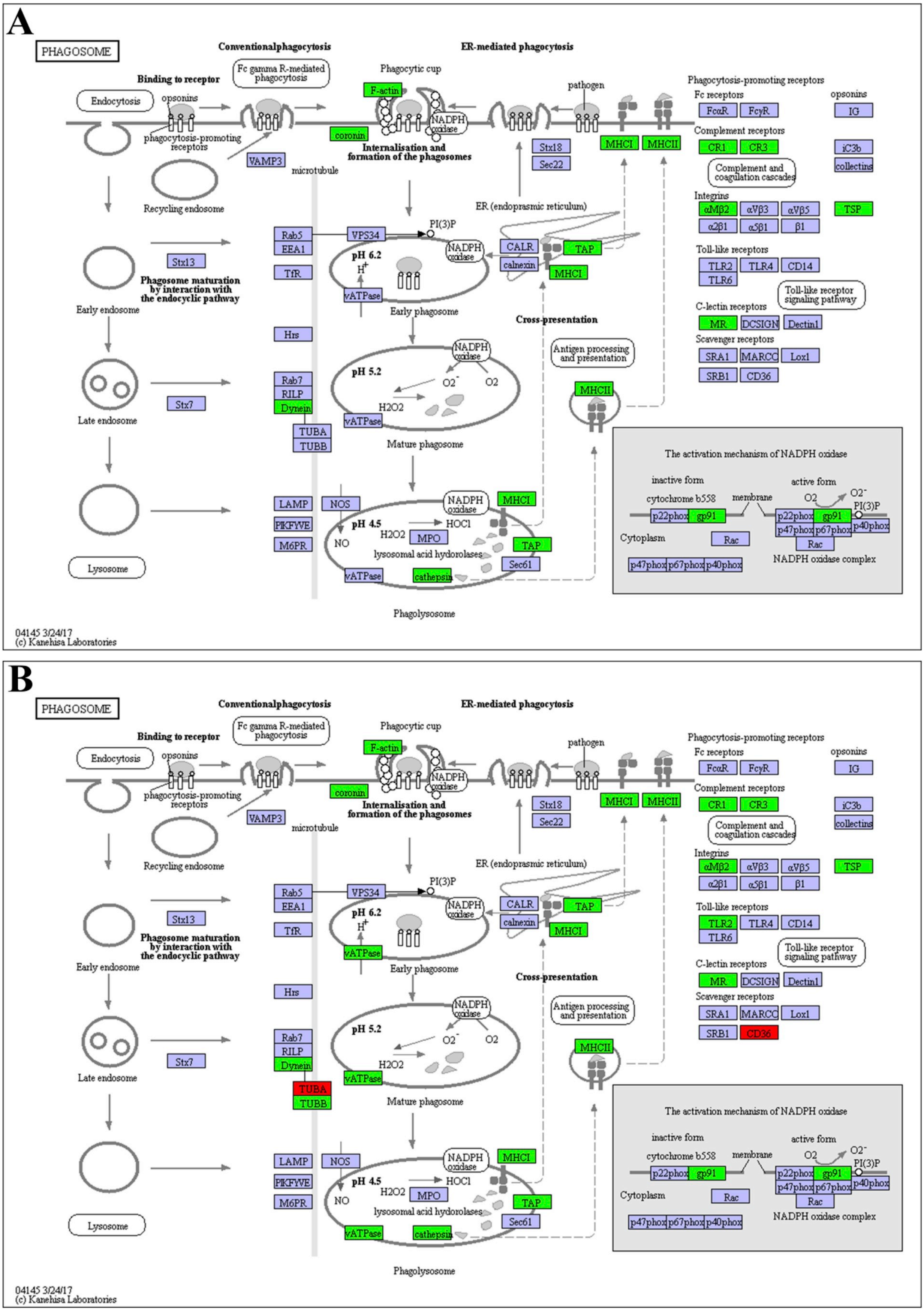

Fig. 6. Color pathway of the phagosome. The green and red background color in pathway represent the down-regulated and up-regulated genes, respectively. (A) Phagosome pathway in A group (B) phagosome pathway in B group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

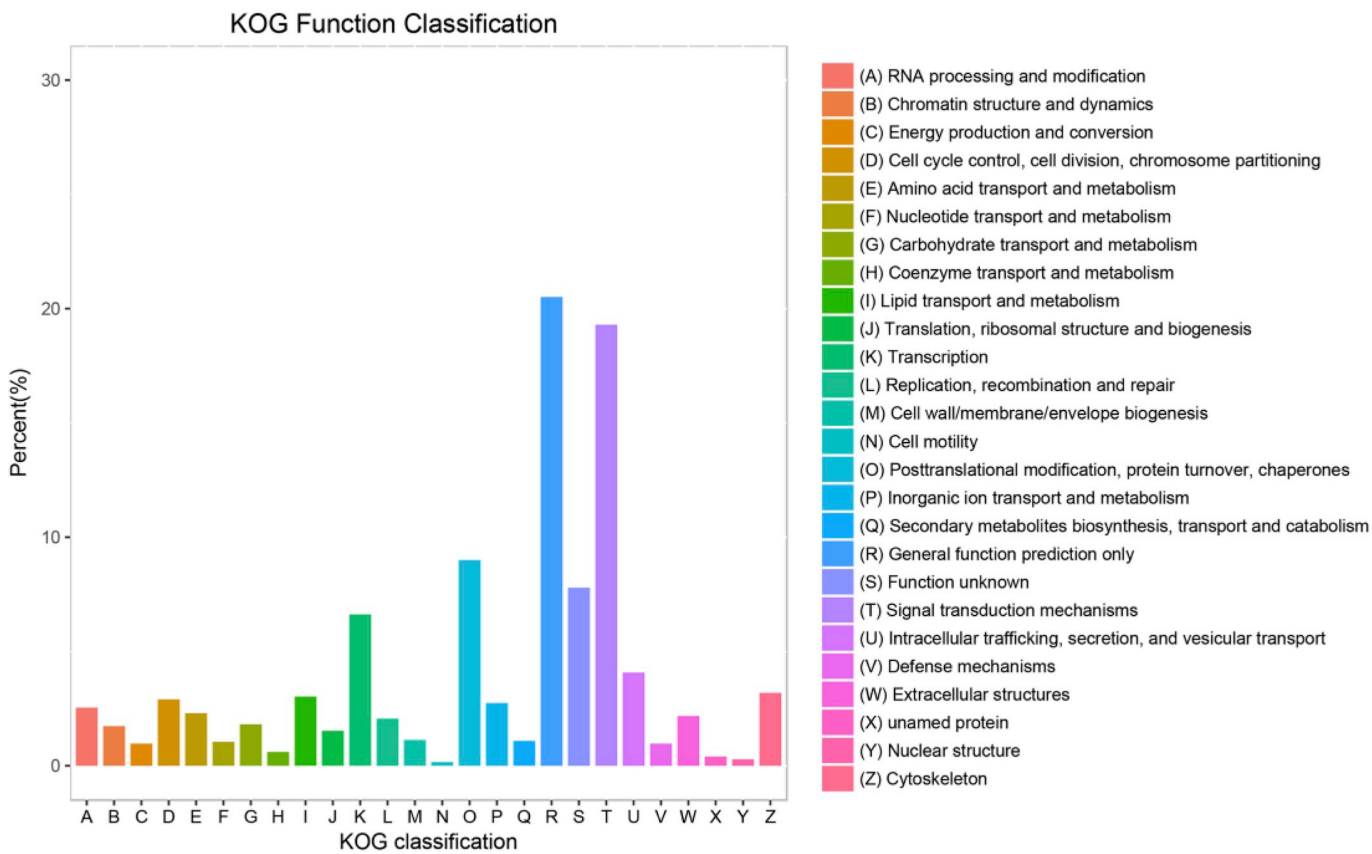

Fig. 7. KOG (euKaryotic Ortholog Groups) classification of putative proteins of DEGs in the kindey transcriptome of *O. niloticus*.

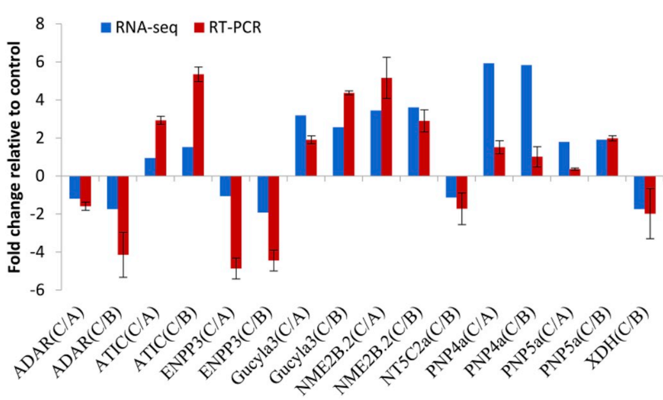

Fig. 8. Validation of RNA-Seq results using RT-PCR. The transcript expression levels of the selected genes were normalized to that of the β-actin gene.

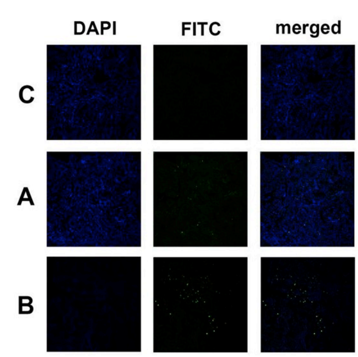

Fig. 9. Immunofluorescence staining of tilapia kidney from three groups (A, B and C). C, control group (28 °C); A, 8 °C/0 h; B, 8 °C/6 h. The "DAPI" represented the DAPI-stained cell nuclei. The "FITC" represented the FITC-stained fractured DNA fragments (a marker for apoptosis). The "merged" represented the combination of cell nuclei and fractured DNA fragments.

exceeds the cell's repair ability.

5. Conclusions

In this study, the transcriptome of tilapia kidney was sequenced via high-throughput technology. It shows the differently expressed genes and the low temperature sensitive GO and KEGG pathways. The levels of urea and uric acid in serum increased significantly associated with kidney damage as cell apoptosis under low temperature were detected. These results indicate that cold stress can cause kidney disfunction and down-regulate the immune-related pathway in the kidney of tilapia.

Conflicts of interest

We declare that we have no financial and personal conflicts with other people or organizations.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.023.

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