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Effect of Lipopolysaccharide (LPS) stimulation on apoptotic process and oxidative stress in fibroblast cell of hybrid crucian carp compared with those of *Carassius cuvieri* and *Carassius auratus* red var

Sheng-Wei Luo *, Ning-Xia Xiong, Zi-Ye Luo, Kai-Kun Luo, Shao-Jun Liu *, Chang Wu, Shi Wang, Ming Wen

State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Science, Hunan Normal University, Changsha 410081, PR China

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ABSTRACT

Bacterial LPS is a heat-stable endotoxin and wall components of gram negative bacteria, which can exhibit a toxicological effect on physiology and biochemical activities of fish. In this study, we investigated the effect of LPS exposure on cell viability, oxidative stress, caspase activity and immune-related gene expressions in cultured fin cell lines of red crucian carp, white crucian carp and their hybrid offspring. LPS stimulation could reduce fish cell viability, whereas gene expression levels and promoter activities in inflammatory signals increased dramatically. Moreover, enhanced levels of intracellular oxidative stress and decreased levels of mitochondrial membrane potential (MMP) were observed in LPS-induced fish cells. N-Acetyl-L-cysteine (NAC) could alleviate LPS-stimulated reactive oxygen species (ROS) generation and caspase-3 activity in fish cells. These results suggested that ROS-mediated cytotoxic stress was involved in LPS-induced inflammation and mitochondrial damage in cultured fish cells.

1. Introduction

Ecological deterioration and environmental pollution can exhibit a suppressive effect on teleostean immunity, thus rendering teleost less resistant against pathogenic infection (Magnadottir, 2010). Evidences are emerging that the exposure to ambient stressors may trigger the occurrences of severe diseases (Qi et al., 2020). In general, biotic or abiotic stressors can result in physiological malfunction in fish (Sung et al., 2011). LPS is one of key components of gram-negative bacteria, which can directly induce inflammatory response by up-regulation of cytokines expressions (Yang et al., 1998). In mammals, the innate immune response to bacterial LPS was regulated by the recruitment of Toll-like receptor 4 (TLR4) complex, whereas pathogens can evade the recognition of cluster of differentiation 14 (CD14) and myeloid differentiation protein 2 (MD-2) molecules by altering LPS structures (Freudenberg et al., 2008; Rosadini and Kagan, 2017). Many studies demonstrated that LPS administration can contribute to cardiovascular collapse (Tien et al., 2010), mediate vascular inflammation in atherosclerosis (Stoll et al., 2004), as well as augment TNFα activation in cardiomyocytes *via* TLR4 signal (Meldrum, 1998). In contrast to LPS-mediated signal activation in mammals, fish are resistant to the toxic effect of LPS exposure and TLR4 orthologues in zebrafish serve as negative modulators of MyD88-dependent signal (Sepulcre et al., 2009). Some studies indicated that TLR2, TLR5M, TLR5S, TLR9, and TLR21 in fish can specifically recognize pathogen-associated molecular patterns (PAMPs) derived from pathogens (Zhang et al., 2014), while TLR4 is lost from the genomes of most fish (Palti, 2011).

Hybridization is a predominant evolutionary event that gives rise to species with novel capabilities and chimeric genes may form through fusion of pieces of various genomes (Rogers et al., 2009). Chimeric genes in hybrid offsprings may generate change of gene structures and occurrence of nonsynonymous variants, subsequently affecting downstream signal transductions in response to *in vitro* stimuli (Koyama et al., 2007; Liu et al., 2016). Recent studies demonstrated that hybrid offspring could exhibit a strong resistance against pathogenic infection in comparison with that of its parental species (Šimková et al., 2013; Šimková et al., 2015). Innate immunity is a basic defense system in teleostean immunity (Magnadóttir, 2006). For instances, developed complemented system and immune-related pathways are discovered in teleost, which exhibits a high similarity to those of mammals (dos Santos

* Corresponding authors at: College of Life Science, Hunan Normal University, Changsha 410081, PR China.
E-mail addresses: swluo@hunnu.edu.cn (S.-W. Luo), lsj@hunnu.edu.cn (S.-J. Liu).

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et al., 2008; Holland and Lambris, 2002; Luo and Wei, 2020). Currently, increasing evidences demonstrate that white crucian carp (*Carassius cuvieri*, WCC) and red crucian carp (*Carassius auratus* red var., RCC) are classified into different species in the genus of *Carassius* (Luo et al., 2014), thus the generation of hybrid crucian carp (WR) by crossing of WCC (2n = 100, ♀) and RCC (2n = 100, ♂) is considered as interspecific hybridization (Liu et al., 2019). Crucian carp is one of the most important commercial freshwater fish and abundant in lakes, rivers and reservoirs in china, which is popular with fish farmers (Li et al., 2018). However, it suffers from environmental deterioration, which may lower their disease resistance (Bowden, 2008; Choo et al., 2018). Occurrence of global climate anomaly may promote a lingering effect in the expansion of water-borne pathogenic diseases, threatening the survival of aquatic animals (Marcogliese, 2008). In addition, pathogenic infection can alter physiological responses in teleost by attenuation of immunity and metabolism (Ellis, 2001).

Cell lines serving as *in vitro* models can be utilized to study virology, pathology, immunology and developmental biology of vertebrates (Lakra et al., 2011; Villena, 2003). Increasing evidences demonstrate that fish fibroblast-like cells can express immune-related genes, playing important roles in regulation of both innate and adaptive immunity (Ingerslev et al., 2010; Wei et al., 2014). Among known fish cells, fibroblast-like cells derived from caudal fin of medaka (*Oryzias latipes*) (Komura et al., 1988), sea bream (*Pagrus major*) (Imajoh et al., 2007) and zebrafish (*Danio rerio*) (Kalaiselvi Sivalingam et al., 2019) can serve as *in vitro* tools to investigate the adverse effects of toxicological factors (Fierro-Castro et al., 2013). However, the data on comparative analysis of LPS-induced apoptotic process and oxidative stress in fish cell lines from hybrid offspring and its parents are sparse.

In the present study, the aims were to characterize the forms of cellular apoptosis and ROS generation in three lines of fish fibroblast cells by various doses of LPS stimulation. We also investigated the regulation of transcript expressions and reporter genes involved in NF-κB signal pathway, which may provide a new insight into the immune regulation of hybrid fish and its parents.

2. Materials and methods

2.1. Chemicals and reagents

Lipopolysaccharide (LPS, *Escherichia coli* O111:B4) and N-Acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (Shanghai, China). Cell counting kit-8 (CCK-8) assay kit, mitochondrial membrane potential (MMP) assay kit, GreenNuc™ caspase-3 assay kit and reactive oxygen species (ROS) assay kit were purchased from Beyotime Biotechnology (Shanghai, China). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and phosphate buffered saline (PBS, pH 7.4) were purchased from Gibco (Shanghai, China). Penicillin-streptomycin and 0.25% trypsin-EDTA were purchased from Hyclone (Shanghai, China). HiPure total RNA mini kit was purchased from Magen (Guangzhou, China). RevertAid RT Reverse Transcription kit and PowerUp SYBR Green Master Mix were purchased from ThermoFisher Scientific (Shanghai, China). Dual-luciferase reporter assay system was purchased from Promega (Shanghai, China).

2.2. Cell culture

RCC fin cells (RCCFCs), WCC fin cells (WCCFCs) and hybrid crucian carp fin cells (WRFCs) were kept in the lab (Luo et al., 2021). Fish cell lines cultured in DMEM supplemented with 15% FBS were maintained at 26 °C in a humidified atmosphere of 5% CO_2.

2.3. Cell viability assay

To detect the potential effect of LPS stimulation on the cell viability, cell counting kit-8 (CCK-8) assay was performed (Xie et al., 2018). Fish cells were seeded in 96-well plates for 24 h. Following cell treatment with LPS (0, 2, 50, 200 and 1000 ng/ml), 10 μl of CCK-8 solution was added to each well. Following 3 h incubation, the optical density was measured at 450 nm. The experiment was performed in triplicate.

2.4. Cell treatment, RNA extraction and cDNA synthesis

Fish cells were seeded in 6-well plates for 18 h. Then, cells were stimulated with various concentrations of LPS (0, 40 and 800 ng/ml) for 24 h and 48 h (Hussain et al., 2020; Zhu et al., 2016). After that, cells were harvested, immediately frozen in liquid nitrogen and preserved in −80 °C. Total RNA was extracted from fish cells by using HiPure Total RNA Mini kit. Concentration and purity quotient of total RNA was determined by measurement of 260/280 nm absorbance, while its integrity was determined by 1% agarose gel electrophoresis (Luo et al., 2019a). According to manufacturer's protocols, purified total RNA was used to synthesize cDNA by using RevertAid RT Reverse Transcription kit.

2.5. Quantitative real-time PCR assay

To investigate the transcript changes of immune-related genes, qRT-PCR assay was performed as previously described (Luo et al., 2015a). qRT-PCR assay was performed in a volume of 20 μl, including 1.0 μl of each primer, 6.0 μl of PCR-grade water, 2.0 μl of 1:10 diluted cDNA, and 10 μl of PowerUp SYBR Green Master Mix (Applied Biosystems, USA). qRT-PCR program contained 1 cycle of 95 °C for 30 s, 40 cycles of 95 °C for 15 s, 60 °C for 35 s, followed by 1 cycle of 95 °C for 30 s, 60 °C for 60 s. At the end of qRT-PCR amplified reactions, melting curve analysis was implemented to confirm credibility of each qRT-PCR analysis (Luo et al., 2020a). The primers of Myeloid differentiation primary response protein 88 (MyD88, KC816578.1), Toll-Interleukin 1 receptor domain containing adaptor protein (TIRAP, MG659314.1), TNF receptor-associated factor 6 (TRAF6, XM_026214981.1), Kelch-like ECH-associated protein-1 (KEAP1, XM_026245355.1) and Bcl-2 associated X protein (Bax, XM_026262399.1) used in this study were shown in Table 1. 18S rRNA (XR_003291850.1) was used as internal control to normalize the results (Luo et al., 2019b). Primer specificity was detected and each sample was analyzed in triplicate to certify the repetitiveness and credibility of experimental results. qRT-PCR results were measured by using Applied Biosystems QuantStudio 5 Real-Time PCR System with $2^{-\triangle\triangle Ct}$ methods (Livak and Schmittgen, 2001).

2.6. Dual-luciferase reporter assay

Based on previous studies, luciferase report assay was performed in fish cells (Gao et al., 2014; Jiang et al., 2019). In brief, RCCFCs, WCCFCs and WRFCs were grown in 24-well plates for 18 h. Cells were co-transfected with PRL-TK and NF-κB Luc/TNFα Luc. Then, the transfected cells were stimulated with LPS (0, 40 and 800 ng/ml) for 6 h. After that, luciferase activity was performed by using a dual-luciferase reporter assay system. Relative folds of luciferase activity were normalized to the amount of Renilla luciferase. The results were repeated in triplicate.

2.7. Measurement of reactive oxygen species (ROS)

To investigate the intracellular ROS content generated by LPS stimulation, DCFH-DA probe was used (Huang et al., 2011). In brief, RCCFCs, WCCFCs and WRFCs were seeded in 24-well plates for 18 h and exposed to LPS stimulation (0, 40 and 800 ng/ml) for 24 h, respectively. Cells were stained with DCFH-DA (10 μM) for 20 min in dark and then washed three times with PBS. ROS signal was detected by fluorescent microscopy. The experiment was performed in triplicate.

2.8. Mitochondrial membrane potential (MMP) detection assay

Based on previous studies, the changes of MMP in fish cells were detected by using a JC-1 assay kit (Luo et al., 2017). In brief, fish cell stimulation by LPS (0, 40 and 800 ng/ml) was performed as above. Cells were stained with JC-1 for 20 min in dark. After washing wit JC-1 buffer, the fluorescence signals were observed by using a fluorescent microscopy. The experiment was performed in triplicate.

2.9. Microplate assay

RCCFCs, WCCFCs and WRFCs were cultured in 96-well black microplates for 18 h. Cells were pretreated with NAC (4 mM) for 30 min and incubated with 300 ng/ml of LPS for 18 h. Then, ROS production and caspase-3 activity were measured by DCFH-DA probe and GreenNuc™ caspase-3 substrate (Qian et al., 2019). Caspase-3 inhibitor Ac-DEVD-CHO was used as the negative control. Following incubation for 20 min, cells were washed twice with PBS. The fluorescence values were detected by using a Synergy 2 multifunctional microplate reader (Bio-Tek, USA). The experiment was performed in triplicate.

2.10. Statistical analyses

The data analysis was measured by using SPSS 18 analysis program and represented as mean ± SD. All of the experimental data analysis was subjected to Student's *t*-test or one-way ANOVA. Further analysis of Duncan's multiple range test, only if the level of P-value <0.05, the differences were considered statistically significant.

3. Results

3.1. Determination of fish cell viability following LPS exposure

To detect the effect of LPS stimulation on fish cell viability, CCK-8 assays were performed. As shown in Fig. 1A–C, cell viability of RCCFCs, WCCFCs and WRFCs began to decrease at 50 ng/ml LPS, whereas they gradually decreased to 77.12%, 71.72% and 73.81% following 1000 ng/ml of LPS stimulation for 24 h, respectively.

3.2. LPS elicited proinflammatory responses in fish cells via MyD88-NF-κB signals

Expression profiles of MyD88, TIRAP and TRAF6 mRNA in RCCFCs, WCCFCs and WRFCs were investigated at 0, 24 and 48 h following the exposure of 40 ng/ml LPS or 800 ng/ml of LPS. In Fig. 2A–C, expression level of MyD88 mRNA in RCCFCs, WCCFCs and WRFCs peaked at 24 h following 800 ng/ml of LPS stimulation with the highest values of 3.85-, 3.77- and 8.38-fold greater than those of the control. In Fig. 2D–F, TIRAP mRNA expression exhibited a 3.31-, 4.31- and 5.48-fold increase in RCCFCs, WCCFCs and WRFCs following 800 ng/ml of LPS stimulation. In Fig. 2G–I, a sharp increase of TRAF6 mRNA expression in RCCFCs, WCCFCs and WRFCs was observed at 24 h following 800 ng/ml of LPS stimulation with a peaked level of 5.71-, 4.25- and 15.16-fold greater than those of the control.

To explore the roles of LPS exposure on NF-κB and TNFα promoter activities, luciferase report assays were performed. In Fig. 3A–C, a gradual increase of NF-κB and TNFα promoter activity in fish cells was observed after 6 h of LPS stimulation. 40 ng/ml of LPS stimulation could cause approximately 1.86-, 1.77- and 1.91-fold increase of NF-κB promoter activity in RCCFCs, WCCFCs and WRFCs, while cells treated with 800 ng/ml of LPS could increase NF-κB activity to 2.52-, 2.13- and 2.46-fold in RCCFCs, WCCFCs and WRFCs, respectively. In Fig. 3D–F, Cells receiving 40 ng/ml of LPS could exhibit approximately 2.71-, 2.45- and 2.55-fold increase of TNFα activity in RCCFCs, WCCFCs and WRFCs, while cells treated with 800 ng/ml of LPS could increase TNFα activity to 3.88-, 3.31- and 3.76-fold in RCCFCs, WCCFCs and WRFCs,

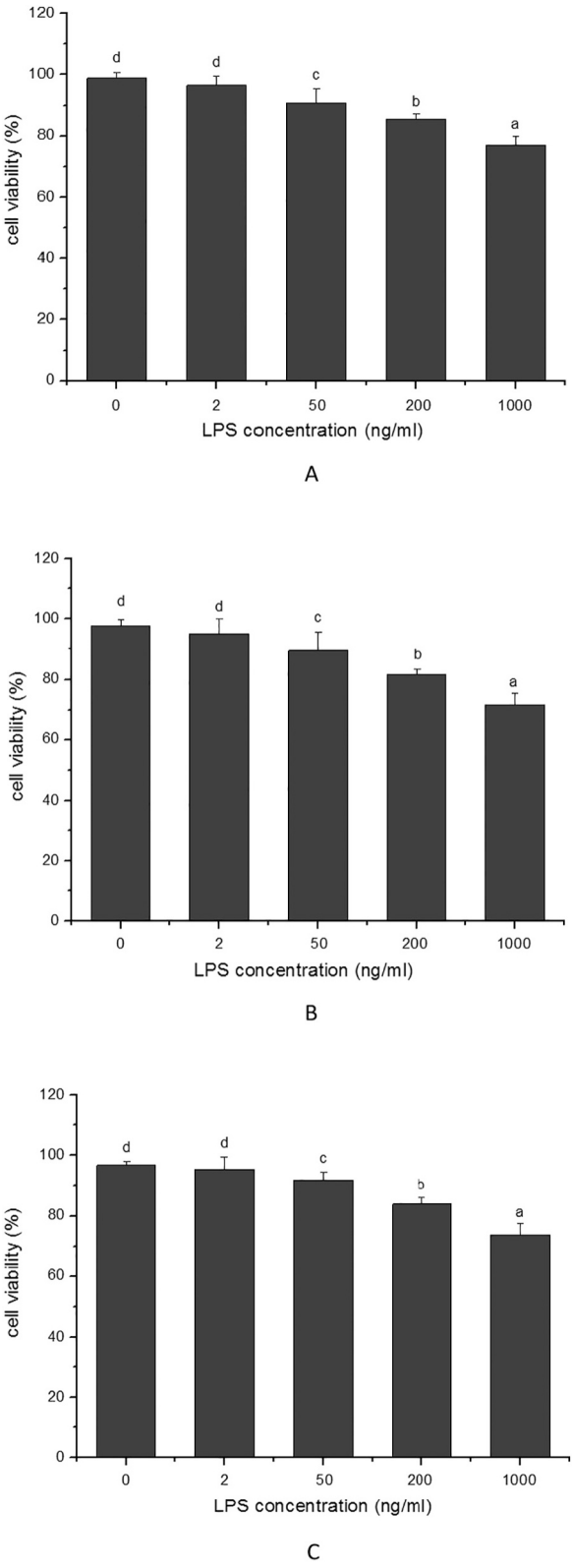


Fig. 1. Cell viability determined by CCK-8 assay. RCCFCs (A), WCCFCs (B) and WRFCs (C) were seeded in 96-well plates for 24 h. Following cell treatment with LPS (0, 2, 50, 200 and 1000 ng/ml), 10 μl of CCK-8 solution was added to each well. The optical density was measured at 450 nm. The calculated data repeated in triplicate (mean ± SD) with different letters were significantly different among the groups.

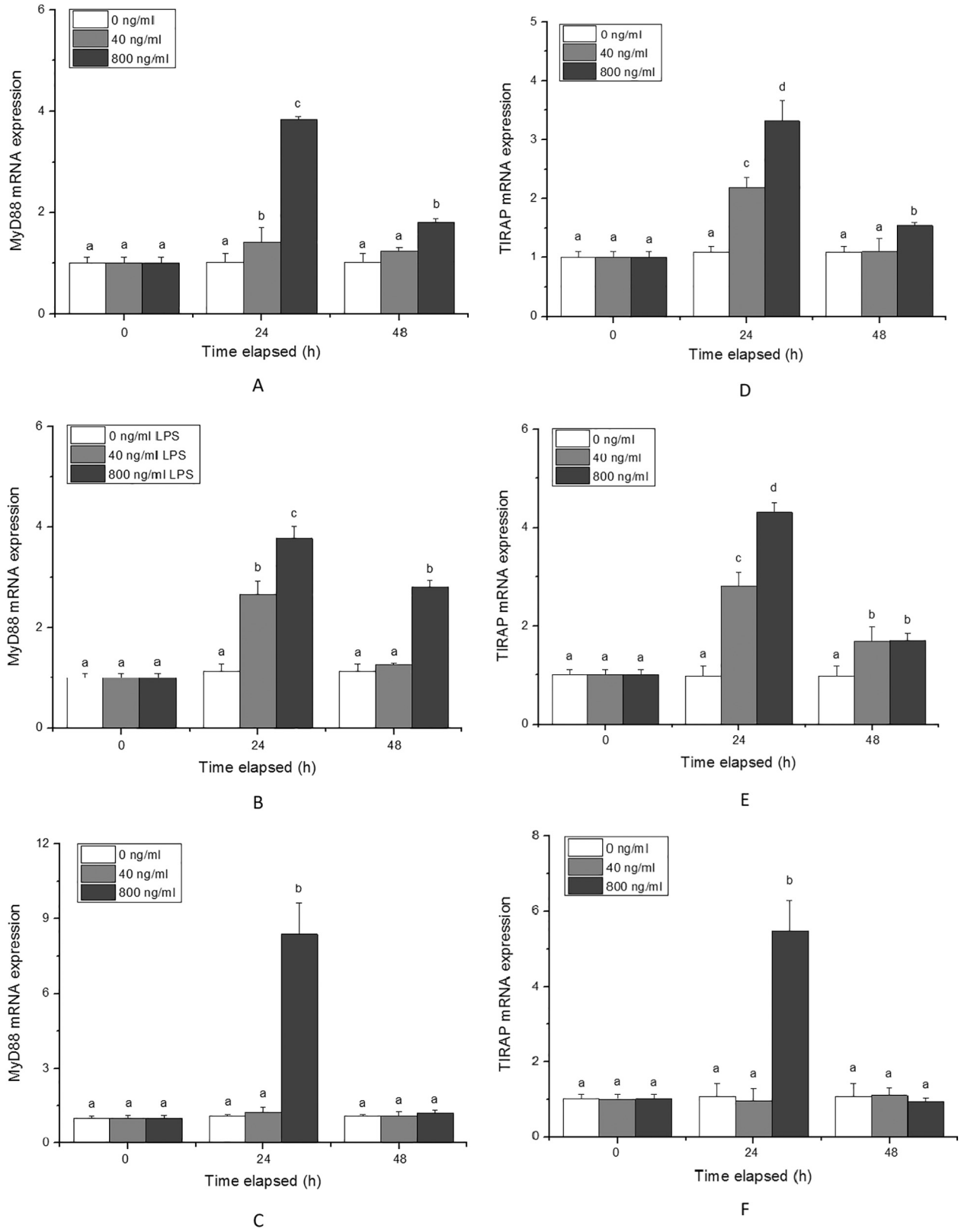


Fig. 2. Gene expressions of MyD88, TIRAP and TRAF6 mRNA in RCCFCs (A, D, G), WCCFCs (B, E, H) and WRFCs (C, F, I) stimulated with various concentrations of LPS were determined by qRT-PCR. The calculated data repeated in triplicate (mean ± SD) with different letters were significantly different among the groups ($P < 0.05$).

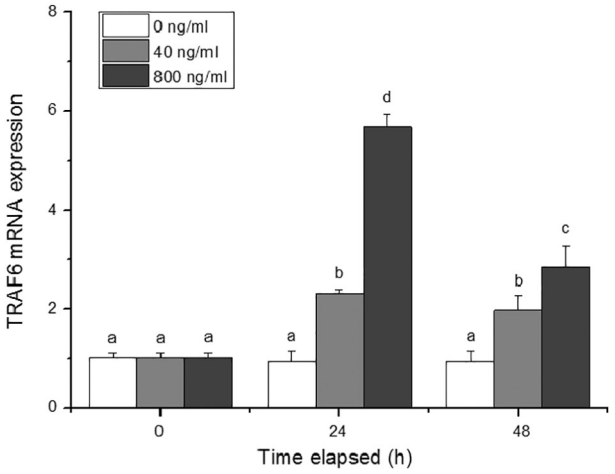


Fig. 2. (*continued*).

respectively.

3.3. Effect of LPS exposure on oxidative stress in fish cells

In this study, LPS-induced intracellular ROS production in fish cells was detected at 24 h post-stimulation. As shown in Fig. 4A, elevated level of green fluorescence signal was observed in RCCFCs, WCCFCs and WRFCs stained with DCFH-DA probe following exposing to various doses of LPS stimulation. In Fig. 4B–D, KEAP1 mRNA expression in RC CFCs, WCCFCs and WRFCs increased dramatically and peaked at 24 h after 800 ng/ml of LPS stimulation with the greatest values of 3.84-, 4.47- and 14.34-fold greater than those of the control, respectively.

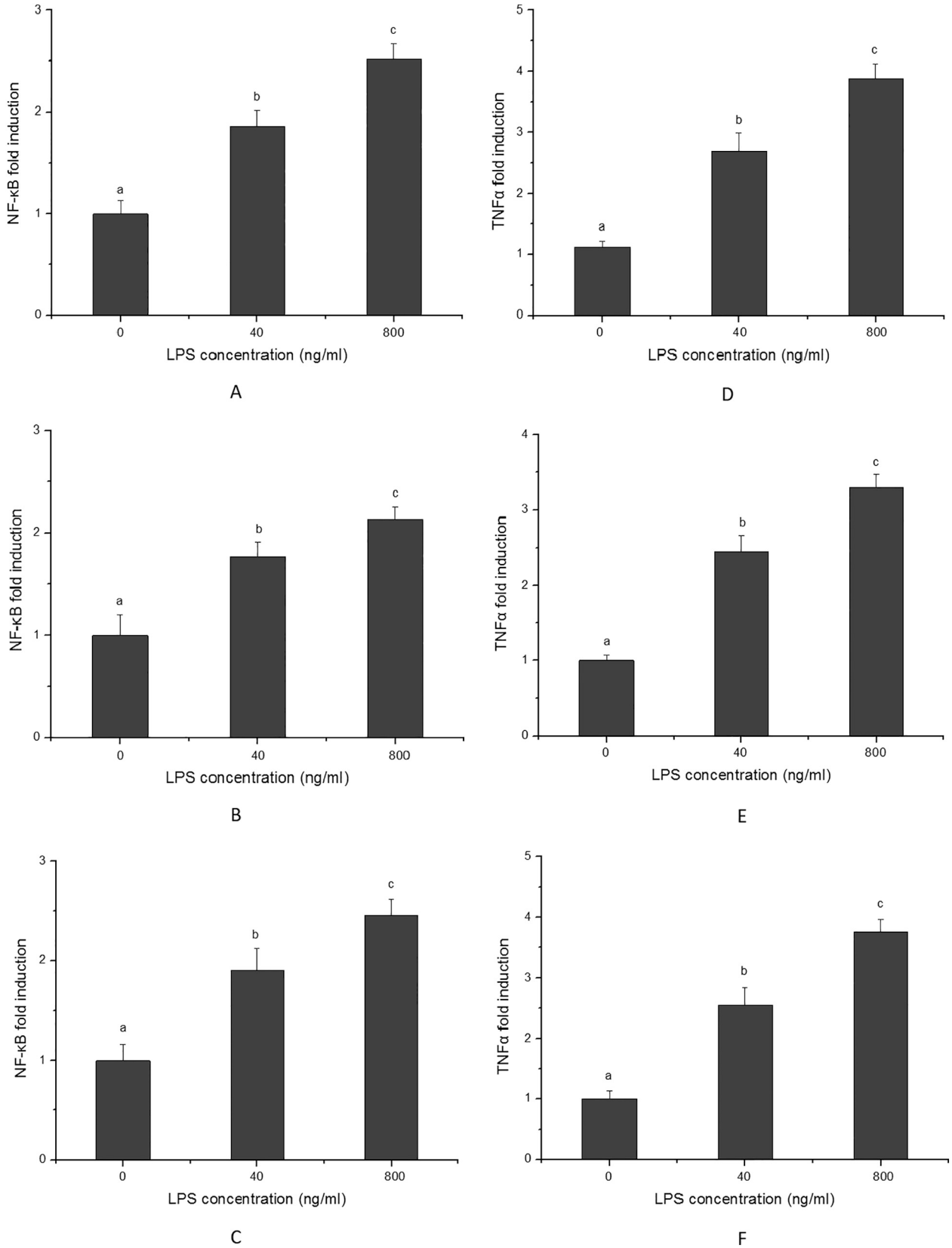


Fig. 3. Effects of LPS stimulation on the promoter activities of NF-κB and TNFα. RCCFCs (A, D), WCCFCs (B, E) and WRFCs (C, F) were co-transfected with pRL-TK and NF-κB-Luc/TNFα-Luc and then exposed to various doses of LPS for 6 h. The calculated data repeated in triplicate (mean ± SD) with different letters were significantly different among the groups ($P < 0.05$).

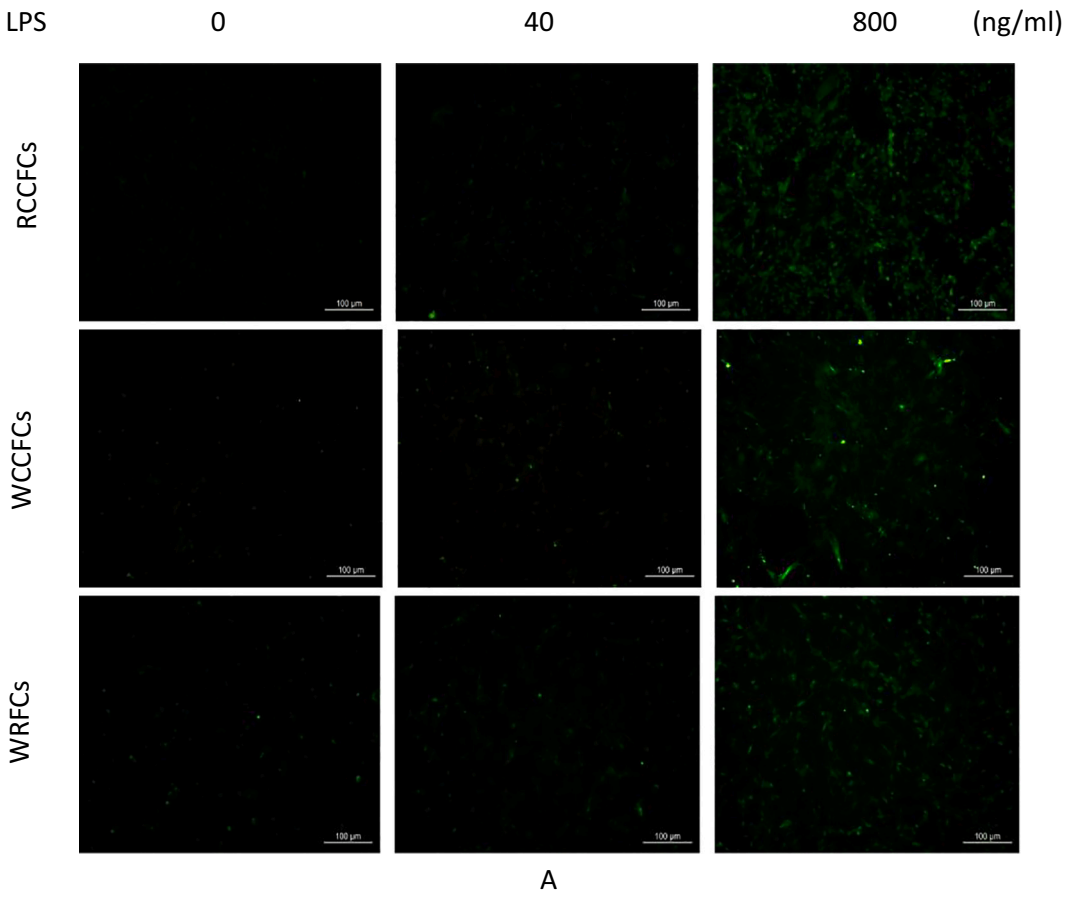


Fig. 4. LPS-stimulated oxidative stress in RCCFCs, WCCFCs and WRFCs. (A) RCCFCs, WCCFCs and WRFCs were seeded in 24-well plates for 18 h and exposed to LPS stimulation (0, 40 and 800 ng/ml) for 24 h, respectively. Cells were stained with DCFH-DA (10 μM) for 20 min in dark and then washed three times with PBS. ROS signal was detected by fluorescent microscopy. (B–D) Gene expressions of KEAP1 mRNA in RCCFCs (B), WCCFCs (C) and WRFCs (D) stimulated with LPS were determined by qRT-PCR. The calculated data repeated in triplicate (mean ± SD) with different letters were significantly different among the groups ($P < 0.05$).

3.4. LPS stimulation induced mitochondrial damage in fish cells

In this study, LPS-induced loss of MMP in fish cells was observed at 24 h following the exposure to various doses of LPS. As shown in Fig. 5A–C, up-regulated levels of JC-1 monomers were detected in RCCFCs, WCCFCs and WRFCs after exposing to various doses of LPS stimulation. In Fig. 5D–F, the ratios of green/red fluorescence intensity in RCCFCs, and WCCFCs and WRFCs exposed to 40 ng/ml increased to 5.64-, 5.08- and 4.94-fold greater than those of the control, while cells simulated with 800 ng/ml of LPS could cause 11.48-, 12.02- and 10.91-fold increase, respectively. In Fig. 5G–I, Bax expression mRNA in RCCFCs, WCCFCs and WRFCs increased dramatically and peaked at 24 h after 800 ng/ml of LPS stimulation with the greatest values of 10.17-, 14.21-, 16.98-fold greater than those of the control, respectively.

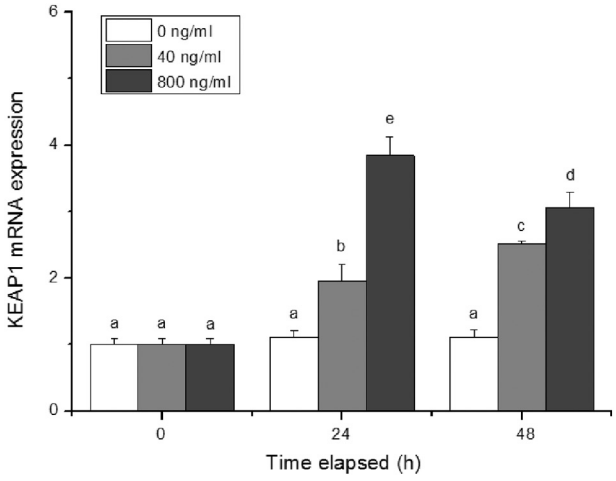
3.5. NAC could diminish LPS-induced ROS generation and caspase-3 activity

The effect of antioxidant NAC on LPS-stimulated ROS production and caspase-3 activity in fish cells were measured. In Fig. 6A–C, NAC could diminish ROS production in LPS-exposed RCCFCs, WCCFCs and WRFCs, respectively. Additionally, LPS stimulation could induce caspase-3 activity in RCCFCs, WCCFCs and WRFCs, while cells pretreated with 4 mM NAC could relieve LPS-stimulated caspase-3 activity (Fig. 6D–F).

4. Discussion

LPS is a heat-stable endotoxin derived from gram-negative bacteria that can mediate immune responses and induce various biological effects *in vitro* and *in vivo* (Lee et al., 2013). Previous studies indicate that LPS administration can diminish cell viability but increase proinflammatory responses in H9c2 cells (Su et al., 2018). In this study, a gradual decrease of fish cell viability was observed following LPS stimulation, suggesting that LPS exposure could decrease proliferation activity of fish cells in a dose-dependent manner.

Recent findings indicate that TIRAP is playing a pivotal role in the connection of LPS-activated TLR signals (Horng et al., 2002), then recruiting MyD88 and triggering the activation of IL-1 receptor receptor-associated kinase 4 (IRAK4) (Akira et al., 2001; Kawai et al., 1999; Wesche et al., 1997). IRAK4 is a pivotal adaptor that can participate in TRAF6 recruitment (Kim et al., 2007; Lye et al., 2004; Ye et al., 2002), thus triggering the induction of proinflammatory cytokines *via* IκB kinases/nuclear factor-κB (IKKs/NF-κB) and mitogen-activated protein kinase/activator protein-1 (MAPK/AP-1) (Gohda et al., 2004; Lu et al., 2008). Our previous studies revealed that gram-negative bacterial infection could activate MyD88-TRAF6 inflammatory signals in fish (Luo et al., 2020b; Luo et al., 2020c). In this study, expression profiles of MyD88, TIRAP and TRAF6 mRNA in RCCFCs, WCCFCs and WRFCs increased significantly following LPS stimulation. In addition, LPS exposure could gradually increase NF-κB and TNFα promoter activity in fish cells. This study indicated that LPS simulation could activate endogenous MyD88-TRAF6 signals in fish cells.



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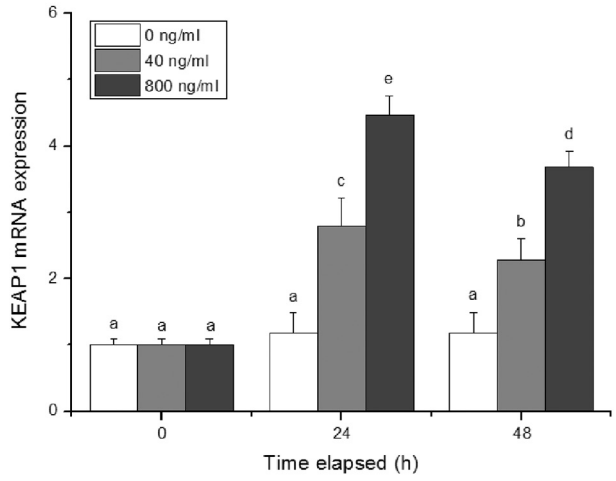

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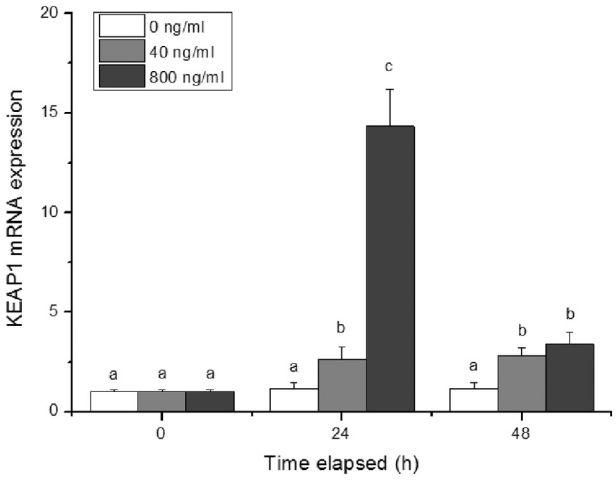

D

Fig. 4. (*continued*).

LPS stimulation can also trigger oxidative stress in mammals (Noworyta-Sokołowska et al., 2013). Oxidative stress may refer to up-regulated level of intracellular ROS accumulation, which may cause damage to biomacromolecules (Schieber and Chandel, 2014). In this study, high concentration of LPS exposure can dramatically increase intracellular ROS generation in fish cells. In general, stress-induced ROS generation appeared to play a pivotal role in bridging innate immunity with adaptive immune response (Suzuki et al., 1997). However,

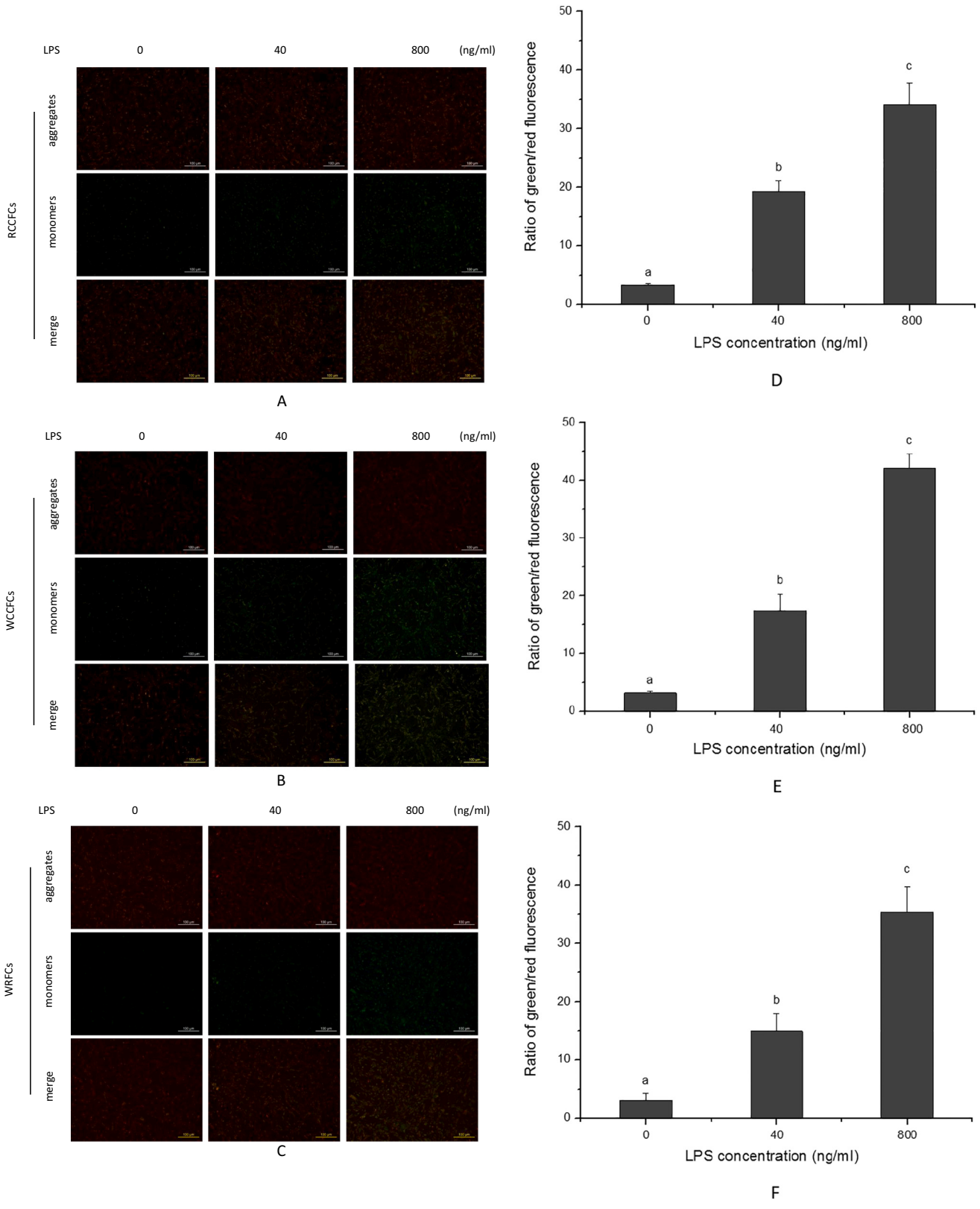

Fig. 5. Effects of LPS stimulation on mitochondrial damage. (A–C) Detection of MMP activity. RCCFCs (A), WCCFCs (B) and WRFCs (C) were stimulated with LPS (0, 40 and 800 ng/ml), stained with JC-1 for 20 min in dark and observed by using a fluorescent microscopy. (D–F) Relative fluorescence intensities of RCCFCs (D), WCCFCs (E) and WRFCs (F) were measured by image J program. The ratios of green fluorescence (JC-1 monomers) and red fluorescence (JC-1 aggregates) were represented as mean ± SD. (G–I) Gene expressions of Bax mRNA in RCCFCs (G), WCCFCs (H) and WRFCs (I) stimulated with LPS were determined by qRT-PCR. The calculated data repeated in triplicate (mean ± SD) with different letters were significantly different among the groups ($P < 0.05$).

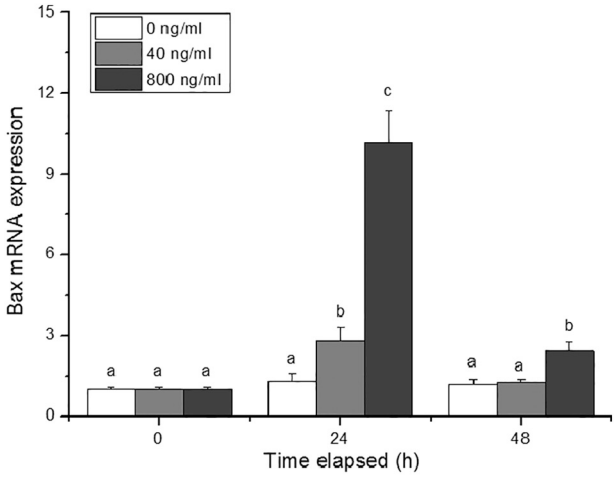

Fig. 5. (*continued*).

excessive level of stress-induced ROS accumulation may elicit an adverse effect on the organisms, leading to antioxidant imbalance, lipid peroxidation and apoptosis induction (Luo et al., 2015b; Simon et al., 2000). KEAP1, a redox-sensitive factor, is a direct target for ROS and other electrophilic molecules, playing a critical role in responding to endogenous or environmental oxidative stress by negatively regulating activity of nuclear factor erythroid-2 related factor 2 (Nrf2) (Kaspar et al., 2009; Sihvola and Levonen, 2017). In this study, the elevated levels of KEAP1 mRNA expression in RCCFCs, WCCFCs and WRFCs were observed following LPS exposure. These results implied that LPS

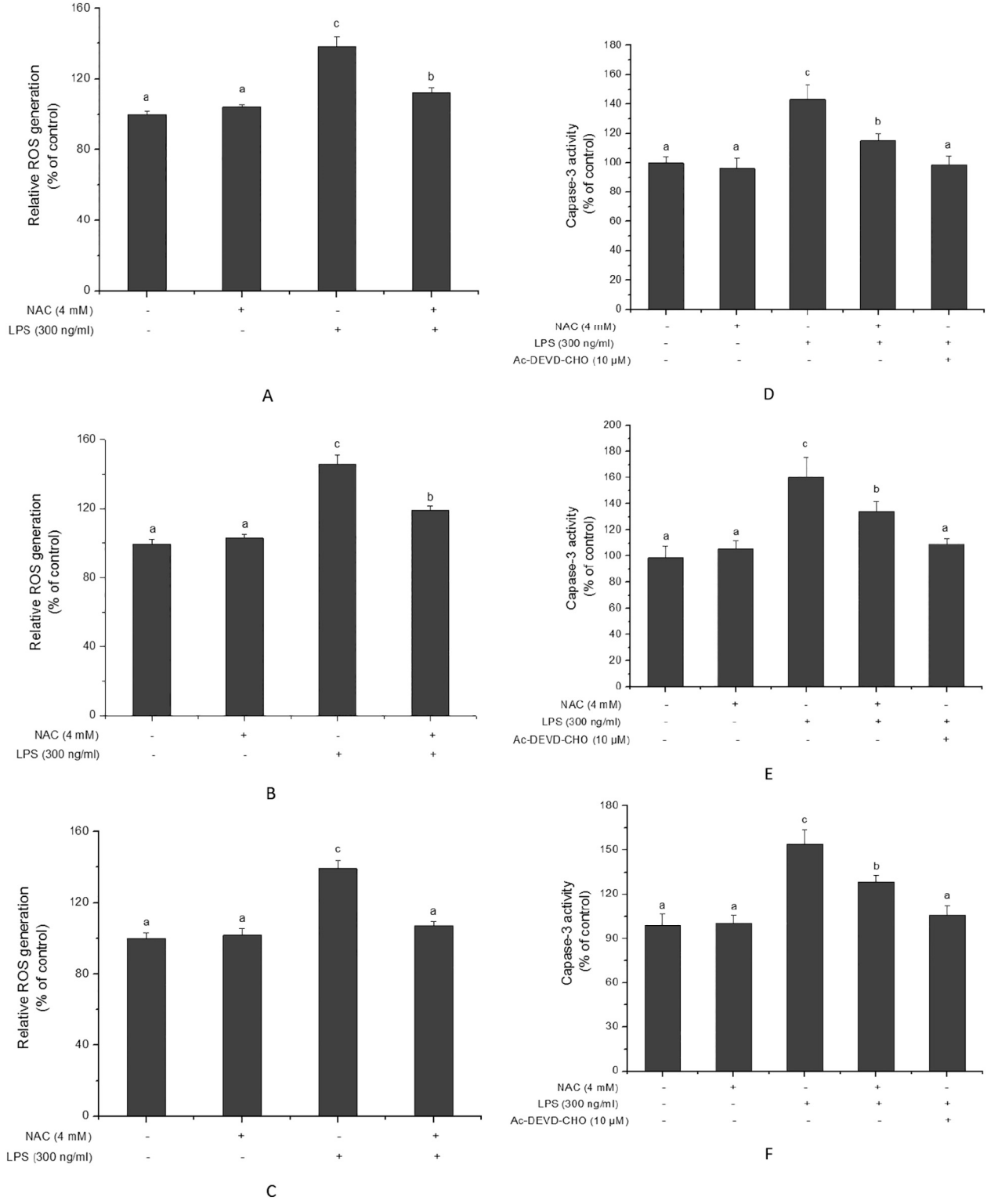

Fig. 6. NAC diminished LPS-induced cytotoxic stress in fish cells. (A–C) Attenuation of ROS production by NAC treatment. After pretreatment with 4 mM NAC, RCCFCs (A), WCCFCs (B) and WRFCs (C) were incubated with LPS for 18h. ROS generation was measured by DCFH-DA probe. (D–F) Attenuation of caspase-3 activity by NAC treatment. After pretreatment with 4 mM NAC, RCCFCs (D), WCCFCs (E) and WRFCs (F) were incubated with LPS for 18 h. Caspase-3 activity was measured by GreenNuc™ caspase-3 substrate, while fish cells treated with caspase-3 inhibitor Ac-DEVD-CHO was used as the negative control. The calculated data repeated in triplicate (mean ± SD) with different letters were significantly different among the groups ($P < 0.05$).

stimulation could cause oxidative stress in fish cells.

Indeed, mitochondrial dysfunction is highly associated with increased levels of ROS generation produced by the organelle or endoplasmic reticulum surface (Murphy, 2013), which is considered as an early event in the apoptotic process (Ly et al., 2003). Apoptosis or programmed cell death (PCD) is highly modulated cell death process in the normal development and homeostasis within the host that can prevent autoimmunity and confer protection against aberrant immune response by mounting immunological self-tolerance, as well as remodel inflamed sites *via* phagocytic clearance of dying cells (Chen et al., 2006; Savill, 1997). Mitochondrial dysfunction can participate in the induction of apoptosis (Ly et al., 2003), while Bax can elicit a pro-apoptotic function in mitochondria with a sharp attenuation of MMP activity (Luo et al., 2016; Smaili et al., 2001). Bax acting as a pro-apoptotic protein can shift its subcellular localization from cytosol to membrane in the induction of apoptosis (Hsu et al., 1997). Previous studies indicate that C-terminal domain of Bax can mediate its relocalization and direct it to mitochondrial targeting (Goping et al., 1998; Schinzel et al., 2004), which can determine mitochondrial integrity (Gross et al., 1999) and facilitate alteration of MMP activity (Jürgensmeier et al., 1998). In addition, activation of caspase cascade plays a central role in the execution of apoptotic processes, being able to cleave poly ADP-ribose polymerase (PARP) and then results in a critical change of cellular apoptosis (Cohen, 1997). Previous findings have revealed that Bax-dependent caspase-3 activation is a predominant determinant in p53-Bax pro-apoptotic signal (Cregan et al., 1999). In this study, increased level of JC-1 monomers and Bax expression was observed in RCCFCs, WCCFCs and WRFCs following LPS stimulation. To further explore the role of ROS generation in LPS-exposed fish cells, NAC, a known antioxidant, was used for ROS elimination. Previous studies demonstrate that NAC can ameliorate LPS-induced DNA damage and caspase activation in A549 cells (Chuang et al., 2011) and confer protection against LPS-stimulated inflammatory response (Beloosesky et al., 2012). The current study demonstrated that pretreatment with NAC could attenuate LPS-induced ROS generation in fish cells. Additionally, LPS stimulation could augment caspase-3 activity in RCCFCs, WCCFCs and WRFCs, while pretreatment with NAC could suppress LPS-stimulated caspase-3 activation in fish cells. Taken together, these results indicated that ROS-induced cytotoxic stress was involved in LPS-stimulated inflammatory response, mitochondrial dysfunction and apoptotic induction in fish cells.

In summary, we characterized the effect of LPS exposure on physiological changes and immune-related signals in fish cells. Our findings revealed that LPS exposure could decrease cell viability but increase inflammatory signals in RCCFCs, WCCFCs and WRFCs. In addition, LPS stimulation also increased oxidative stress and mitochondrial damage in fish cells, whereas NAC pretreatment could diminish LPS-induced ROS generation and caspse-3 activation. Thus, the information presented in this study could give a new insight into the mechanism of ROS-mediated cytotoxic stress by LPS stimulation in fish cells.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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