Cold Shock Induced Oxidative Stress, Apoptosis and Genome-wide Gene Expression Perturbation in the Eyes of Zebrafish and the Mitigation of Blue Wavelength Light

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Abstract

As global climate changes, severely temperature variations have significant impacts on survival and development of fish. While the potential effects of light wavelength on cold shock and associated mechanisms remain largely unknown in fish. Here, zebrafish were pre-exposed to white LEDs (an irradiance of 0.9 W/m^2) and blue LEDs (LDB, peak at 450 nm, 0.9 W/m^2) for 2 weeks, and then exposed to 26°C or 11°C for 48 hours, respectively. Cold shock led to low survival rate. Cold shock altered retinal structure, increased the number of apoptotic cells and Caspase-3 activity, inhibited superoxide dismutase (SOD) and catalase (CAT) activities, up-regulated mRNA expression of (NF-E2-related factor 2) *nrf2*, *p53*, *casp3* and *casp9*, and down-regulated *cat* expression in fish eyes. These results demonstrated that acute cold exposure induced oxidative stress and apoptosis in zebrafish eyes, which may lead to mortality. However, cold shock in combination with LDB apparently mitigated these negative effects, which might be involved in the up-regulation of antioxidant response and down-regulation of apoptotic responses at transcriptional and translational levels. Furthermore, cold shock also caused dysregulation of genome-wide gene expression involved in circadian rhythm, phototransduction and il-17 signaling pathway, indicating that cold shock disturbed phototransduction cascade and circadian rhythm signals and caused inflammatory responses. Ten key genes involved in circadian rhythm, phototransduction, cell cycle arrest, RNA processing or inflammatory responses were identified, including *muc5d*, *rnps1*, *si:dkey-243i1.1*, *opn1mw1*, *gadd45ba*, *cebpd*, *btg2*, *si:dkey-242g16.2*, *nr1d1* and *zgc:122979*, which may play an important role in the protection of LDB against cold shock. Finally, our study suggested the relationship between spectrum and cold stress and demonstrated LDB could protect fish against the negative effect of cold stress in the eyes.

1. Introduction

Water temperature is considered as the “abiotic master factor” for fish, which controls nearly all life activities, including growth, development, behavior, and reproduction (Donaldson et al., 2008). Although fish species own the ability to adapt to gradual temperature variations (Wang et al., 2022), sharp changes commonly lead to sublethal disorders and even mortality (Bacchetta et al., 2020). A rapid decline in water temperature, namely cold shock, has negative effects on metabolic processes (Islam et al., 2021; Jiao et al., 2020; Zhang et al., 2021), swimming ability (Szekeres et al., 2014), antioxidant and immune systems (Ale et al., 2021; Reda et al., 2020; Wen et al., 2018; Singh et al., 2021), and ultimately result in cellular apoptosis (Cheng et al., 2017). Furthermore, several documented events, referred to as “winterkills”, have occurred in wild and cultured fish populations (Doménech et al., 1997; Hoag, 2003; Conover et al., 2006), which lead to episodic die-off of fish and great economic losses.

In natural fish habitat, penetrability of different colors or wavelengths of light varies greatly, fish vision and spectrum perception match their living ethology (Brüning et al., 2016). This means that each species has an optimal wavelength where fish prefer to aggregate, grow and develop (Villamizar et al., 2011). Light emitting diodes (LEDs) have a longer life span and lower electrical running costs than traditional metal bulbs, in particular, could provide much more efficient lighting systems by using high energy short

wavelength in fish farming (Migaud et al., 2007). Several studies have reported effects of different colors of LEDs on growth and development in juvenile Atlantic cod and turbot (Sierra-Flores et al., 2016), European seabass larvae (Villamizar et al., 2009), and yellowtail damselfish (Shin et al., 2013). Other researches have confirmed the interactive effects between light spectrum and other environmental factors such as starvation, bacteria or virus (Choi et al., 2012; Roh et al., 2018a; Roh et al., 2018b; Ho et al., 2020). However, to the best of knowledge, few researches investigated the relationship between light spectrum composition and cold shock in fish (Kim et al., 2014).

Among the functional organs in fish, the eyes are an extension of the brain (Jiang et al., 2019), and are important sensory organs which provide approximately 80% of the information from all external stimuli (Carrasco, 2018). No eyelids and fixed pupils make the fish retina more vulnerable to the external environment (Park et al., 2013). The damage of eyes and retinal cells results in disorders of brain neuromodulation and hormone secretion, reduces finding/intake and predator avoidance, and ultimately results in a reduction of fish survival (Baumann et al., 2016). Although the morphological damage of eyes has been well known during cold stress and specific light spectrum for a long time in fish (O'Day and Young, 1979; Pointer et al., 2005), the underlying mechanisms are largely unknown in fish eyes.

Zebrafish are an excellent model for understanding the mechanisms of effects of cold shock or light spectrum on fish (Wang et al., 2014). The molecular mechanisms of cold stress have well been elucidated by RNA-seq in zebrafish larvae (Long et al., 2013; 2015). Our studies have suggested that blue LEDs (LDB) play a positive role in immune and antioxidant systems (Zheng et al., 2016a; Yuan et al., 2017a), and mitigated cadmium-induced toxicity in the liver of zebrafish (Yuan et al., 2017b). To test the hypothesis that LDB can protect fish against cold shock in fish, zebrafish were pre-exposed to white LEDs and blue LEDs for 2 weeks, and then exposed to 26°C or 11°C, respectively. Body weight, body length, and survival rate were measured. Eyes were sampled to examine histological changes, oxidative stress, apoptosis and transcriptomic responses. Oxidative stress was evaluated by measuring lipid peroxidation and the activity and mRNA levels of antioxidant enzymes. Cellular apoptosis was confirmed by detecting the activity levels of several caspases and mRNA expression of genes involved in apoptosis pathway, as well as TUNEL staining. RNA-seq was used to identify some key genes or pathways which were sensitively responsive to LDB and/or cold shock. The present study investigated the effects of LDB on cold shock and associated mechanisms in fish, which would provide a new insight for improving the overwintering ability of fish in aquaculture.

2. Materials And Methods

2.1. Experimental design

Three months old zebrafish (AB strain) were initially acclimated to white LEDs (LDW, control) for 2 weeks at $26.2 \pm 0.5^\circ\text{C}$ with a photoperiod of 14 h light and 10 h darkness before the experiment. The fish were fed twice daily (10.00 h and 18.00 h) with *Artemia* nauplii and commercially manufactured feeds. At the beginning of the experiment, healthy and uniform zebrafish were subjected to white LEDs (an irradiance

of 0.9 W/m²) and blue LEDs (LDB, peak at 450 nm, 0.9 W/m²) for 2 weeks, and then exposed to 26°C or 11°C for 48 hours, respectively. The pre-acclimation to LDB for 2 weeks was selected because it could induce fish resistance to cold shock. The setting of irradiance is determined according to our previous research (Yuan et al., 2017b; Zheng et al., 2016a). The low temperature at 11°C was selected for the cold shock experiment because fish started to die at this temperature after 12 h. The low water temperature was maintained by a constant circulation of cold water by using a thermostat. There were four groups in the experiment including the control, LDB, Cold, and LDB + Cold. Each treatment was assigned to 3 tanks with 40 fish per tank. In the present study. Survival rate was monitored at 6, 12, 24, 36 and 48 h. Body weight and body length were recorded at 24 h to confirm the effect of LDB on growth. Fish displaying no gill movement and no response to touch were considered as dead and removed. Eyes were sampled at 6 h and divided into three parts. The first part was immediately stored in RNAlater solution (Takara) for RNA extraction. The second part was fixed for histological and apoptotic observation. The final part is immediately placed in dry ice and transferred into refrigerator at -80 °C for biochemical parameters.

2.2. Histological and apoptotic observation

Eyes were fixed in eyeball fixed liquid (Servicebio), embedded in paraffin wax, stained with hematoxylin/eosin (H & E), and prepared for light microscopy according to our previous studies (Zheng et al., 2011). TUNEL (terminal deoxynucleotidyl transferase (TdT) - mediated dUTP nick end labeling) staining was performed using In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany) following the instruction of manufacturer. Generally, cells labeled with green fluorescence were considered to be apoptotic.

2.3. Biochemical analysis

Malondialdehyde (MDA) and activity levels of superoxide dismutase (SOD) and catalase (CAT) were investigated by commercial kits (Nanjing Jiancheng Biological Engineering Company), referred to our recent study (Zheng et al., 2016b). Caspase activities were examined by cleavage of colourless substrates specific for caspase-3 (Ac-DEVD-pNA), caspase-8 (Ac-IETD-pNA) and caspase-9 (Ac-LEHD-pNA) and release of the chromophorep nitroaniline (pNA). The examination methods were performed to the methods provided by Zheng et al (2021). Generally, aliquots of homogenate (10µL) were incubated at 37°C with assay buffer including 50 mm HEPES pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA and 10% glycerol. The reaction mixture was incubated at 37°C for 5 h and absorbance of the product was measured at 405 nm in a 96-well plate reader (Infinite 200 PRO, TECAN). Enzyme activity and protein content were calculated by normalizing the values to protein levels which were examined using Bradford method (Bradford, 1976). Enzyme activity was expressed as fold change relative to the control.

2.4. Real-time PCR

Examination of mRNA expression was carried out according to our recent study (Zhu et al., 2019), including the following steps: RNA extraction, cDNA synthesis, and real-time PCR. The primer sequences of all genes were referred to Supplementary Material in Table S1. The specificity of primer pair sequences

was confirmed by electrophoresis detection and melting curve analysis with a single DNA band or peak. Amplification efficiency was stable among all genes and different treatments. Relative mRNA levels of genes were calculated according to the $2^{-\Delta\Delta C_t}$ method, normalizing to the geometric mean of the best combination of *β -actin* and *gapdh* (Zheng et al., 2015).

2.5. Transcriptome analysis

The entire operation process of transcriptome analysis includes the following steps: RNA preparation, sequencing, assembly, annotation, identification of DEGs and KEGG enrichment pathway, as suggested by Zhu et al., (2020a). Generally, total RNA was isolated using TRIzol reagent (Takara Bio Inc., Tokyo, Japan). RNA quality analysis was performed using Bioanalyzer 2100 (Agilent, USA) ($RIN \geq 7.0$). Twelve cDNA libraries (three replicates per treatment) were constructed using the RNA Library Prep Kit for Illumina and sequenced on an Illumina HiSeq platform by Beijing Genomics Institute (BGI, China). Raw data were filtered to obtain clean reads by removal of adaptors, low-quality reads, and ambiguous reads. A total of 24932 non-redundant unigenes and their FPKM values (Fragments PerKilobase Million) were provided in Supplementary Material. Genes that met the cut-off criteria of $FDR < 0.01$ and a $|\log_2 \text{fold-change}| > 1.0$ were assigned as DEGs. FPKM values determined by RNAseq analysis significantly correlated with expression levels analyzed by qPCR ($R^2 = 0.8734$, $p < 0.05$) (Fig. S1), which supported the reliability of transcriptome data. To find target enrichment pathways, the DEGs were further carried out using online database (<http://www.webgestalt.org>). Functions with $FDR \leq 0.05$ were considered as significant enrichment.

2.6. Statistical analysis

Results are showed as mean $\pm$ standard error (SEM). Prior to statistical analysis, all data were tested for normality of distribution using the Kolmogorov–Smirnov test. Data were analyzed using a two-way ANOVA to identify the main effects of LDB and cold shock and their interactions. Pairwise comparisons were carried out by Bonferroni t-test if significance was found ($p < 0.05$). Analyses were performed using SPSS 18.0 for Windows (SPSS, Michigan Avenue, Chicago, IL, USA).

3. Results

3.1. Growth and survival

Body weight (Fig. 1A) and body length (Fig. 1B) were not affected by LDB, cold shock, and their interaction ($p > 0.05$) (Table 1). On the contrary, survival rate was significantly impacted by LDB, cold shock, and their interaction at 12 h, 24 h, 36 h and 48 h ($p > 0.05$) (Table 1). Generally, cold shock sharply reduced survival rate at 24 h, 36 h and 48 h under LDW and LDB ($p < 0.01$), however, the LDB + Cold group showed higher survival rate than the Cold group ($p < 0.05$).

Table 1

Summary of two-way ANOVA between LDB and cold shock on growth, survival, and thickness of different retina layers in zebrafish

Parameters	Source of variation	<i>F</i>	<i>P</i>
Body weight	Cold	0.838	0.440
	LDB	0.008	0.931
	Cold × LDB	0.071	0.795
Body length	Cold	2.052	0.178
	LDB	0.082	0.779
	Cold × LDB	0.009	0.926
Survival rate (12 h)	Cold	16	0.0040**
	LDB	16	0.0040**
	Cold × LDB	16	0.0040**
Survival rate (24 h)	Cold	384	<0.001***
	LDB	384	<0.001***
	Cold × LDB	96	<0.001***
Survival rate (36 h)	Cold	1633	<0.001***
	LDB	37.5	<0.001***
	Cold × LDB	37.7	<0.001***
Survival rate (48 h)	Cold	4107	<0.001***
	LDB	27	0.001**
	Cold × LDB	27	0.001**
OS thickness	Cold	13.437	0.001**
	LDB	0.024	0.977
	Cold × LDB	2.944	0.097
ONL thickness	Cold	16.216	<0.001***
	LDB	0.288	0.596
	Cold × LDB	1.459	0.237
OPL thickness	Cold	6.698	0.015*

Data are means ± SEM (n = 3–12). *, ** and *** means significant differences ($p < .05$), ($p < .01$) and ($p < .001$)

Parameters	Source of variation	<i>F</i>	<i>P</i>
INL thickness	LDB	7.860	0.009**
	Cold × LDB	1.674	0.206
	Cold	0.010	0.921
IPL thickness	LDB	0.251	0.620
	Cold × LDB	4.041	0.055
	Cold	9.830	0.004**
	LDB	0.988	0.329
	Cold × LDB	9.767	0.004**
Data are means ± SEM (n = 3–12). *, ** and *** means significant differences ($p < .05$), ($p < .01$) and ($p < .001$)			

3.2 Histological changes

Histological alterations of retina were shown in Fig. 2. The retinal cross-section of zebrafish from inside to outside is inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nucleus layer (ONL), and outer segment (OS), respectively (E) (Fig. 2E). There were high cell densities in the different layers of retina in the control and LDB groups, particularly in the ONL and INL (Fig. 2A and 2C). The cell densities in the ONL and INL were low in the Cold group (Fig. 2B), but were not apparent in the LDB + Cold group (Fig. 2D). The thickness of different retina layers including OS, ONL, OPL and IPL increased in the Cold group ($p < 0.05$), and remained relatively unchanged in the LDB group compared with the control ($p > 0.05$) (Fig. 2F). However, cold shock with LDB reduced the thickness of OPL and IPL compared with cold shock singly ($p > 0.05$). Two-way ANOVA analysis confirmed that the thickness of OS, ONL, OPL and IPL was strongly associated with cold shock ($p < 0.05$) (Table 1). Besides, LDB and the combination of LDB and cold exhibited a significant difference on the OPL and IPL thickness, respectively ($p < 0.05$) (Table 1).

3.3 Apoptotic response

Apoptotic cells were found in the OS of retina in the all groups including the control (Fig. 3A), Cold (Fig. 3B), LDB (Fig. 3C), and LDB + Cold (Fig. 3D). However, the Cold group showed more apoptotic cells than the other groups (Fig. 3E).

3.4 Oxidative stress and apoptotic responses

The MDA content, the activity levels of SOD, CAT, Caspase-3, Caspase-8 and Caspase-9, and the mRNA levels of *nrf2*, *sod1*, *sod2*, *cat*, *p53*, *casp3* and *casp9* were significantly responsive to LDB or cold or their interaction (Table 2). Compared with the control, cold shock reduced *cat* expression and activity levels of SOD and CAT, and increased Caspase-3 activity and mRNA levels of *nrf2*, *p53*, *casp3* and *casp9* ($p < 0.05$).

(Fig. 4). Compared with the single cold stress, cold shock in combination with LDB decreased MDA content, *casp9* expression, and activity levels of Caspase-3, Caspase-8 and Caspase-9, and enhanced mRNA expression of *sod1* and *cat* and activity levels of SOD and CAT ($p < 0.05$). Compared with the control, LDB up-regulated mRNA expression of *nrf2*, *sod2*, *cat*, *casp3* and *casp9* ($p < 0.05$).

Table 2

Summary of two-way ANOVA between LDB and cold shock on parameters involved in oxidative stress and apoptosis in the eyes of zebrafish

Parameters	Source of variation	<i>F</i>	<i>P</i>
MDA	Cold	0.969	0.354
	LDB	10.407	0.012*
	Cold × LDB	1.651	0.235
SOD activity	Cold	0.932	0.362
	LDB	10.178	0.013*
	Cold × LDB	6.813	0.031*
CAT activity	Cold	6.490	0.034*
	LDB	0.597	0.462
	Cold × LDB	17.594	0.003**
Caspase-3 activity	Cold	9.990	0.014*
	LDB	14.809	0.006**
	Cold × LDB	5.414	0.048*
Caspase-8 activity	Cold	0.027	0.873
	LDB	9.718	0.014*
	Cold × LDB	7.932	0.013*
Caspase-9 activity	Cold	0.814	0.393
	LDB	8.610	0.019*
	Cold × LDB	10.556	0.012*
<i>nrf2</i> expression	Cold	14.238	0.005**
	LDB	4.668	0.063
	Cold × LDB	1.041	0.337
<i>sod1</i> expression	Cold	9.248	0.016*
	LDB	15.241	0.005**
	Cold × LDB	2.282	0.169
<i>sod2</i> expression	Cold	0.005	0.944

Data are means ± SEM (n = 3). *, ** and *** means significant differences ($p < .05$), ($p < .01$) and ($p < .001$)

Parameters	Source of variation	<i>F</i>	<i>P</i>
	LDB	7.676	0.024*
	Cold × LDB	0.438	0.527
<i>cat</i> expression	Cold	2.556	0.147
	LDB	55.084	<0.001***
	Cold × LDB	3.675	0.092
<i>p53</i> expression	Cold	5.991	0.040*
	LDB	0.869	0.378
	Cold × LDB	0.486	0.505
<i>casp3</i> expression	Cold	20.632	0.002**
	LDB	0.347	0.572
	Cold × LDB	15.571	0.004**
<i>casp8</i> expression	Cold	0.130	0.728
	LDB	0.540	0.483
	Cold × LDB	1.184	0.308
<i>casp9</i> expression	Cold	29.150	0.001**
	LDB	0.132	0.726
	Cold × LDB	24.874	0.001**
Data are means ± SEM (n = 3). *, ** and *** means significant differences ($p < .05$), ($p < .01$) and ($p < .001$)			

3.5. Transcriptomic analysis

A total of 1246, 11 and 6 differently expressed genes (DEGs) were observed in the eyes of zebrafish from sample groups Cold/Control, LDB/Control, and LDB + Cold/Cold respectively (Fig. 5A). The number of up-regulated DEGs was 838, 3 and 5 while the number of down-regulated DEGs was 408, 8 and 1 for Cold/Control, LDB/Control, and LDB + Cold/Cold respectively (Fig. 5B). A total of 3 and 7 DEGs were shared between LDB + Cold/Cold and Cold/Control and between LDB/Control and Cold/Control, respectively (Fig. 5C). Among these genes, *muc5d* (mucin 5d) was up-regulated in the Cold group compared with the control ($p < 0.001$) while LDB with cold shock reduced its expression compared with the single cold exposure ($p < 0.01$) (Fig. 5D). Expression levels of *mnp1* (RNA-binding protein with serine-rich domain 1) and *si:dkey-243i1.1* declined in the Cold group compared with the control ($p < 0.01$) while both genes were up-regulated by LDB + Cold compared with cold shock alone ($p < 0.01$) (Fig. 5E and 5F).

Cold shock sharply reduced mRNA expression of *opn1mw1* (medium-wave-sensitive opsin 3) under LDW and LDB ($p < 0.001$) while LDB up-regulated its expression under normal temperature ($p < 0.001$) and cold conditions ($p < 0.05$) (Fig. 5G). The other genes were up-regulated by cold stress under LDW and LDB and were down-regulated by LDB under normal temperature condition including *gadd45ba* (growth arrest and DNA damage-inducible protein) (Fig. 5H), *cebpd* (Fig. 5I), *btg2* (anti-proliferative cofactor) (Fig. 5J), *si:dkey-242g16.2* (Fig. 5K), *nr1d1* (Fig. 5L) and *zgc:122979* (Fig. 5M).

Several significantly enriched pathways were observed by KEGG and Gene Ontology (GO) analysis of DEGs in zebrafish exposed to cold shock (Fig. 6). DEGs are mainly related to “circadian rhythm”, “il-17 signaling pathway” and “phototransduction” according to KEGG enrichment (Fig. 6A), and several Go terms namely “circadian regulation of gene expression”, “photoperiodism”, “visual perception” and “response to light stimulus” according to GO enrichment (Fig. 6B). For circadian rhythm pathway, a total of 17 DEGs were confirmed (Fig. 6C). Among these these, 9 genes were up-regulated including *per1a* (period circadian clock 1a), *per1b*, *per2*, *per3*, *bhlhe40* (BHLH protein DEC1), *bhlhe41*, *nr1d1* (Nuclear receptor subfamily 1 group D member 1) and *cry2* (cryptochrome circadian regulator 2) while 8 genes were down-regulated including *clocka* (clock circadian regulator a), *clockb*, *cry3a*, *cry4*, *arntl1a* (Aryl hydrocarbon receptor nuclear translocator-like 1a), *arntl1b*, *fbxl3l* (F-box and leucine-rich repeat protein 3, like) and *npas2* (neuronal PAS domain-containing protein 2). For il-17 signaling pathway, 17 DEGs were up-regulated including *il1b* (Interleukin-1 beta), *il17c*, *cxc18a* (Interleukin-8), *fosb* (FBJ murine osteosarcoma viral oncogene homolog B), *fosaa*, *fosab*, *jun* (proto-oncogene c-Jun), *mapk6* (mitogen-activated protein kinase), *ccl20a.3* (C-C motif chemokine), *ptgs2a* (cyclooxygenase-2), *ptgs2b*, *cebpb* (CCAAT/enhancer-binding protein), *jund* (transcription factor jun-D), *nfkbiaa* (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha a), *nfkbiab*, and *fosl1a* (FOS-like 1, AP-1 transcription factor subunit a) (Fig. 6D). For phototransduction, a total of 10 DEGs were confirmed (Fig. 6E). Among these these, 4 genes were up-regulated including *guca1a* (guanylyl cyclase-activating protein 1), *guca1b*, *saga* (retina and pineal gland a), and *grk7a* (rhodopsin kinase), and 6 genes were down-regulated including *opn1mw1*, *opn1mw3*, *opn1mw4*, *gngt1* (guanine nucleotide-binding protein subunit gamma), *grk1b* (G protein-coupled receptor kinase), and *gnb1a* (guanine nucleotide-binding protein subunit beta-1).

4. Discussion

Acute decreases in water temperature have led to large losses of production and economy for many fish species in aquaculture and wild environments (Wu et al., 2011), which results in that studying the mechanisms of cold resistance are fascinating. In the present study, we found that cold shock damaged retinal structure and reduced survival rate in zebrafish, and demonstrated that the related mechanisms were involved in oxidative stress, apoptosis, and genome-wide gene expression dysregulation. Furthermore, we found that LDB could mitigate cold stress and improve fish survival, which may provide a cheap and effective method to improve cold tolerance of fish in aquaculture.

The injury of eyes may be due to excessive reactive oxygen species (ROS), as many existing studies have shown that ROS is produced when fish are subjected to cold stress (Wu et al., 2015; Cheng et al., 2017;

Ghisaura et al., 2019). To scavenge excess ROS and reduce its toxic effects, organisms have evolved an antioxidant system. SOD promotes the conversion of $\cdot\text{O}^{2-}$ into H_2O_2 which is subsequently catalyzed into H_2O by CAT, playing a vital role in the removal of ROS. However, when ROS-scavenging system cannot effectively neutralize or eliminate the excess of ROS, oxidative damage would happen (Wang et al., 2019). In the present study, the reduction in SOD and CAT activities and *cat* expression were observed in the eyes of zebrafish exposed to cold shock, which may be associated with oxidative damage of eyes. Similarly, several studies have reported that cold shock changed activity and mRNA expression of antioxidant genes and caused oxidative damage in the liver, gill and brain of zebrafish (Tseng et al., 2011; Wu et al., 2015). This concept was further supported by that cold shock induced apoptosis as reflected by increases in Caspase-3 activity and mRNA expression of *casp3*, *casp9* and *p53*, as well as the increased apoptotic cells in the eyes of zebrafish. Among apoptosis related genes, *p53* was the first tumor suppressor gene linked to apoptosis, and can promotes apoptosis via up-regulating pro-apoptosis genes such as *casp3* and *casp9* (Perfettini et al., 2004). Low temperature induced apoptosis by up-regulating *casp3*, *casp9* and *p53* in the liver of pufferfish and grouper (Cheng et al., 2017; Sun et al., 2019). In the present study, the positive relationships between CAT activity and *cat* expression and between Caspase-3 activity and *casp3* expression were confirmed during cold stress, suggesting these genes were transcriptionally regulated. On the contrary, enzyme activity levels did not always parallel with corresponding gene expression such as Caspase-9 activity and *casp9* expression. This may be associated with time-lag effect between transcription and translation, post-translational protein modification or RNA stability, as suggested by Zheng et al (2016c). Therefore, our results indicate that cold shock may cause eye damage by inducing oxidative stress and apoptosis at transcriptional and post-transcriptional levels.

Up to now, no published data is available on the relationship between between light spectrum and cold stress and the potential mechanisms that LDB could mitigate cold stress. However, several previous studies have indicated that there are positive effects of LDB on growth, development, immune and antioxidant responses (Villamizar et al., 2011; Shin et al., 2013; Kim et al., 2014; Sierra-Flores et al., 2016; Choi et al., 2017; Ho et al., 2020). The present study observed demonstrated that LDB alleviated oxidative stress and apoptosis induced by cold shock. For example, compared with the

This may be another possible mechanism that enabled fish to respond more rapidly to subsequent cold stress. The increased *nrf2* expression paralleled with the up-regulated mRNA levels of *sod2* and *cat*, suggesting that both antioxidant genes are transcriptionally regulated by *nrf2* (Osburn and Kensler, 2008; Zheng et al., 2019). However, the potential negative effects of LDB on fish should be not neglected. Although low intensity blue light cannot cause acute stress and histological damage of eyes, the retina may be damaged during chronic irradiation for a long time or high intensity blue light (Migaud et al., 2007). In the present study, LDB alone did not significant affect growth, lipid peroxidation and caspase activity, but the *casp3* and *casp9* were significantly up-regulated compared with the control. This may be an early warning sign of eye injury. Therefore, light intensity and irradiation time should be considered with precautions when LEDs are used as a technique to improve cold resistance in fish.

To understand the potential mechanisms of cold shock and the protective role of LDB on cold stress, comparative transcriptomic profiles were examined. Studies on transcriptomic responses have focused on the liver and larvae when fish are subjected to cold stress (Long et al., 2012; Ge et al., 2020; Zhu et al., 2020b; Song and McDowell, 2021; Zhuo et al., 2021), with little information on effects of cold stress or light spectrum on transcriptome of eyes. In the present study, DEGs were significantly enriched in the pathways of circadian rhythm in the eye of zebrafish exposed to cold shock. It has well been known that eyes and brain are responsive to circadian oscillators (Zhao and Fent, 2016). Circadian rhythm genes in eyes exhibit higher intensity of oscillations than brain in zebrafish (Whitmore et al., 1998). There are a few transcription factors or core circadian genes which control all circadian gene expression, including *clock*, *arntl*, *per*, *cry*, *nr1d*, *npas2*, *bhlhe40/41* in mammals (Ko and Takahashi, 2006). CLOCK or NPAS2 and ARNTLs heterodimers activate the transcription of core clock genes and clock-controlled genes and enhance metabolic processes by a positive limb of the feedback loop. The heterodimers also activate nuclear receptors which repress transcription of CLOCK/ARNTL and CLOCK/NPAS2 heterodimers by a negative limb of the feedback loop. Similarly, the core clock genes PER and CRY which are transcriptional repressors interact with the heterodimers by a negative limb of the feedback loop and inhibit its activity and thereby negatively regulate their own expression (Sen and Hoffmann, 2020). BHLHE40 represses the activity of CLOCK/ARNTL heterodimer by competing for the binding to E-box elements in the promoters of its target genes (Sato et al., 2004). Therefore, the up-regulation of *per1a*, *per1b*, *per2*, *per3*, *bhlhe40*, *bhlhe41*, *nr1d1* and *cry2* and the down-regulation of *clocka*, *clockb*, *arntl1a*, *arntl1b*, *npas2* may indicate a negative regulation of circadian rhythm in the eyes of zebrafish exposed to cold stress. Furthermore, we also found that DEGs in phototransduction were dysregulated and DEGs in il-17 signaling pathways were up-regulated in zebrafish exposed to cold shock, possibly indicating that cold stress disturbed phototransduction cascade signal and caused inflammatory responses.

In the present study, 10 genes may play an key role in the protection of LDB against cold shock by transcriptomic analysis, including *muc5d*, *rnps1*, *si:dkey-243i1.1*, *opn1mw1*, *gadd45ba*, *cebpd*, *btg2*, *si:dkey-242g16.2*, *nr1d1* and *zgc:122979*. Zebrafish *muc5d* is a gel-forming secreted mucin gene, which is highly homologous to other vertebrate mucins in their genomic and protein domain organization (Jevtov et al., 2014). Transcription of *muc5* results in mucus hypersecretion, which are closely associated with oxidative stress and many chronic inflammatory airway diseases in mammals (Rada et al., 2011). RNPS1 is a subunit of the postslicing complex and directly serves as a mark for mRNA surveillance which subjects aberrant mRNAs to nonsense-mediated decay (Lykke-Andersen et al., 2001). *si:dkey-243i1.1* participates in centriole replication which is important for cell polarization and division (Howe (Howe et al., 2013; Firat-Karalar and Stearns, 2014). In the present study, LDB cold shock up-regulated *muc5d* and down-regulated *rnps1* and *si:dkey-243i1.1*, indicating that cold stress may cause inflammation and inhibit RNA processing and cell division. However, LDB mitigated the negative effects of cold stress on gene expression. The opsin encoded by *opn1mw1* is a light-sensitive protein (Lamb, 2013), which is important for maintaining the normal function in the visual cycle in zebrafish (Shi et al., 2019). Although *opn1mw1* was dramatically down-regulated by cold stress, LDB significantly increased *opn1mw1* expression under normal temperature and cold shock conditions compared with cold shock alone, suggesting that LDB

could alleviate phototransduction damage induced by cold stress. The other 6 genes such as *gadd45ba*, *cebpd*, *btg2*, *si:dkey-242g16.2*, *nr1d1* and *zgc:122979* showed a similar change that cold stress up-regulated these genes under LDW and LDB while LDB significantly down-regulated their expression under normal temperature condition. CEBPD is a transcription activator regulating the expression of genes involved in inflammatory responses (Kinoshita et al., 1992). GADD45 and BTG2 negatively regulate of mitotic cell cycle (Jin et al., 2000; Gaudet et al., 2011). NR1D1 negatively regulates transcription of CLOCK/ARNTL and CLOCK/NPAS2 heterodimers to inhibit metabolomic processes (Ko and Takahashi, 2006). Therefore, the down-regulation of *gadd45ba*, *cebpd*, *btg2* and *nr1d1* induced by LDB may contribute to reduce inflammation and increase cell division and metabolism, which is possibly involved in the protection of LDB against cold shock.

5. Conclusion

The use of LED technology has increased considerably in the last few years because LEDs have a long-life span and low electrical running costs, in particularly, can be manufactured to yield specific wavelengths according to a species' environmental requirements (Sánchez-Vázquez et al., 2019). For zebrafish, blue LEDs are an effective light source, which can be used to mitigate cold stress and increase survival rate by inhibiting oxidative damage and apoptosis and regulating genome-wide gene expression in the eyes. The present study confirmed the importance of light spectrum in resisting cold shock in fish. However, further studies are required to investigate whether other cultured fish species have a preference for a specific light spectrum and whether the light spectrum can increase the tolerance to cold shock.

Declarations

Author Declarations

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Conflicts of interest

The authors declare that they have no conflict of interest.

Ethics declarations

This animal experiment was permitted by Animal Experimental Ethical Inspection, Institutional Animals Care and Use Committee of Zhejiang Ocean University (2021023).

Authors' contributions

P LB, operated the experiment, collected samples, extracted data, carried out the data analysis, interpreted results, and prepared the manuscript draft; H T, C X, W D, Z QL and W Z: operated the experiment and collected samples; W P and Z JL: developed the protocol and search strategy, supervised the study, screened all samples for eligibility, reviewed and revised the manuscript, and provided financial support.

Data availability

The data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability

Not applicable.

Informed consent

Not applicable.

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Figures

Figure 1

Effects of treatment with blue LEDs (LDB) irradiation on body weight (A), body length (B), and survival rate (C) of zebrafish subjected to cold shock. Data are means $\pm$ SEM (n = 3-12). *, ** and *** means significant differences (p < 0.05), (p < 0.01) and (p < 0.001) between Cold and Control or between LDB and LDB + Cold. #, ## and ### means significant differences (p < 0.05), (p < 0.01) and (p < 0.001) between LDB and Control or between Cold and LDB + Cold.

Figure 2

Effects of treatment with blue LEDs (LDB) irradiation on histopathological alterations in the eyes of zebrafish subjected to cold shock. Transverse sections of retina in zebrafish subjected to Control (A), Cold (B), LDB (C), LDB + Cold (D) groups. The retinal cross-section of zebrafish is inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nucleus layer (ONL), and outer segment (OS), respectively (E). Histopathological alterations in the retina in the eyes of zebrafish (F). Data are means $\pm$ SEM (n = 8). *, ** and *** means significant differences ($p < 0.05$), ($p < 0.01$) and ($p < 0.001$) between Cold and Control or between LDB and LDB + Cold. #, ## and ### means significant differences ($p < 0.05$), ($p < 0.01$) and ($p < 0.001$) between LDB and Control or between Cold and LDB + Cold.

Figure 3

Effects of treatment with blue LEDs (LDB) irradiation on apoptosis in the eyes of zebrafish subjected to cold shock. Transverse sections of retina in zebrafish subjected to Control (A), Cold (B), LDB (C), LDB + Cold (D) groups. Green fluorescence by TUNEL staining denotes apoptosis in the outer segment (OS) of eyes of zebrafish. E, number of apoptotic cells relative to the control. *, ** and *** means significant differences ($p < 0.05$), ($p < 0.01$) and ($p < 0.001$) between Cold and Control or between LDB and LDB +

Cold. #, ## and ### means significant differences ($p < 0.05$), ($p < 0.01$) and ($p < 0.001$) between LDB and Control or between Cold and LDB + Cold.

Figure 4

Effects of treatment with blue LEDs (LDB) irradiation on oxidative stress and apoptosis in the eyes of zebrafish subjected to cold shock. Data are means $\pm$ SEM ($n = 3$). *, ** and *** means significant differences ($p < 0.05$), ($p < 0.01$) and ($p < 0.001$) between Cold and Control or between LDB and LDB + Cold. #, ## and ### means significant differences ($p < 0.05$), ($p < 0.01$) and ($p < 0.001$) between LDB and Control or between Cold and LDB + Cold.

Figure 5

Brief summary of DEGs in the eyes of zebrafish subjected to cold shock with or without treatment of blue LEDs (LDB) irradiation. A, heatmap showing the overall changes of DEGs among groups; B, histogram showing number of up-regulated or down-regulated DEGs in each group; C-M, Venn diagram and histogram showing key genes that are responsive to both LDB and cold shock. *, ** and *** means significant differences ($p < 0.05$), ($p < 0.01$) and ($p < 0.001$) between Cold and Control or between LDB

and LDB + Cold. #, ## and ### means significant differences ($p < 0.05$), ($p < 0.01$) and ($p < 0.001$) between LDB and Control or between Cold and LDB + Cold.

Figure 6

Pathways of KEGG (A) and Gene Ontology (B) of significant enrichment in the eyes of zebrafish subjected to cold shock with or without treatment of blue LEDs (LDB) irradiation. Heatmap showing the expression of genes involved in the KEGG analysis, mainly related to circadian rhythm (C), IL-17 signaling pathway (D), and phototransduction (E).