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Transcriptomic and cortisol analysis reveals differences in stress alleviation by different methods of anesthesia in Crucian carp (*Carassius auratus*)

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ABSTRACT

Stress response has negative effect on fish in aquaculture and research, which can be alleviated with anesthetic. To determine the optimal anesthetic, we investigated the physiological response of crucian carp (*Carassius auratus*) treated with three different anti-stress treatments: MS-222, eugenol and percussive stunning. Stress responses were evaluated by analyzing serum cortisol level and gene expression in blood. We determined the optimal concentrations of MS-222 (100 mg L^{-1}) and eugenol (20 mg L^{-1}) by dose selection. We found that the control group had significantly higher cortisol levels (172.78 ± 19.95 ng mL^{-1}) compared to the MS-222 treated group (46.85 ± 3.22 ng mL^{-1}), the eugenol treated group (72.78 ± 9.07 ng mL^{-1}), and the stunning treatment group (82.78 ± 8.16 ng mL^{-1}). Transcriptome analysis revealed 1572 differentially expressed genes (DEGs), including 155 DEGs related to the stress response, mainly involved in oxidative-stress response, heat shock proteins, and cold shock domain-containing protein. The heat shock protein genes were the primary DEGs in response to stress. RT-qPCR analysis confirmed differential expression of Hsps. We analyzed the function of the DEGs, which were enriched in genes involved in cellular response to stress and antigen processing and presentation. Combining the results from biochemical, transcriptome, and gene expression analysis, our data suggest that eugenol is more effective than MS-222 and percussive stunning in alleviating stress in crucian carp.

1. Introduction

The stress response induces a state of tolerance to external stimuli, and is a defensive response to challenges by specific environmental factors. Some routine procedures, such as transportation, sampling and artificial reproduction, could induce physiological response, including changes in energy metabolism, plasma hormones, and electrolyte balance [1,2] in various fish species. Stress can have a negative impact on the health and growth of fish, even cause mortality. To avoid these problems, it is necessary to reduce the damage to fish caused by routine stresses [3].

In modern aquaculture and research, anesthesia is used during routine handling of fish as an anti-stress agent to prevent physical injury and to reduce metabolism [4], which including chemical anesthetics and non-chemical methods. Fish are typically anesthetized by immersion in a bath containing an appropriate concentration of anesthetic agent, which is absorbed through the gills and rapidly enters the bloodstream. The drug initially inhibit the cortex of the brain (tactile loss), then act on the basal ganglia and cerebellum (excitement), and finally the spinal cord (anesthesia) [4], however, excessive or persistent contact with the drug can cause penetration into the medulla, vasomotor central paralysis and possibly death [5]. MS-222 and eugenol are commonly used to anesthetize fish in both aquaculture and research [6,7], and exhibit low risks for toxicity and mortality [8]. MS-222, the

Abbreviations: MS-222, tricaine methanesulfonate; HPI, hypothalamus-pituitary-interrenal; HSP, heat shock protein; Hsp, heat shock protein gene; CSD, cold shock protein; GCR, glucocorticoid receptor; MT, metallothionein; PEPCK, phosphoenolpyruvate carboxykinase; RT-qPCR, Real-time quantitative polymerase chain reaction; DEG, differentially expressed gene; COG, Clusters of Orthologous Groups; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; ELISA, Enzyme-linked Immunosorbent Assay; RT-qPCR, Real-time quantitative polymerase chain reaction

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only anesthetic approved for use in food fish in the USA, is a white, crystalline powder that can be dissolved in water. Eugenol is a colorless or pale-yellow liquid with a strong aroma that is insoluble in water. Eugenol is extracted from the buds, leaves, and stems of clove trees, and has an excellent safety profile. However, eugenol requires a relatively long recovery time compared to MS-222 [9]. Non-chemical methods of anesthesia include hypothermia [10], electro-anesthesia [11], CO_2 anesthesia [12], and percussive stunning [13]. Percussive stunning involves delivering a blow to the fish to induce a reaction to touch stimuli [14], so this method is convenient, efficacious, and cost effective [15–17]. In addition, percussive stunning does not pose a risk of residual drug in the flesh, and the technique presents no environmental risks or risks to users.

To understand the influence of different treatments on fish stress response, we investigated changes in the expression of blood chemistry factors following treatment with anesthetic agents. Several indices are applied to assess the stress in fish, including hemoglobin, hematocrit, glucose, hydrocortisone, and cortisol [18]. Cortisol is one of the hormones involved in the stress response, and serum cortisol concentrations increase following stress [19,20]. Studies have shown that responses to stressor exposure involve activation of the hypothalamus–pituitary–interrenal (HPI) axis in fish, leading to cortisol release into the circulation [21]. The cortisol of red porgy remained significantly higher in crowded fish compared to controls [22], and the same discovery in zebrafish [23]. However, after using an anesthetic, cortisol of salmon was lower compared to control [24]. Anesthetics are used to inhibit the effects of cortisol; we therefore elected to investigate the role of serum cortisol in the adaptive stress response [25–27]. Various stress-related genes [28,29], such as heat shock proteins (HSPs) [30–32], oxidative stress genes [33,34], cold shock protein (CSP) genes [35,36], glucocorticoid receptor (GCR) [37,38], and metallothionein (MT) [39,40], are expressed in response to specific stressors. HSPs make up a family of highly conserved cellular proteins that are found in all organisms, and be induced in response to a variety of stress conditions and metabolic insults. Upon encountering a stressor, such as transportation, synthesis of HSPs increases; the accumulation of HSPs provides added cellular protection. Accumulated HSPs may serve as a biomarker of stress and environmental insult in aquatic organisms [41,42]. Xu et al. [43] showed HSP70 significant up-regulation under stress, and the rapid and persistent response of HSP70 implies its critical role in the heat shock response, and other researchers put forward the same point on the lake trout [44] and blunt snout bream [45] of HSP70 and HSP90. Here, we studied the implications of gene expression changes observed in response to stress in fish, and verified changes in gene expression by real-time quantitative polymerase chain reaction (RT-qPCR).

In the present work, we aimed to ascertain the effect of different anti-stress agents on stress responses in the crucian carp, in order to determine the most suitable method of anesthesia for aquaculture and scientific research. We evaluated stress responses by routine handling of crucian carp in the context of anesthesia with MS-222, eugenol, and percussive stunning. The objectives of this study were to determine the optimum concentrations of MS-222 and eugenol to alleviate stress responses and to compare the efficacy of MS-222, eugenol, and percussive stunning by assessing survival of fish after exposure to an environmental stress. Serum cortisol level was assessed as a biochemical indicator of stress, and gene expression was evaluated to identify molecular biomarkers that would reflect the effect of each treatment. Comparing the stress-reducing effects of the different treatments enabled us to recommend an economical and effective anti-stress method that can be used during routine fish handling in aquaculture.

2. Methodology

2.1. Fish and experimental conditions

The crucian carp in this study had an average body weight of 65.8 ± 5.7 g (mean ± standard deviation) and an average length of 15.2 ± 2.0 cm. Crucian carp were cultured in a laboratory at Ningbo University. Experimental fish were placed into 1000 L fiberglass-reinforced plastic tanks at ambient water temperature (25 ± 1 °C) and natural photoperiod to acclimate for one week prior to the start of the experiment. During the acclimation period, crucian carp were fed a commercial mixed feed diet (Tianbang, Ningbo) twice a day; this was equivalent to 1.5% of their weight. The aquaculture water (containing 80% dissolved oxygen, maintained at pH: 7.8 ± 0.2) was exchanged ~80% daily. Fish fasted for 24 h before experimental procedures.

2.2. Selection of anesthesia concentrations

For this study, the optimum anesthetic doses of MS-222 (Cantin, Shanghai) and eugenol (Aladdin, Shanghai) for handling of crucian carp were established based on the minimum dose that the desired effects of rapid immobility, total loss of equilibrium, and cessation of locomotion. Fish were exposed to different concentrations of anesthetics, and the time to anesthetization, recovery time, and survival were recorded. The concentrations of anesthetics were as follows: MS-222 (25, 50, 100, and 200 mg L^{-1}) and eugenol (10, 20, 40, and 80 mg L^{-1}). Fish were quickly netted from the plastic tank and exposed to anesthesia. After exhibiting total loss of equilibrium and no reaction to touch stimuli, fish were moved to water without anesthetic agent for recovery. The appropriate concentration of anesthesia used was able to anesthetize fish within three minutes, and fish would be able to recover within 5 min without dying [46].

2.3. Determining appropriate force of percussive stunning

Crucian carp were stunned by the mechanical hammer with an oscilloscope (Tektronix, TDS 3014B, USA). After stunning, the fish were placed in a tank of water and monitored for signs of recovery over using tactile stimuli, such as a pinch on the caudal peduncle. No sign of recovery within 10 min was considered an indication of mortality. According to reference, an appropriate force of percussive stunning would allow the fish to recover within 4 min [14]. Through a series of pre-study evaluations, we determined that a force of 6 ± 0.1 N was the most suitable.

2.4. Sampling

Four experimental groups (five fish each) were established: fish stressed without anesthetic (control group), fish stressed and subject to percussive stunning (stunning group), fish stressed and treated with MS-222 (MS-222 group), and fish stressed and treated with eugenol (eugenol group). Fish were retrieved from the stock tanks and transferred randomly into experimental tanks with 2 L of water (25 ± 1 °C). Fish from each experimental group were netted, and the experimental groups received stunning treatment or were administered anesthesia before handling and sampling. For percussive stunning, the fish head is hit by a mechanical hammer, resulting in syncope but not death. For MS-222 and eugenol treatment, the appropriate doses (100 mg L^{-1} MS-222, 20 mg L^{-1} eugenol, as determined in pre-experimentation) were used to calm the fish. Blood samples (n = 5) were immediately collected after anesthetization through syringes inserted into the caudal vein. A0.5 mL portion of the blood sample was maintained at 4 °C for 2 h and then centrifuged at 3500 × *g* for 10 min to collect serum. The serum supernatant was transferred to a 1.5 mL centrifuge tube and stored at −20 °C for analysis. The other 1 mL portion of the blood sample was stored at −80 °C for sequencing and RT-qPCR. The experiments were undertaken in triplicates for each treatment.

2.5. Determination of serum cortisol levels

The serum cortisol levels of crucian carp during stress were

measured. Samples were determined using the Fish Cortisol Enzyme-linked Immunosorbent Assay Kit® (Xin Yu Biotech, Shanghai) following the manufacturer's instructions. This cortisol ELISA Kit employs the quantitative sandwich enzyme immunoassay technique. The microplate provided in the kit is pre-coated with a monoclonal antibody specific for cortisol.

2.6. Transcriptome analysis

2.6.1. RNA extraction

Total RNA was extracted from blood obtained from crucian carps using TRIzol® Reagent (Invitrogen, USA) following the manufacturer's instructions. RNA purity was assessed using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentrations were measured using the Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). The average RIN of samples was 8.9, as assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

2.6.2. Sequencing library preparation and transcript assembly

Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA), following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. A total of 1.5 μg of RNA per sample was used as input material for the RNA sample preparations. For gene expression profiling, fragmented mRNA was used as the template, and first strand cDNA was synthesized using random hexamer primers and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. The short fragments were purified and resolved with EB buffer for end-repair and single nucleotide A (adenine) addition, and were then connected to adapters. Suitable fragments were selected as templates for PCR amplification. For QC, the Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System were used to quantify and assess the quality of the sample library. Finally, the libraries were sequenced on an Illumina HiSeq™ 2000 system. To obtain unigenes, transcriptome *de novo* assembly was conducted using the short-read assembly program Trinity (release-20121005). Clean reads were obtained by removing reads containing adapter sequences, reads containing poly-N sequences, and low quality reads from raw data. At the same time, Q20, Q30, GC-content, and sequence duplication levels of the clean data were calculated. All downstream analyses were based on high-quality clean data.

2.6.3. Functional annotation of unigenes

The assembled unigenes of the crucian carp transcriptome were input into BLASTx searches and annotated against the NCBI non-redundant protein sequence database (NR-NCBI), using an e-value cut-off of 0.00001. BLASTx alignments (e-value < 0.00001) were then performed between the unigenes and several protein databases, including Swiss-Prot and the Clusters of Orthologous Groups (COG) database. With NR-NCBI annotation, the Blast2GO program was used to predict Gene Ontology (GO) terms related to molecular functions, cellular components and biological processes. After obtaining GO annotations for every unigene, we used the GOseq R package and Wallenius non-central hyper-geometric distribution to conduct GO functional classification of all unigenes and to understand the overall distribution of gene functions in this species. We used KOBAS software to test the statistical enrichment of differentially expressed genes among KEGG pathways for the assigned carp orthologs. KEGG orthology (KO) assignments were performed based on the bi-directional best hit (BHH) of the BLAST.

2.6.4. Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis was performed using the Mastercycler ep gradient realplex instrument (Eppendorf, Germany). SYBR Green (Roche, USA) was used as the fluorescent dye, and the procedure followed the manufacturer's protocol. RT-qPCR primers were designed based on each identified gene sequence from the blood transcriptome library using Primer Premier 5.0 (Table 1). The reliability of all primers were checked. The GC% of the primers were 50%, 47.8%, 51.2%, 55.6%, and the length of the target segment were 186bp, 172bp, 129bp and 157bp. The efficiency of primers were 95%, 92%, 95% and 90% respectively. Prior to qPCR analysis, every primer was validated by conventional PCR to demonstrate that no primers formed dimers in the melting curves and that a single band was observed on the agarose gels. Total RNA was extracted from the blood samples from the four different treatment groups. First-strand cDNA was synthesized from 1 μg of total RNA using the PrimeScript™ RT Reagent Kit (TransGen, China) and subsequently used as template for qPCR analysis using gene specific primers. qPCR analysis was performed using a total reaction volume of 20 μL. The cycling conditions were as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 20 s. Each qPCR reaction was performed in triplicate, and the data from each sample were expressed relative to the expression levels of β-actin by using the $2^{-\Delta\Delta CT}$ method. Statistical significance was determined by using independent-sample *t*-test, provided in the SPSS software (Version 22).

Table 1
Primers of Heat Shock Protein genes used for qPCR.

Gene Name	Primer (5′→3′)
β-actin-F	CCAGATCATGTTCGAGACCTTC
β-actin-R	GAACCTCTCATTGCCAATGGTG
Hsp60-2F	TTAGAACTGAAACTCCTCCACCC
Hsp60-2R	AAGGCTACCGCTGACATCATACA
hsp70-1F	TGATGGAGGGAAGCCGAAAG
Hsp70-1R	GAAGTAGGCAGGAACTGTGAT
hsp90-3F	GCCTACCTGGTGGCTGAA
hsp90-3R	CCGATGAACTGGGAATGT

3. Results

3.1. Behavioral effects of anesthesia

After anesthesia, the average recovery time and percent of survival for individuals treated with chemical anesthesia were determined for each concentration. The optimal concentration was selected based on the concentration that caused the fish to lose consciousness within three minutes and to recover recovery in five minutes without dying. The most effective concentration of MS-222 was 100 mg L^{-1}. Lower doses of MS-222, such as 25 mg L^{-1}, did not achieve anesthetization in all fish, and 50 mg L^{-1} required an extended time to induced anesthesia and recovery. The recovery rate was decreased when the concentration reached 200 mg L^{-1}. Similar results were observed in the eugenol group; when the concentration increased to 40 mg L^{-1} even 80 mg L^{-1}, anesthesia time was reduced, and the mortality rate increased. The optimal concentration of eugenol was found to be 20 mg L^{-1} (Table 2).

3.2. Parameters of serum cortisol

Serum cortisol levels are an important indicator of stress response. Serum cortisol concentrations in the control group (172.78 ± 19.95 ng mL^{-1}) were significantly higher than the other groups. Cortisol concentrations were similar in the percussive stunning group and eugenol group (percussive stunning 82.78 ± 8.16 ng mL^{-1}; eugenol 72.78 ± 9.07 ng mL^{-1}). The MS-222 group exhibited the lowest concentration of serum cortisol (46.85 ± 3.22 ng mL^{-1}) (Fig. 1). Comparison of serum cortisol concentrations in crucian carp exposed to stress with and without anesthetic were significantly significant ($P < 0.05$).

Table 2
Efficacy of two different anesthetics for Crucian Carp.

Concentration (mg L^{-1})	Anesthesia time (min)	Recovery time (min)	Rate of surgical anaesthesia (%)	Survival (%)
MS-222				
25	–	–	0	100
50	6.2 ± 0.7	3.2 ± 0.4	50	100
100	2.4 ± 0.3	4.3 ± 0.5	100	100
200	1.7 ± 0.4	7.2 ± 0.3	100	60
Eugenol				
10	5.8 ± 0.6	2.8 ± 0.7	60	100
20	2.1 ± 0.4	3.7 ± 0.6	100	100
40	1.5 ± 0.3	6.9 ± 0.2	100	100
80	1.1 ± 0.5	8.2 ± 0.3	100	50

3.3. Transcriptome sequencing and de novo assembly

To better compare the molecular changes in the stress response of different anesthetic methods, cDNA libraries were constructed. After conducting replicate experiments and ensuring quality of the library preparations, Illumina HiSeq™ 2000 sequencing was performed to sequence the libraries. High-throughput paired-end sequencing yielded 186,763,882 total raw reads, and 177,716,724 clean reads were obtained after filtering the raw reads. The clean reads were assembled into contigs using Trinity software. The distribution of the length of transcripts and unigenes obtained from the libraries is shown in Fig. 2; the total number of transcripts and unigenes was 214,736 and 97,530, respectively.

3.4. Analysis of differentially expressed genes (DEGs) and identification of stressed-related genes

To identify stress-related DEGs when fish were subject to different methods of anesthesia, transcriptome data from fish blood was analyzed by DEGSeq (1.12.0). The criteria of q-value < 0.005 & $|\log_2(\text{fold change})| > 1$ were used to identify genes significantly up-regulated or down-regulated during handling. Using these criteria, we identified 877 DEGs in the percussive stunning group (300 up-regulated and 577 down-regulated), 487 DEGs in the MS-222 group (179 up-regulated genes and 308 down-regulated), and 208 DEGs in the eugenol group (65 up-regulated and 143 down-regulated) (Fig. 3).

We identified 155 DEGs related to stress response, including heat shock protein genes (n = 126, 81.3%), oxidative stress genes (n = 11, 7.10%), cold shock protein genes (n = 8, 5.16%), stress-associated endoplasmic reticulum protein genes (n = 6, 3.87%), and a series of stress-activated protein kinase genes (n = 4, 2.58%). Most of differentially expressed genes were HSPs, indicating that changes in HSPs had the most direct correlation with stress. From Table 3, in total, 12 DEGs identified in stunning group were stress response genes, including three oxidative-stress responsive genes, two cold shock protein genes, and nine heat shock protein genes. In the MS-222 group, five heat shock protein genes were down-regulated (Table 3), and in the eugenol group, one cold shock protein gene and two heat shock protein genes were differentially expressed (Table 3); genes in the heat shock protein 70 family were down-regulated in all three groups.

3.5. Functional classification by Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG)

To generate an overview of the functions of the DEGs identified by DESeq (1.10.1), GO analysis of these genes was performed using GOseq (1.10.0). According to the GO terms, 943,789 genes were classified into three major functional categories, including 645,817 genes in 'biological process', 192,464 genes in 'cellular component', and 105,508 genes in 'molecular function'. The genes in the biological processes were mainly related to 'cellular process' (270,265, 41.85%), 'metabolic process' (140,051, 21.69%), and 'single-organism process' (60,793, 9.41%). The main subcategories of the cellular component included 'organelle' (45,582, 23.68%), 'cell part' (54,176, 28.15%), and 'organelle part' (15,879, 8.25%). In the category of 'molecular function', the significant subcategories were 'binding' (76021, 72.05%) and 'catalytic activity' (18,590, 17.62%). Comparing the different treatments (Fig. 4), most genes are enriched in the 'biological process', and the least amount of enriched genes was found in the 'cellular component' category. The stunning group contained more DEGs compared with other groups, and the DEGs in the eugenol fewer than those found in the MS-222 group. We found 7128 stress-related genes (including 2073 DGEs) classified into five GO terms (Fig. 5), including 'response to stimulus'

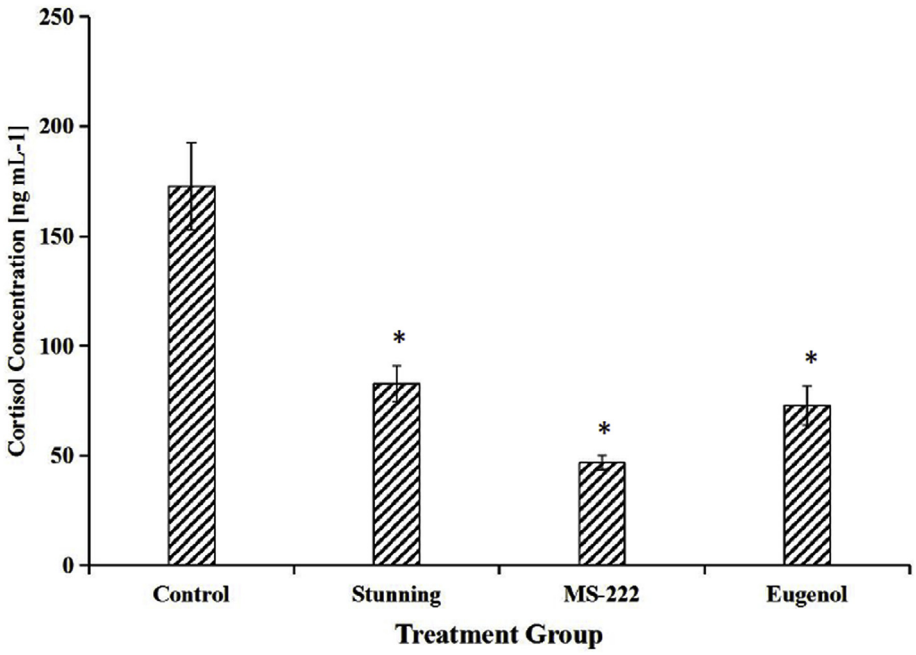

Fig. 1. The serum cortisol concentration of crucian carp in four groups (x-axis). Values (y-axis) are means ± SD, and significant differences among four groups are indicated by different letters (*, p < 0.05).

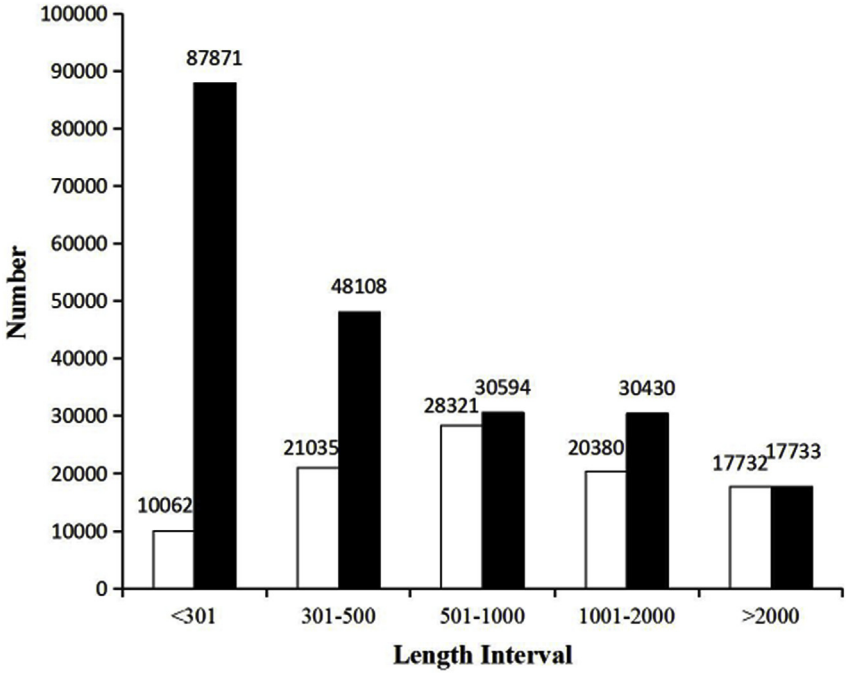

Fig. 2. Length statistics of transcripts and unigenes obtained from the blood of crucian carp transcriptome libraries.

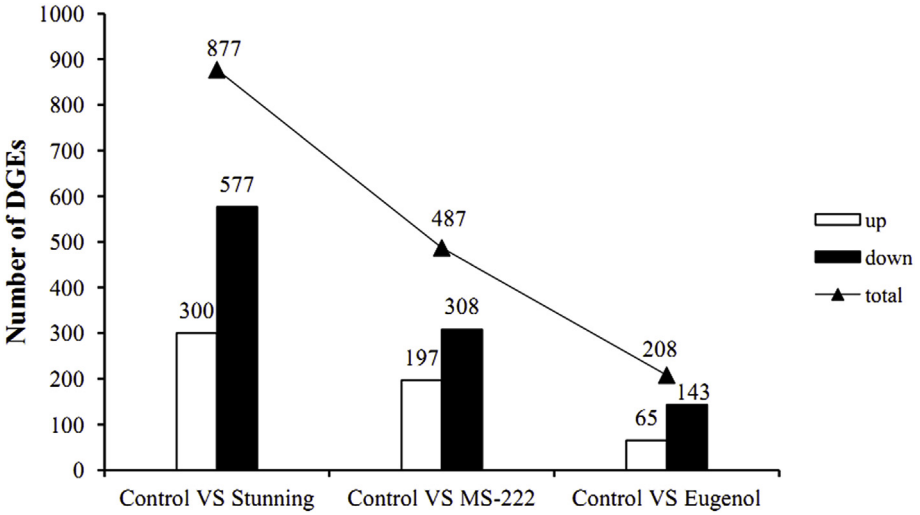

Fig. 3. DEGs identified in four treatment groups. Vertice of polyline was total DEGs. White bar represent up-regulated genes and black bar indicate down-regulated genes. The height of *s* mean the number of DEGs.

(GO:0050896, 96 up, 229 down), 'positive regulation of biological process' (GO:0048518, 9 up, 20 down), 'cellular process' (GO:0009987, 295 up, 600 down), 'regulation of biological process' (GO:0050789, 100 up, 280 down), and 'biological regulation' (GO:0065007, 112 up, 296 down). We found 333 up-regulated DGEs and 832 down-regulated DGEs in the percussive stunning group, 75 up-regulated DGEs and 194 down-regulated DGEs in the eugenol group, and 204 up-regulated DGEs and 399 down-regulated DGEs in the MS-222 group.

The KEGG pathways can be represented as collections of manually drawn pathway maps that enhance the understanding of the biological functions and interactions of genes. To identify the biological pathways that were active in the crucian carp during handling, a total of 31,400 genes were mapped to 230 statistically significant categories. These categories are related to several biological functions, including 'intracellular process', 'material and energy metabolism', 'immunoregulation', and 'endocrine metabolism'. KEGG pathway analysis identified the functions and interactions of DEGs responding to anesthetic treatment. A total of 1690 unigenes were mapped onto 229 pathways, and 35 categories were highly enriched in the stunning group; 353 unigenes were mapped onto 141 pathways, and 27 categories were highly enriched in the eugenol group; 836 unigenes were mapped onto 208 pathways, and 37 categories were highly enriched in the MS-222 group. The top five most significant KEGG classifications of crucian carp genes in the different groups are shown in Table 4. According to the enrichment level and q-value ($q < 0.05$), two categories were highly enriched for the stress protein Hsp70 from the percussive stunning group, including the 'Antigen processing and presentation (ko04612)' pathway and the 'Spliceosome (ko03040) pathway'. The category highly enriched for the stress protein Hsp70 in the eugenol group was the 'Antigen processing and presentation (ko04612) pathway'. Three categories were highly enriched for the stress protein Hsp70 in the MS-222 group, including the 'Spliceosome (ko03040) pathway', the 'Antigen processing and presentation (ko04612) pathway', and the 'Influenza A (ko05164) pathway'. There were 20

Table 3
Differentially expressed stress response genes in different treatment groups.

Gene_id	NR Description	Log2Fold change	P-value
Stunning VS Control			
Cluster-11905.21062	oxidative-stress responsive 1b	−2.4342	7.72E-06
Cluster-11905.24678	oxidative-stress responsive 1b	−2.3951	2.42E-05
Cluster-11905.32393	oxidative stress-responsive serine-rich protein 1	1.5769	8.33E-07
Cluster-11905.31522	cold shock domain-containing protein E1 isoform X2	−1.543	5.09E-13
Cluster-11905.45696	cold shock domain-containing protein E1 isoform X2	−1.2674	7.43E-06
Cluster-11905.31935	heat shock protein HSP 90-alpha	−1.2226	2.10E-49
Cluster-11905.32394	60 kDa heat shock protein	−1.5535	4.02E-26
Cluster-11905.34134	heat shock protein 4a	−2.3469	9.91E-20
Cluster-11905.35133	heat shock protein	−1.7773	5.69E-08
Cluster-11905.36710	heat shock protein 4a	−1.5122	5.20E-10
Cluster-11905.39142	heat shock protein 70 kDa	−2.5523	8.33E-24
Cluster-11905.47390	heat shock 70 kDa protein	−2.5402	7.18E-14
Cluster-11905.51203	heat shock factor protein 1-like	−2.3469	7.07E-07
Cluster-11905.52272	heat shock factor protein 1-like	−1.6996	2.78E-08
MS-222 VS Control			
Cluster-11905.32394	60 kDa heat shock protein	−1.0682	1.5206E-16
Cluster-11905.34134	heat shock protein 4a	−1.4085	5.0555E-12
Cluster-11905.35133	heat shock protein	−1.2133	0.000019685
Cluster-11905.39142	heat shock protein 70 kDa	−2.7949	8.4134E-24
Cluster-11905.47390	heat shock 70 kDa protein	−2.0236	5.1913E-11
Eugenol VS Control			
Cluster-11905.31522	cold shock domain-containing protein E1 isoform X2	−2.2066	2.0487E-15
Cluster-11905.39142	heat shock protein 70 kDa	−1.9548	8.6366E-18
Cluster-11905.47390	heat shock 70 kDa protein	−1.7185	1.7494E-09

DEGs (9 down-regulated, 11 up-regulated) that were enriched in the 'Antigen processing and presentation (ko04612) pathway', and 30 DEGs (17 down-regulated, 13 up-regulated) enriched in the 'Spliceosome (ko03040) pathway'. Two Hsps, Hsp40 and Hsp70, were both down-regulated in the 'Influenza A pathway'.

3.6. qPCR analysis

We analyzed expression of three types of heat shock protein genes by qPCR to verify the results of DEG analysis from the RNA-seq analysis. The results of the RT-qPCR were mostly consistent with the RNA-seq analysis, and the key genes, Hsp60, Hsp70, and Hsp90, were all significantly differentially regulated in the three treatment groups (Fig. 6).

4. Discussion

The stress of handling fish can often result in poor performance and increased mortality, and can confound experimental results [47]. The physiological responses to stressors include associated tissue responsiveness, changes in hormonal profiles, and the induction of molecular mechanisms associated with an adaptive response [21,48]. Fish anesthetics are used for applications ranging from mild sedation during transport to total anesthetization for surgical procedures, such as sampling blood from the body [49]. Calming the fish prior to experimental procedures is consistent with humanitarian principles.

4.1. Effects of anesthesia

The goal of this part of the study was to evaluate the efficacy of two

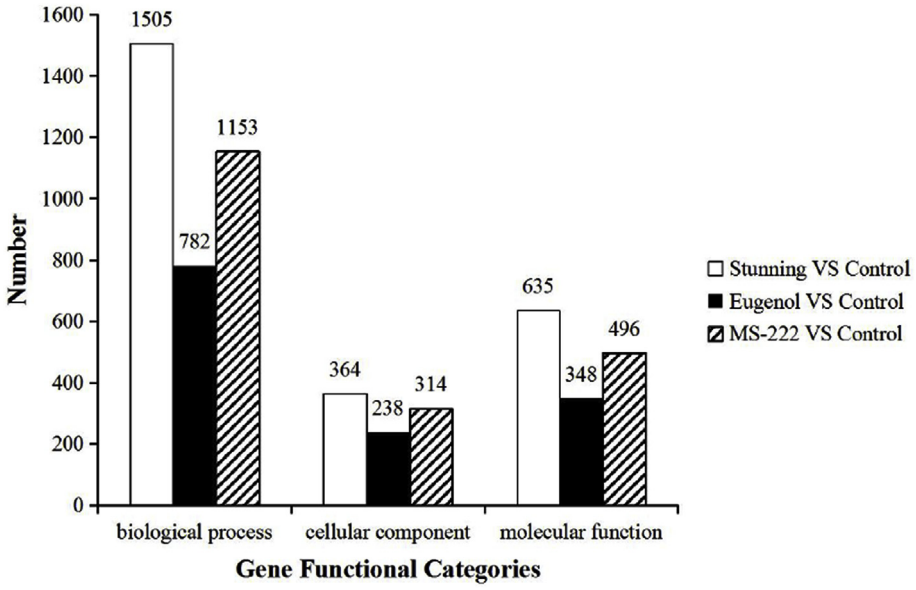

Fig. 4. GO analysis of stress-related DEGs in five terms. The height of bars mean the number of DEGs.

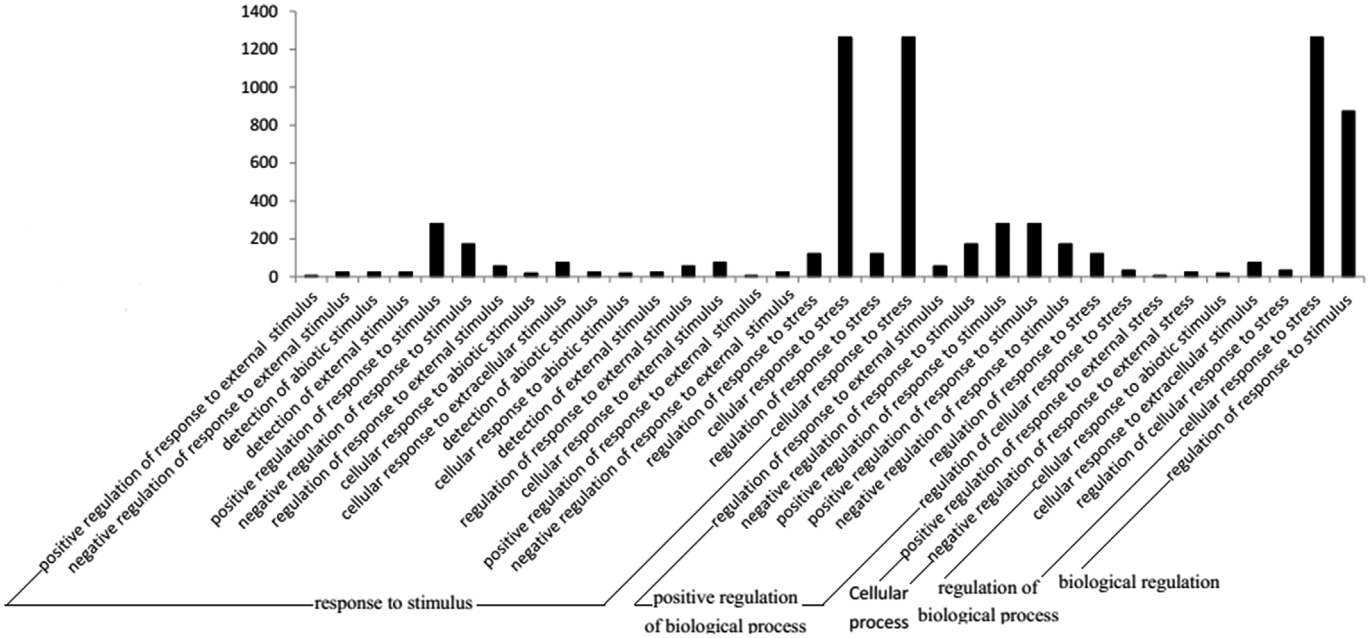

Fig. 5. DEGs identified from the three comparisons are distributed into functional categories. White bar represent stunning, black bar indicate eugenol and stripe bar was MS-222. The height of bars mean the number of DEGs.

Table 4
Statistically significant KEGG classifications of DEGs in crucian carp blood (Top 6).

Pathway	Gene Number	P-Value	Pathway ID
Stunning VS Control			
Ribosome	60	1.99E-26	ko03010
Butirosin and neomycin biosynthesis	7	1.74E-06	ko00524
Antigen processing and presentation	20	1.26E-05	ko04612
Parkinson's disease	23	9.10E-05	ko05012
Huntington's disease	30	0.000106	ko05016
Spliceosome	30	0.000115	ko03040
Eugenol VS Control			
Staphylococcus aureus infection	5	9.46E-05	ko05150
Allograft rejection	5	0.000116	ko05330
Asthma	4	0.00016	ko05310
Autoimmune thyroid disease	5	0.000163	ko05320
Cell adhesion molecules (CAMs)	6	0.000364	ko04514
Antigen processing and presentation	7	0.000423	ko04612
MS-222 VS Control			
Spliceosome	23	8.98E-07	ko03040
Antigen processing and presentation	15	1.17E-06	ko04612
Cardiac muscle contraction	11	2.67E-05	ko04260
Purine metabolism	16	0.000112	ko00230
Fructose and mannose metabolism	9	0.000123	ko00051
Butirosin and neomycin biosynthesis	4	0.000141	ko00524

anesthetics in treating crucian carp during stress and to establish a minimum dose that produces an optimal anesthetic state [50–52]. In this study, both MS-222 and eugenol were found to be effective anesthetics in crucian carp. In contrast to the optimum concentration of MS-22 (100 mg L^{-1}), eugenol (20 mg L^{-1}) was efficient for anesthesia. It seemed that MS-222 was less efficient than eugenol at the same dose, and this phenomenon support previous reports in red swamp crayfish [53], zebrafish [54] and sturgeon [55], demonstrating that MS-222-treated fish were indeed less sensitive to the stress than eugenol. Even though recovery time with eugenol may take 6 to 10 times longer than MS-222 [9,56], it induced anesthesia faster and at lower concentrations than MS-222 in this study. Therefore, we recommend that the eugenol was a reasonable alternative to MS-222 for crucian carp, which could apply to future anesthesia studies of other fish species.

4.2. Serum cortisol parameters

Exposure to stressors can cause a significant increase in serum cortisol concentration for fish [57–60]. Measurement of serum cortisol concentration is conventionally used to evaluate the effects of stress with and without anesthetics [61,62]. In this study, we investigated and analyzed the serum cortisol levels induced by stress under different anesthetic treatments. The data demonstrated that using MS-222, eugenol, or percussive stunning before handling can effectively maintain serum cortisol at low level in crucian carp. For fish that were not anesthetized before handling, the cortisol concentrations were approximately 3.7, 2.4 and 2.1 fold higher than fish anesthetized with MS-222, eugenol and percussive stunning concentrations respectively. The cortisol recorded for MS-222 was lower than eugenol was reported for steelhead trout by Pirhonen et al. [9], and contrary result was reported by Wagner [63] and Kebus [64]. In this study, MS-222 treatment had better performance than percussive stunning and eugenol treatment, while the above result of anesthesia effects showed eugenol was better than MS-222. Taken those into consideration, we thought the cortisol could be influenced by plenty of factors and some other parameters, such as hemoglobin, hematocrit, glucose and hydrocortisone [65], should also be used to evaluate anesthetic efficiency for further research.

4.3. Analysis of stress-related genes

To further research the mechanism of anesthesia, we performed transcriptome analysis of different anesthesia. In this study, mainly DGEs were related to oxidative-stress response genes, Csps, and Hsps in all treatment groups. Among them, the Hsps was a class of stress-related genes, which would be extremely changed during handling [66]. The relative expression of Hsp90 demonstrated the MS-222 group had the lowest level, which was similar with the cortisol result. Some other studies have reported that cortisol is a stress indicator, and Hsp90 is a downstream product of cortisol [67,68]. So we speculated that cortisol levels modulate the cellular stress response by affecting the transcription of Hsp90 in fish. In addition, the relative expression of Hsp70 and Hsp60 in control group had significantly higher level during stress than fish treated under anesthesia. It has been established that the

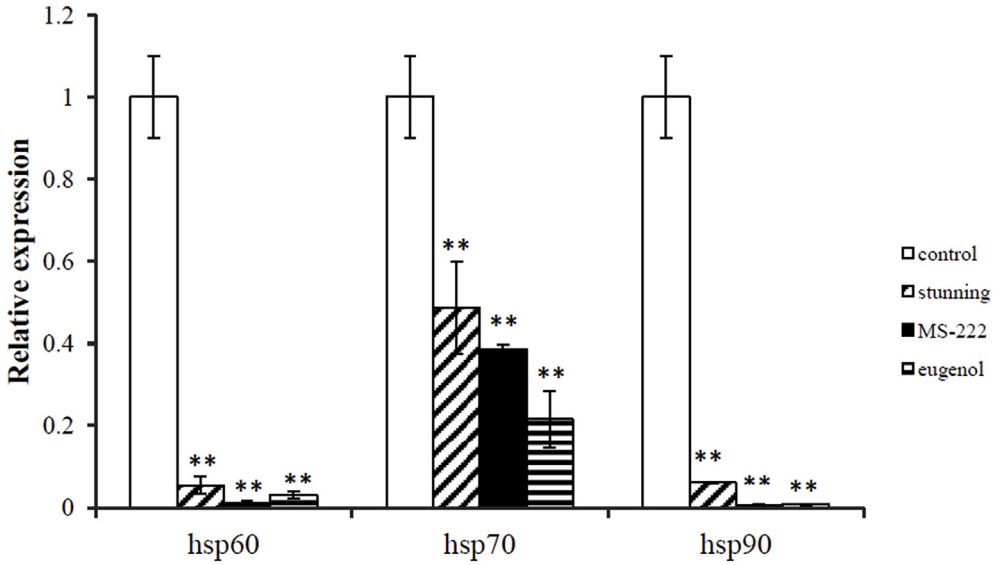

Fig. 6. RT-qPCR analysis for Hsp60, Hsp70, Hsp90 in crucian carp blood during handling. Relative expression levels were calculated according to the $2^{-\Delta\Delta CT}$ using β-actin as an internal reference gene (**p < 0.01).

variational expression of Hsp70 and Hsp60 were detected under stress with or without anesthesia [69–71]. In this study, Hsp90, Hsp70, and Hsp60 were all down-regulated in the three treatment groups, and confirmed by qPCR (Fig. 6), therefore Hsps could be considered as a marker for stress response in fish [72]. Based on this result, we believed that eugenol was more effective in relieving stress by reducing Hsps expression compared with others.

Oxidative stress, through the production of reactive oxygen species, is a natural consequence of aerobic metabolism [73]. We found that only percussive stunning resulted in differential expression of oxidative-stress responsive genes, suggesting that delivering a blow to the head might cause oxygen shock, and stunning fish to alleviate stress may have side effects [74]. The Csps are implicated in various cellular processes and appear to function as RNA-chaperones [75]. In this study, they were differentially expressed in the percussive stunning group and the MS-222 group, and more total DEGs in this two groups than in the eugenol group. So we speculated that Csps might be involved in post-transcriptional gene regulating these DEGs. In conclusion, engenol has less side effect in processes of anesthesia。

Our results suggested that a physiological response as well as an inducible cellular stress response occurred in crucian carp subjected to stress; the down-regulation of Hsps provided molecular evidence of the stress mechanism induced during stress. These data demonstrated that anesthesia had a significant effect on relieving stress, and the eugenol worked best to alleviate stress in crucian carp.

4.4. Function analysis of DEGs

The category of the most high enriched DEGs in the current study was ‘cellular response to stress’, which indicated a cellular response characterized by a modification in the genetic expression and physiology of the cell induced by environmental changes [76], demonstrating that handling caused the stress response. Meanwhile, the ‘regulation of response to stimulus’ category was also activated, indicating a concerted mechanism to alleviate stress. A three-way comparison of the three treatment groups indicated that the fewest DEGs were found in the eugenol group, suggesting that eugenol is better in relieving stress. In the three treatment groups, Hsp70 was enriched in the ‘Antigen processing and presentation pathway’ [77–80]. The molecular chaperones Hsp70 and Hsp90 were involved in the ‘antigen processing immune response pathway’ (Fig. 7) [81–83]. These data confirmed that increasing expression of Hsp70 and Hsp90 enhance resistance to stress, and could be considered as physiological response marker in stress research.

5. Conclusions

The present study established the optimal anesthetic dose of MS-222 and eugenol for research and aquaculture of crucian carp. We evaluated the effects of stress using serum cortisol concentration in carp handled with and without anesthesia. Meanwhile, transcriptome analysis revealed that three types of genes are involved in the stress-response, especially heat shock protein genes, which were are involved in cellular response to stress, antigen processing and presentation. Combining the results from biochemical, transcriptome, and gene expression analysis, our data suggest that eugenol is more effective than MS-222 and percussive stunning in alleviating stress in crucian carp. Although percussive stunning is cost effective, it has side effects, and the use of anesthetic agents is more consistent with humanitarianism and the Animal Welfare Act. By associating blood biochemistry and gene expression analysis in response to stressors under anesthesia, this study establishes a new model for studying stress in aquaculture, and enhances our understanding of anesthetic mechanisms and the optimal method of anesthesia for relieving stress.

Data availability

Raw sequencing data are available through the NCBI Sequence Read Archive (Accession: RJNA406987).

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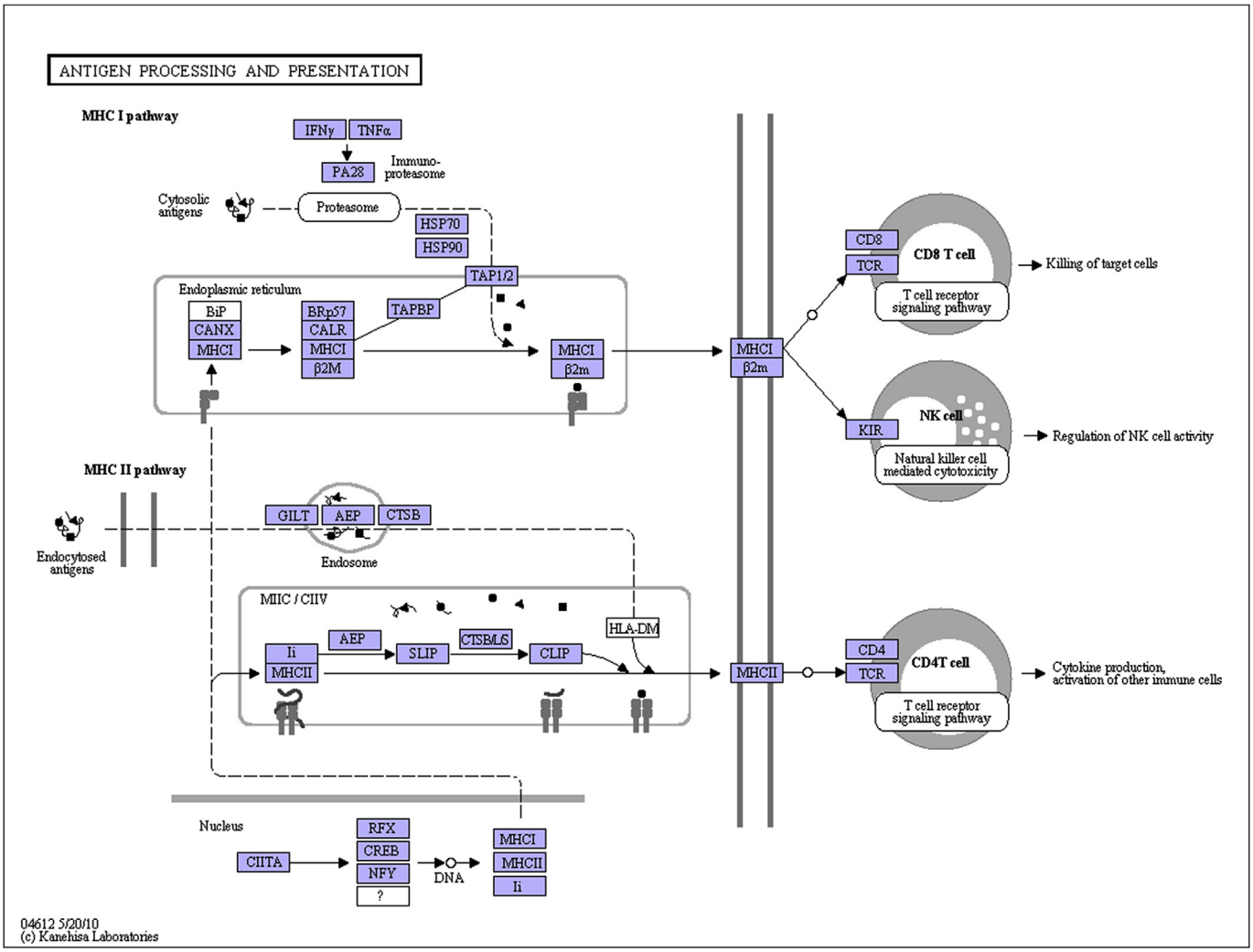

Fig. 7. Antigen processing immune response pathway of crucian carp.

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