RESEARCH ARTICLE

Enhanced Resistance of Triploid Crucian Carp to Cadmium-induced Oxidative and Endoplasmic Reticulum Stresses

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Abstract: Cadmium (Cd) is highly toxic to aquatic organisms. In this study, we found a new variety of triploid crucian carp with strong Cd resistance. Under Cd stress, the mortality and abnormality rates in triploid crucian carp were lower than those of diploid strains. To explore the molecular mechanisms underlying Cd stress, the liver transcriptomes of triploid crucian carp were obtained. The expression of 5,797 unigenes in Cd-treated triploid crucian carp differed significantly from those of the control group. These differential expression genes are mainly involved in endoplasmic reticulum (ER) associated ubiquitin-dependent proteins, membrane proteins of ER and mitochondria. Quantitative real-time PCR and enzyme activity analysis all confirmed that triploid crucian carp resistance to Cd stress was regulated by both oxidative stress and ER stress responses. Underlying Cd stress, the enhanced expression of sestrin-1 gene may increase triploid crucian carp survival compared to diploid fish. This study also provides an important clue that IRE-1 and PERK, but not ATF-6, were involved in the enhancement of Cd resistance in triploid crucian carp. Moreover, our results showed that egg envelopes of crucian carp had a strong ability to block Cd and could protect embryos from Cd stress damage.

Keywords: Cadmium, triploid crucian carp, ER stress, oxidative stress.

1. INTRODUCTION

Cadmium (Cd) is a major heavy metal pollutant. By the end of the last century, increased extraction, smelting and commercial manufacturing had resulted in the global release of 22,000 tons of Cd annually. Even at low concentrations in wastewater Cd can accumulate in algae and sediments, and it is absorbed by both plants and aquatic animals, like shellfish and fish [1]. Once it enters the soil-crop system, it readily accumulates in roots, stems, leaves and grains, and cannot be easily removed.

Cd can seriously affect crop yield and, via food chains, human health. The "Itai-Itai Disease", a well-known event in Japan, was caused by long-term consumption of "Cd rice". Cd overdose can lead to osteoporosis, bone disease, kidney damage, and long-term Cd poisoning can cause emphysema, gastrointestinal diseases, high blood pressure and cancer [2, 3]. Cd can accumulate in the human kidney for 17 to 30 years without degrading [4].

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At least three main pathways exist for fish to absorb Cd: 1) through filtration by the fish gill, with absorption into and circulation in blood through the body; 2) bioaccumulation through the food chain; and 3), osmosis [4]. Given the importance of fish to aquatic ecosystems, investigation of their resistance to Cd stress in polluted waters is warranted.

The triploid crucian carp, known alternatively as the Xiangyun crucian carp, was generated by mating diploid female crucian carp with allotetraploid hybrid males of a carp and crucian carp cross [5]. The triploid crucian carp has several enhanced traits, such as faster growth, higher anti-disease ability and good flesh quality. Given its infertility, this triploid strain could avoid genetic disorders, and have reduced ecological impact in the event of escape from aquaculture facilities. Triploid crucian carp is an important economic fish and have been farmed on a large scale in China [6].

In the current study, we reported toxic effects of Cd on triploid and diploid crucian carp. Under Cd stress, Cd^{2+} levels in muscle tissues prove lower than in kidney and liver tissues. Larval and adult stage mortality and abnormality rates in triploid crucian carp were lower than those of diploid strains. Using Illumina sequencing

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technology, we performed transcriptomic analysis on triploid crucian carp liver tissues to understand the molecular mechanisms of adaptation and tolerance under Cd stress. Our study provides novel insights into potential uses of carp strains in the use and remediation of polluted water bodies.

2. MATERIALS AND METHODS

2.1. Ethics Statement

All experiments performed from 2015–2017 were approved by the Animal Care Committee of Hunan Normal University. All sampling procedures were conducted in accordance with standards and ethical guidelines established by the Animal Ethical Review Committee, Hunan Normal University, Changsha, China.

2.2. Fish

Triploid and diploid crucian carp were sourced from the State Key Laboratory of Developmental Biology of Freshwater Fish, Hunan Normal University, China. Embryos were raised in laboratories at 23–24°C, and larvae and adults were cultured in separate concrete ponds.

2.3. Acute Toxicity Tests

We used Cd sulfate, $3CdSO_4 \cdot 8H_2O$ (Tianjin Kermel Chemical Reagent Co. Ltd.), as our source of Cd. Toxicity experiments were performed on embryos, larvae and adults of triploid and diploid crucian carp. For embryos, 100 blastocysts were treated with Cd solution until hatching. For larvae, 50 seven-day-old individuals were incubated in Cd solution for 96 h. For adults, 10 one-year-old fish, average length and weight of diploid and triploid crucian carp was 14.81 cm, 11.31 cm, 51.319 g and 21.639 g, were pretreated in aerated water at 22°C (without feeding) for one week, then, continuously exposed to Cd solution for 96 h. For each group, toxicology experiments were replicated at least three times.

2.4. Analysis of Cd^{2+} Content in Fish

Embryos and one-year-old triploid and diploid crucian carp were treated with 9 mg/L Cd^{2+}. Egg envelopes and embryophytes were collected separately from somatic pigment stage embryos (60 h after fertilization). Tissues of liver, kidney, skin, gill and muscle were collected from diploid and triploid crucian carp which were treated with 9 mg/L Cd^{2+} for 96 h. After anesthetizing with 2-phenoxyethanol, the egg envelopes, embryophytes and tissues were treated by microwave digestion. Cd^{2+} contents were tested by atomic absorption spectrophotometry [7]. The untreated group was used as a control.

2.5. Construction of Regression Models for the Mortality Rate

Student's T-test is a statistical hypothesis test where the test statistic follows a Student's t-distribution if the null hypothesis is supported. It can be used to determine whether the coefficients in a regression model differ significantly from 0. We used Student's T-test to determine whether the confidence level of the regression coefficients in the model of the mortality rates in triploid and diploid crucian carp embryos. Based on acute toxicity experimental data, regression models were constructed using SPSS (v17.0) for Windows to express the dependence relation of mortality rate on Cd-treated concentration and time. Cd^{2+} concentration and exposure duration (time) were chosen as the predictors of regression models; mortality rate was the dependent variable.

2.6. Egg Envelopes Proteins Analysis

Egg envelopes were collected separately from eye pigmentation stage embryos 60 hours after fertilization, then, frozen at −80°C. Egg envelopes were subsequently digested in SDS-PAGE sample loading buffer by boiling in water and ultrasonic cell crusher; then, using 10% SDS-PAGE to isolate the buffer and stained the sample with Coomassie Blue; finally, captured with Image Scanner III.

2.7. Histological Observation

After anesthetizing the fishes with 2-phenoxyethanol, liver tissues were excised carefully to avoid contamination. These tissues were fixed in Bouin's solution for less than 24 h. After dehydrated, tissues were embedded in paraffin wax, cut into 5 μm sections using a Leica RM2015 Microtome, and transferred to slides, stained using hematoxylin and eosin (H.E.) following standard procedures [8-10]. For electron microscopy, liver tissues and egg envelopes were fixed in 1% OsO_4 in PBS (pH 7.0) for 2 h, washed three times in PBS, then dehydrated with series of ethanol concentrations (50, 70, 90, and 100%) for 15 min intervals. Samples were subsequently incubated in a mixture of alcohol and isoamyl acetate (v:v=1:1) for 30 min, followed by incubation with pure isoamyl acetate for 1 h, before finally being cut into ultrathin sections and observed using a transmission electron microscope (Hitachi, Tokyo, Japan) [10].

2.8. Construction of cDNA Library and Sequencing

Total RNAs were isolated from liver tissues using Trizol Reagent (Invitrogen, USA) following manufacturer protocols. After removing genomic DNA with DNase I (Fermentas, Vilnius, Lithuania), cDNA was constructed from 2 μg of total RNA per sample. Total RNA was purified using RNA Trizol (Invitrogen) and quantified with the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Complementary DNA (cDNA) libraries were synthesized using mixed messenger RNA (mRNA) fragments as templates with the TruSeq RNA sample preparation kit V2 (Illumina, San Diego, CA, USA) according to manufacturer instructions. Libraries were sequenced using Illumina HiSeq 2500 at Shanghai OE Biotech. Co., Ltd, China [11].

Raw reads were filtered using FastQC software to obtain paired-end clean reads, and all clean reads were used for assembly with Trinity. Assembled transcripts were annotated by BLASTx (National Center for Biotechnology Information [NCBI], Bethesda, MD, USA) against the protein databases of the NCBI, nonredundant (Nr), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups (COG), and Gene Ontology (GO) with an e-value of 1e5 [11]. GO annotation was performed according to the Nr annotation using Blast2GO software, and the GO functional classification was classified using WEGO software [12]. Relevant biological pathways were identified through gene enrichment analyses of the KEGG categories and annotation. The numbers of genes included in each KEGG pathway were counted and the significance of gene enrichment for each KEGG pathway calculated using the hypergeometric distribution test.

2.9. Quantitative Real-time PCR (qPCR) Analysis of Gene Expression

Analyses of gene transcript levels were conducted by qPCR [13]. cDNA was synthesized using a PrimeScript^{TM} RT reagent Kit with gDNA Eraser (Takara) and RNA extracted from the fresh liver tissues of triploid crucian carp (including control and Cd-treated groups) [11]. Primer sequences used in analyses are detailed in Supplementary Table **S1**, designed using Primer Premier 5.0 software. PCR was carried out on the Prism 7500 Sequence Detection 140 System [11]. For each sample, qPCR analysis was done on three replicate samples (Table **S1**). The relative expression ratio of target genes versus the β-actin gene was calculated using the $2^{-\triangle\triangle Ct}$ method [13].

2.10. Enzyme Activity Assay

Catalase (CAT) activity was estimated by CAT Detection Kit (Visible light) (Nanjing Jiancheng Bioengineering Institute, China). The activity of CAT could be calculated by measuring its change at 405 nm.

The activity of superoxide dismutase (SOD) was assayed using a SOD Detection Kit (Nanjing Jiancheng Bioengineering Institute). All reagents required for superoxide production and detection with Water Soluble Tetrazolium-1 (WST-1) were mixed in bulk. Rates of WST-1 reduction were measured using a microplate reader at 450 nm. The sample activity was calculated by relating its I50 to that of the standard SOD [14].

3. RESULTS

3.1. Effects of Cd-treatment on Embryos

In both of diploid and triploid crucian carp, only a few of embryos died (6.17%) at lower Cd^{2+} concentrations (≤3 mg/L). However, as the Cd^{2+} concentration exceeded 4 mg/L, the corresponding mortality rate increased dramatically (Fig. **1**). Embryos pre-fertilization were embedded in a gelatinous egg envelope (Fig. **2A, B**), and the Gel electrophoresis revealed no significant difference in triploid and diploid crucian carp egg envelope proteins (Fig. **2C**). For both triploid and diploid crucian carp the egg envelope Cd^{2+} contents of Cd-treated embryos was significantly higher than the Cd^{2+} content of Cd-treated embryophytes (Fig. **3**). To further understand the effects of egg envelope on Cd stress, we analyzed the mortality rates of embryos with or without egg envelopes (Table **S2**). Regression models were constructed using SPSS to express the confidence level of regression coefficients of embryo mortality rates with (1) and without (2) an egg envelope, where: rd is mortality rate, d is Cd^{2+} concentration, and t is the duration of exposure (time):

$$rd = \ln(4.60 \times 10{-}4dt + 0.018d + 1.217) \quad (1)$$

$$rd = 0.167d + 0.008t + 0.217 \quad (2)$$

Model 2 reveals increased Cd^{2+} concentration is the main reason for embryo mortality. The mortality rate of embryos with an envelope (model 1) is significantly dependent on the interaction between Cd^{2+} concentration and exposure time, and Cd^{2+} concentration. However, the mortality rate of embryos without an envelope (model 2) is not affected by an interaction between Cd^{2+} concentration and exposure time. Further statistical differences in these regression models are presented in Table **1**.

3.2. Effects of Cd Treatment on Larvae

Increased Cd^{2+} concentration led to increased mortality rate, though for any concentration the mortality rates of larval triploid crucian carp were significantly lower than diploid crucian carp (Fig. **4**). Cd^{2+} levels in larvae in acute toxicological groups were higher than in control groups, and also increased as Cd^{2+} concentrations increased (Fig. **5**). For any given

Table 1. Paired samples test.

W		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Embryo without film Normal Embryo	0.4587	0.2311	0.0770	0.2810	0.6364	5.953	8	0

concentration, Cd^{2+} levels in triploid crucian carp were also higher than those in diploid crucian carp (Fig. **5**).

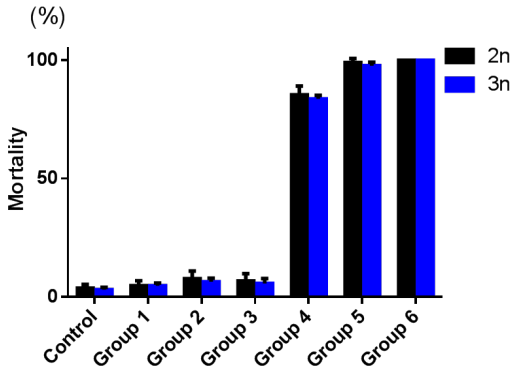

Fig. (1). Effect of different Cd^{2+} concentrations on embryos of triploid (3n) and diploid (2n) crucian carps. 1–6 represent groups treated with six different Cd^{2+} concentrations (1, 2, 3, 4, 6, 9 mg/L).

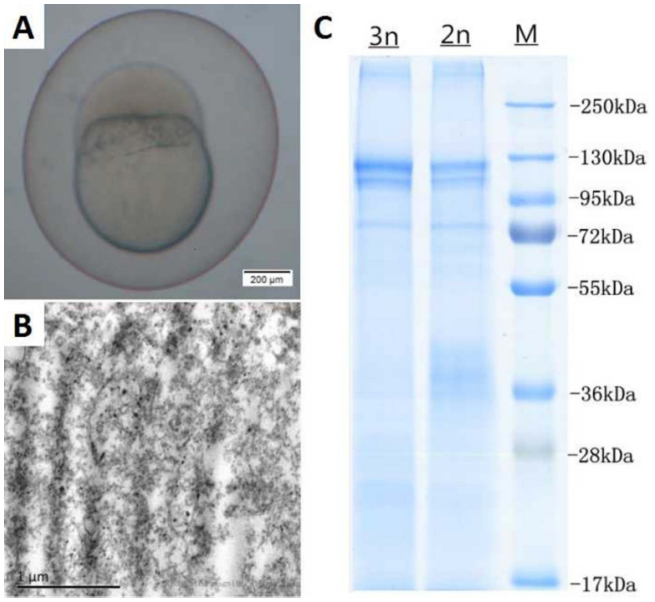

Fig. (2). Egg envelope structure in triploid crucian carp (**A**, **B**); gel electrophoresis analyses of egg envelope proteins from diploid (2n) and triploid (3n) crucian carps (**C**).

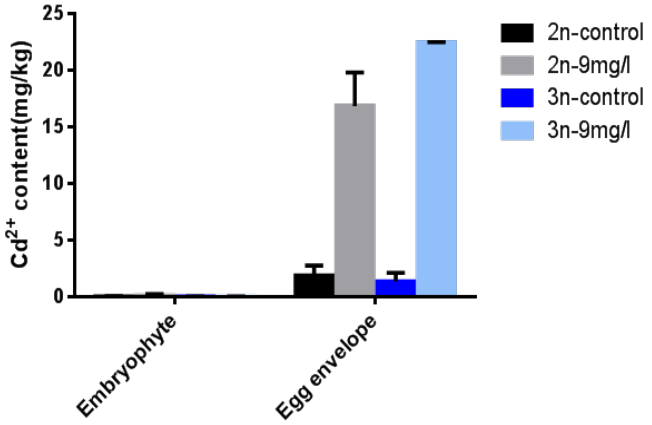

Fig. (3). Cd^{2+} level in the embryophyte and egg envelope.

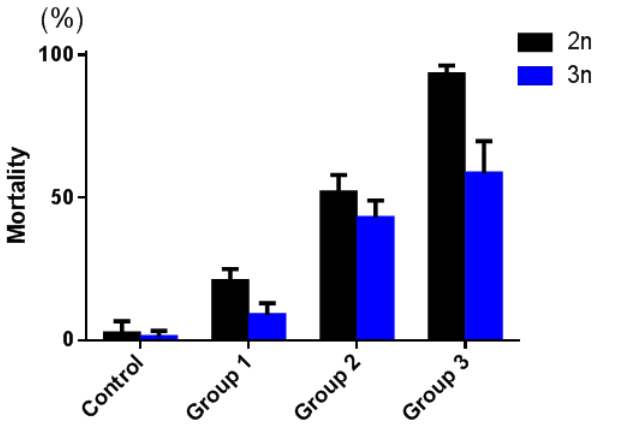

Fig. (4). Acute toxicological experiment of seven-day-old larvae of diploid (2n) and triploid (3n) crucian carps. 1–3 represent groups treated with three different Cd^{2+} concentrations (3, 6, 9 mg/L).

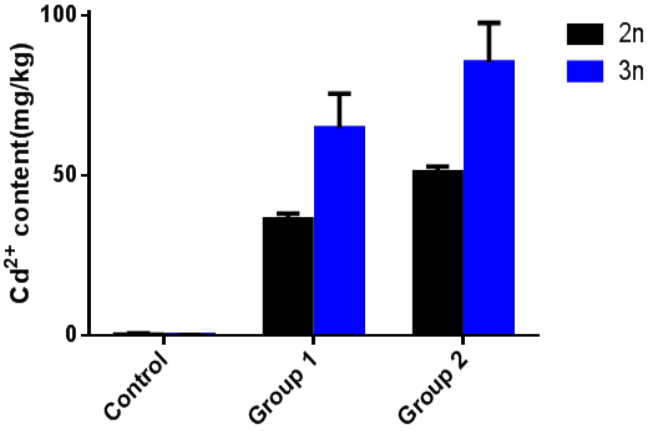

Fig. (5). The Cd^{2+} levels in larvae examined in acute toxicological experiments. 1 and 2 represent groups treated with two different Cd^{2+} concentrations (6 and 9 mg/L).

3.3. Effects of Cd Treatment on Adult Fish

Adult triploid crucian carp mortality was lower than for diploid individuals (Fig. **6**). Following acute toxicological experiments, the Cd^{2+} content of triploid and diploid crucian carp liver, gill, kidney, muscle and skin tissues differed (Fig. **7**). Post Cd treatment, histology revealed swollen liver cells, vacuolization (Fig. **8A–D**), and significant enlargement endoplasmic reticulum cavities (Fig. **8E, F**).

3.4. Transcription Analysis of Triploid Crucian Carp Liver Tissue Under Cd Stress

After filtering low quality and short sequences, clean reads were obtained for each raw read (Table **S3**). Unigenes were obtained by paired-end methods using Trinity and TGICL clustering, with maximum (26,769 bps) and minimum (301 bps) lengths, and averages 887.97 bps and N50:1315 bps.

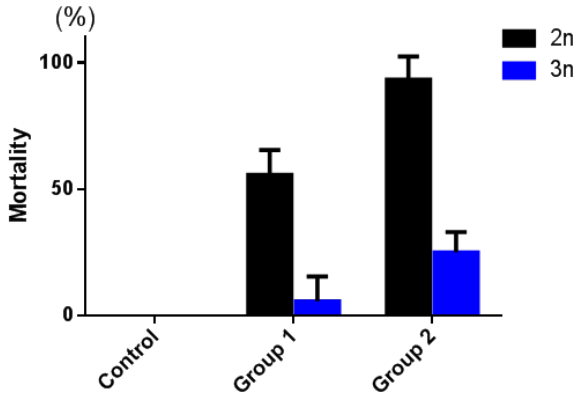

Fig. (6). Acute toxicological experiments of one-year-old fish of diploid (2n) and triploid (3n) crucian carps. 1 and 2 represent groups treated with two different Cd^{2+} concentrations (9 and 15 mg/L).

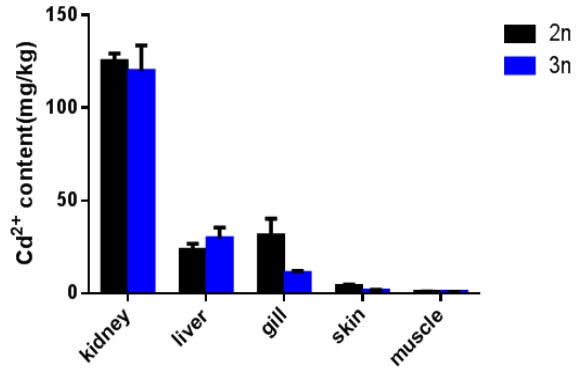

Fig. (7). Cd^{2+}content in crucian carp tissues in acute toxicological experiments.

Differentially expressed unigenes (DEGs) in control and treatment groups were counted using the FPKM method. We defined an expression level with fold change threshold value = 2 and FDR test (P<0.05) to filter genes with significant differential expression, and found the expression of 5,797 unigenes differed significantly between groups (2,785 up-regulated in treated groups, and 3,012 down-regulated relative to control-group levels).

GO enrichment significant analysis of the DEGs was implemented by hypergeometric distribution and the number of DEGs was counted in each GO term (Fig. **S1**). In biological process, ER-associated ubiquitin-dependent protein catabolic process (GO:0030433), rRNA processing (GO:0006364), and ER unfolded protein response (GO:0030968) were the most prominent terms; ER membrane (GO:0005789) was the most prominent within cellular component followed by nucleolus (GO:0005730), and mitochondrial inner membrane (GO:0005743).

To explore the regulating pathway under Cd stress in triploid crucian carp, significant DEGs were annotated to KEGG (1,236, of which 473 were

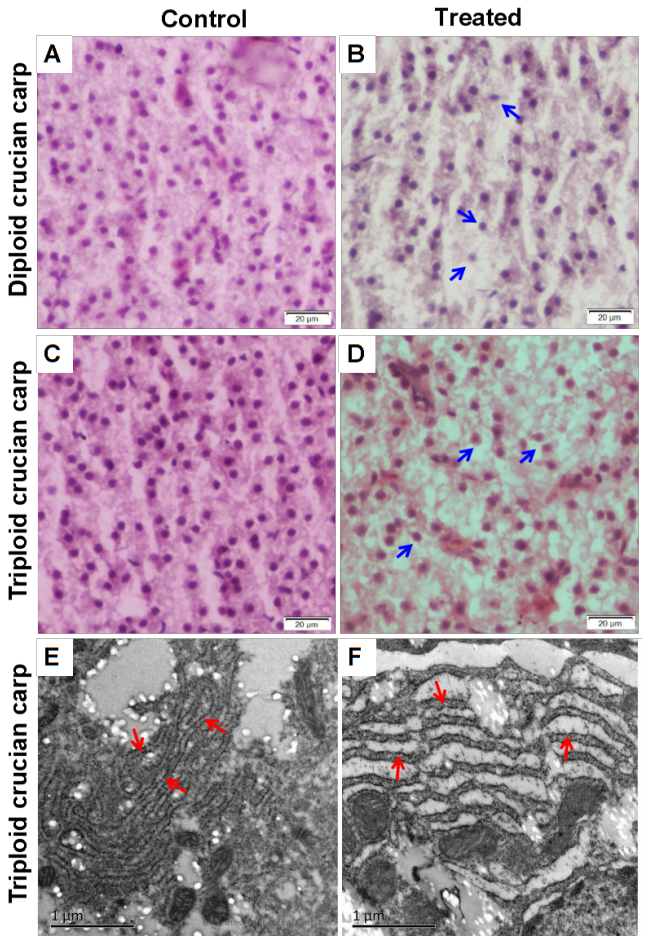

Fig. (8). Liver tissues of crucian carp exposed to Cd stress. Paraffin section of liver tissue. (**A**) diploid crucian carp control group, (**B**) diploid crucian carp Cd^{2+}-treated group, (**C**) triploid crucian carp control group, and (**D**) triploid crucian carp Cd^{2+}-treated group; scale bar = 20 um. (**E**, **F**) Electron microscopy section of liver tissue: (**E**) triploid crucian carp control, (**F**) triploid crucian carp Cd-treated group; blue arrows indicate vacuolated cells; red arrows indicate enlarged ER cavities; scale bar = 1um.

up-regulated and 763 were down-regulated after exposure of Cd) for enrichment analysis (Table **S4**). Results reveal 336 annotated pathways, of which a series (e.g., protein processing in ER, proteasome, protein export, fatty acid metabolism, PPAR signaling) were significantly enriched. In order to judge the DEGs identified by transcriptomic analysis, 14 DEGs with different expression patterns in the control and treatment groups were randomly selected for further qPCR assay on biological replicates. Of these DEGs, 12 qPCR expression patterns were consistent with data obtained from RNA-seq, where 10 unigenes were up-regulated and 2 unigenes were down-regulated (Fig. **9**).

3.5. Analysis of Related Genes of Oxidative Stress and ER Stress Expression Under Cd Treatment

From the GO and KEGG for enrichment analysis of DEGs, the regulation information of Cd stress in triploid

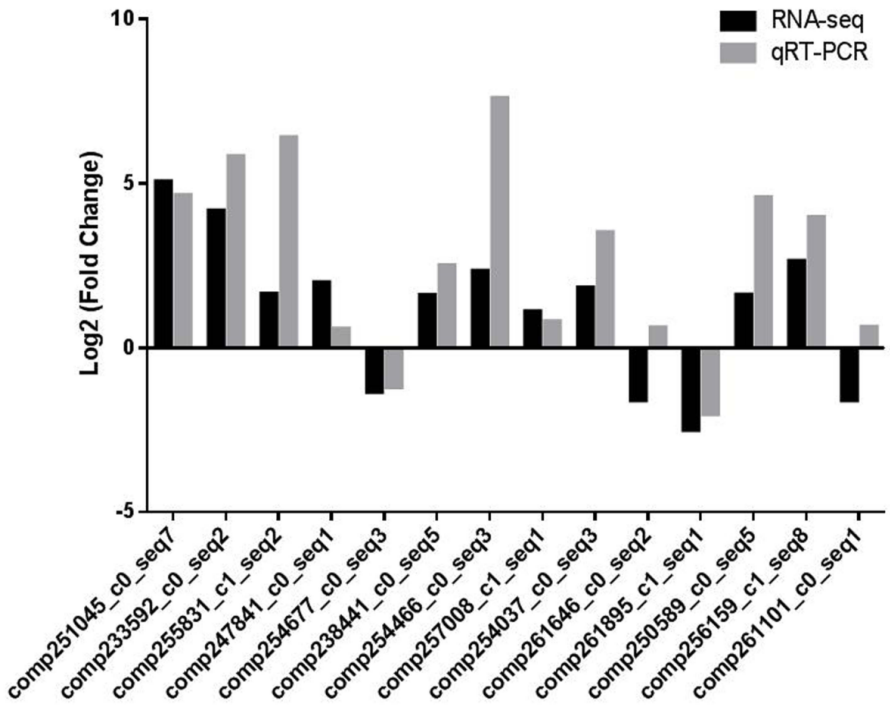

Fig. (9). Validation of RNA-seq results by qPCR.

crucian carp liver showed that Cd treatments caused series of changes in triploid crucian carp liver, for example, ER membrane proteins and the mitochondrial membrane. Further analysis of DEGs from transcriptional data found that many DEGs in triploid crucian carp were closely related to oxidative stress and ER stress response during Cd exposure (Table **S5**). Accordingly, we selected five key genes in oxidative stress (Sestrin-1, Nrf2, CAT, SOD1, GST) and four key genes in ER stress (ATF6, IRE1, PERK, XBP1) for the analysis of expression patterns in Cd-treated triploid and diploid crucian carp liver tissues.

In diploid crucian carp, four (Nrf-2, GST, CAT, SOD1) oxidative stress gene mRNA levels were up-regulated relative to levels in control carp, but in triploid crucian carp, all five were up-regulated (Fig. **10**). Of ER stress response genes, mRNA levels of ATF6 and PERK were down-regulated, IRE-1 was not significantly different in diploid crucian carp compared to the control group, while in triploid crucian carp, the two (IRE-1, PERK) gene mRNA levels up-regulated except ATF6. CAT activity in liver tissues of diploid crucian carp post-Cd exposure decreased significantly relative to levels in control carp, while that in liver tissues of triploid crucian carp was significantly up-regulated; SOD activities in liver tissues of both diploid and triploid crucian carp were significantly lower than levels in control carp (Fig. **12**).

4. DISCLOSURE

In the present study, we obtained following important results:1) Under Cd stress, the mortality and abnormality rates in triploid crucian carp were lower than those of diploid strains and the effects of Cd treatment on the liver were revealed by histological observation; 2) the liver transcriptomes of triploid crucian carp were obtained and the data was analyzed; 3) 14 genes related to Cd stress were screened from transcriptome data, and the expression of these genes was verified by Q-PCR. The results were consistent with the gene results in transcriptome data; 4) Q-PCR and enzyme activity analysis all confirmed triploid crucian carp resistance to Cd stress was regulated by both oxidative stress and ER stress responses; 5) We found that IRE-1 and PERK, not ATF-6, were involved in the enhance Cd resistance of triploid crucian carp. Together, these results demonstrate that triploid crucian carp have strong resistance.

Nuclear factor-erythroid 2-related factor 2 (Nrf2), a member of the Cap 'n' Collar family of transcription factors, plays an important role in the expression of antioxidant genes mediated by antioxidative response element (ARE) [15]. EFOX, which relies on COX-2, is added to the protein residue of thiol, cysteine and histidine to activate Nrf2, and could regulate the transcription of protective genes like GST and SOD to resist oxidative stress damage caused by various stimuli, thus protecting normal physiological functions of cells and tissues [16-20]. After exposure to Cd, we found antioxidant (e.g., Nrf2, CAT, SOD, GST) expression in both diploid and triploid crucian carp to be up-regulated, demonstrating Cd exposure caused changes in levels of oxidative stress in carp, with the biological response involving induction of antioxidant defense systems.

Expression of antioxidants (e.g. Nrf2, CAT, SOD, GST) in triploid crucian carp was greater than in diploid crucian carp. Additionally, levels of Sestrin-1 were down-regulated in diploid but significantly up-regulated

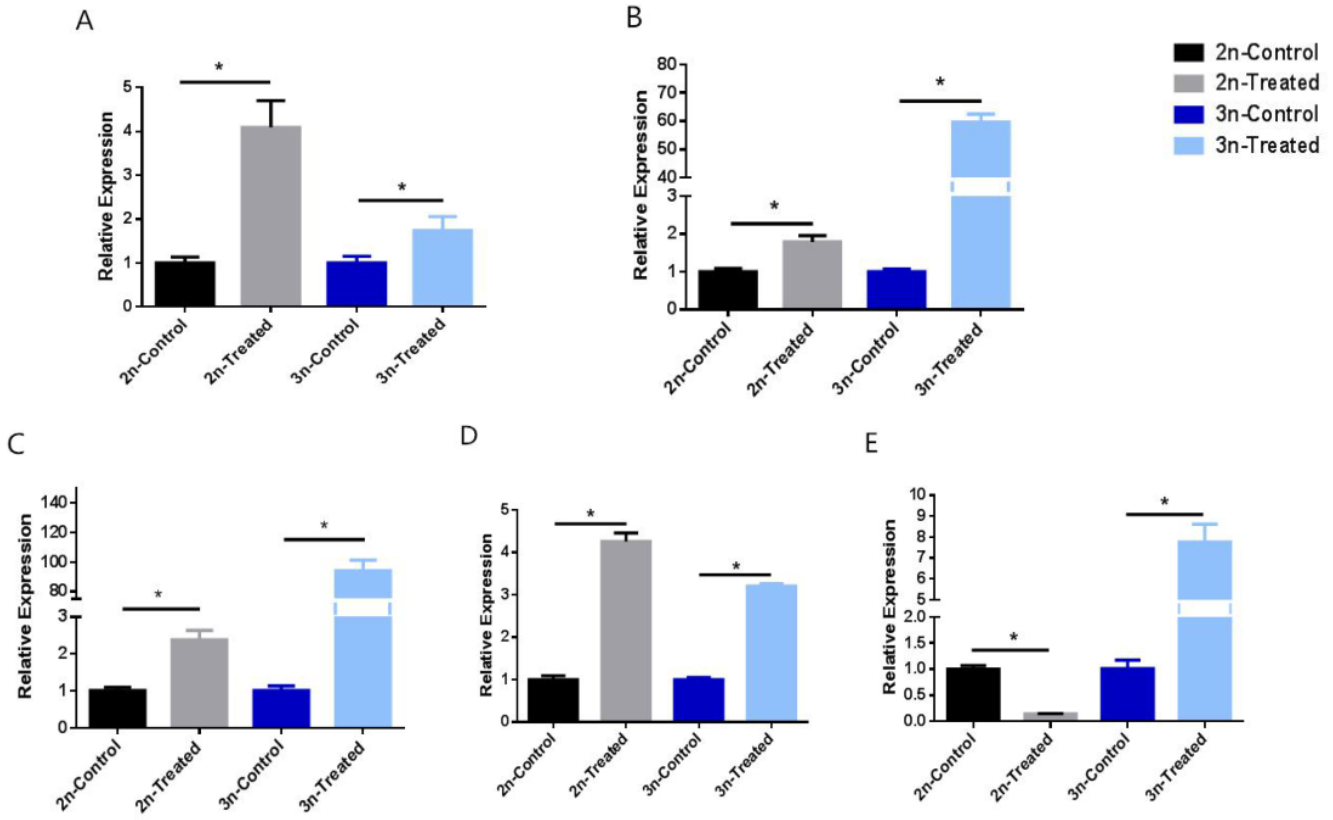

Fig. (10). Gene expression levels of oxidative stress in liver tissues of triploid and diploid crucian carp: (**A**) Nrf2, (**B**) CAT, (**C**) GST, (**D**) SOD1, (**E**) Sestrin-1.

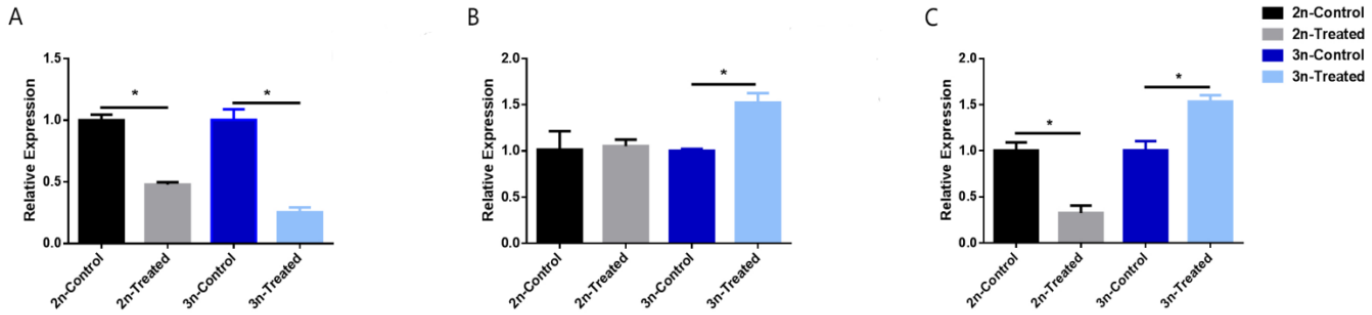

Fig. (11). Gene expression levels of ERS in liver tissues of triploid and diploid crucian carp: (**A**) ATF6, (**B**) IRE1, (**C**) PERK.

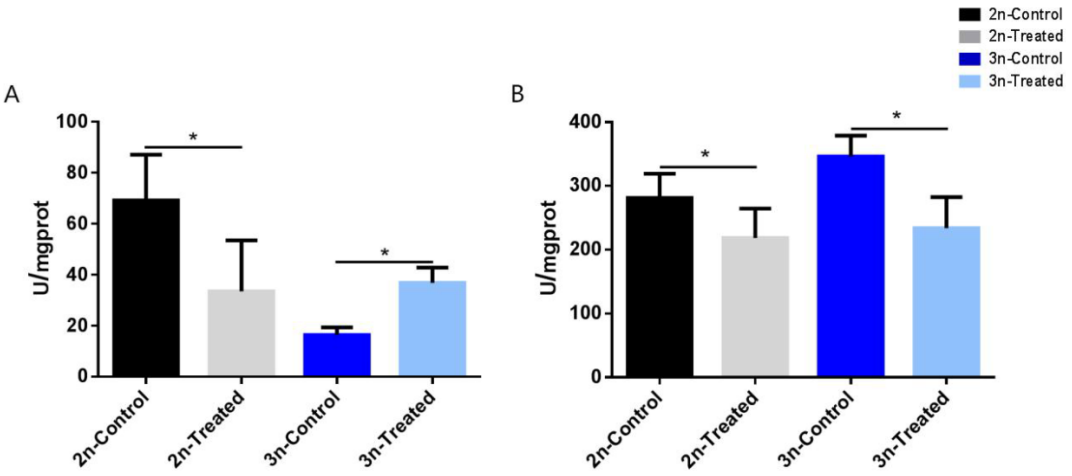

Fig. (12). CAT (**A**) and SOD (**B**) activity in triploid and diploid crucian carp liver tissues.

in triploid crucian carp. Sestrin, a highly conserved protein, is up-regulated when cells are exposed to various environmental stresses, such as DNA damage, hypoxia and oxidative stress [21]. Sestrin can promote autophagic degeneration of Keap1 and accelerate transcription of Nrf2-dependent antioxidant genes [21]. Genes silence of any member of the Sestrin family in a variety of cell lines could lead to ROS accumulation, DNA damage, chromosomal instability or cell death [22]. Sestrin-1 and Keap1 were combined to promote the autophagic degradation of Keap1 and to accelerate the transcription of Nrf2-dependent antioxidant genes, like GST, SOD, NQO1. This pathway could resist the oxidative stress damage caused by Cd exposure, protected the normal function of cells and tissues, thus, promote the triploid crucian carp survival. In contrast, lowered Sestrin-1 expression could result in Nrf2 not being dissociated from Keap1, possibly explaining the reduced survival of diploid compared with triploid crucian carp.

The ER is important for protein synthesis, lipid formation, and for storage of calcium ions in eukaryotic cells. Under conditions of ischemic hypoxia, calcium ion disorder, and viral infection, the wrong folding or unfolded proteins aggregate in the ER, disrupting the ERs physiological function and causing endoplasmic reticulum stress (ERS) which can stimulate the unfolded protein response (UPR) [13, 23]. However, excessive and prolonged ERS can lead to cell apoptosis [24]. UPR also requires expression of three kinds of stress receptor proteins (inositol-requiring enzyme 1 (IRE-1), activating transcription factor 6 (ATF-6), and pancreatic ER kinase (PKR)-like ER kinase (PERK)), down-regulation of the ERS signal, up-regulation of the stress-related gene [24]. When ERS does not occur, IRE-1, ATF6, and PERK are inactive for combined with GRP78/Bip. However, when ERS occurs, the accumulation of unfolded proteins causes GRP78/Bip to dissociate from three membrane proteins and turn to combine with unfolded proteins [25]. ERS is activated when free IRE-1 and PERK pass through the dimerization and sel-phosphorylation of the intracellular domains respectively, the ATF6 is transferred to the Golgi body after dissociation, inducing the transmission of the downstream information of ERS and expression of related genes [26, 27]. Our results demonstrate that ATF-6 was down-regulated in both triploid and diploid crucian carp following Cd exposure. However, the expressions of IRE-1 and PERK were significantly up-regulated in triploid crucian carp, but down-regulated in diploid crucian carp. Thus, we speculate that IRE-1 and PERK (but not ATF-6) are involved in relieving ERS induced by Cd exposure to enhance the Cd resistance of triploid crucian carp.

In addition, given the egg envelopes strongly adsorbed Cd, it might prove an effective bioremediation tool for reducing cadmium concentrations in polluted waters. This envelope might explain the apparent immunity of fish embryos to external stresses. We can further study the ultrastructure of the egg envelopes, explore its possible factors in protecting the embryo, and provide relevant information for future related research.

In a word, we should study the mechanism of aquatic fish resistance to Cd toxicity, look for new strains with strong resistance and low uptake, and popularize aquaculture in heavy metal polluted waters. At the same time, we should study the genetic mechanism and gene mapping of low uptake, and cultivate new fish varieties with strong resistance, low uptake, high yield and good quality through molecular biological technology such as genetic engineering. Ensuring safe production and aquaculture of aquatic products under Cd pollution and reducing the migration and harm of Cd to humans and livestock are of great theoretical and practical significance to study in healthy fish culture.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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