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Cytotoxic effects, inflammatory response and apoptosis induction of cyclophosphamide in the peripheral blood leukocyte of blunt snout bream (*Megalobrama amblycephala*)

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ABSTRACT

The present study was aimed to evaluate the effects of the cyclophosphamide (CY) exposure (Control, 0.032, 0.32, 1.0, 1.6 and 3.2 mg/mL) on the damage in the peripheral blood leukocytes of blunt snout bream for 24 h, which including cell viability, apoptosis, lactate dehydrogenase (LDH) release, mitochondrial membrane potential (Δψm), ROS, antioxidant enzyme activity and the relative mRNA levels of apoptosis. Results showed that cell viability and Δψm effects of CY were greatly reduced, and occurred in a dose-dependent manner. CY exposure (0.32–3.2 mg/mL) significantly increased the LDH release and induced apoptosis accompanied by ΔΨm disruption and ROS generation compared to the control. The cellular ROS was significantly increased with increase of CY level from 0.032 mg/mL to 1 mg/mL and the plateau occurred at 0.32 mg/mL. Additionally CY exposure led to oxidative stress as evidenced by significantly the decrease of SOD and CAT and increase of MDA concentration after treating cells with 3.2 mg/mL of CY. Besides, the relative mRNA levels of caspase-3 in the dose of 0.032, 0.32 mg/mL CY, caspase-9 and interleukins-1β (IL-1β) in the dose of 0.32 mg/mL CY, tumor necrosis factor-alpha (TNF-α) in the dose of 0.032 mg/mL CY significantly higher than that of the control. In conclusion, 0.32–3.2 mg/mL CY could lead to cytotoxic effect, inflammatory response and induce the apoptosis of the peripheral blood leukocyte of *Megalobrama amblycephala*.

1. Introduction

Blunt snout bream, *Megalobrama amblycephala*, is one of the conventional freshwater fish farmed in China, which has a long history of cultivation because of its excellent flesh quality, rapid growth performance and high larval survival rate [1]. It plays an important role in aquaculture with 0.82 million tons in 2017 [2]. However, cultured blunt snout bream have suffered environmental stressors which impacted the immunity and led to the disease of *M. amblycephala* [3]. In order to control the disease, a large amount of antibiotics have been used in intensive aquaculture. Therefore, it is necessary and important to screen non-chemical and natural therapeutics, such as immune-stimulants, natural from plants for a better controlling of fish disease in aquaculture.

Peripheral blood leukocytes (PBLs) are the main defensive cells of the body. In addition to blood and lymph, leukocytes are also widely found in tissues other than blood vessels and lymphatic vessels and are the first line of defense against invasion by foreign microorganisms [4]. Leukocytes have resting, ready, and activated states; in the activated state, leukocytes can kill pathogens through degranulation and respiratory burst function. Their change demonstrates the state of body's defense against potentially harmful factors such as stress factor, bacteria and viruses [5]. In addition, abnormal PBLs may be associated with autoimmune disorders [6] or hematologic malignancies [7]. Therefore, PBLs are important diagnostic indices for a number of diseases and disorders.

Apoptosis or programmed cell death, is characterized by cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and apoptotic bodies [8]. There are several factors involved in apoptosis and diverse apoptotic stimuli converge on a common apoptotic

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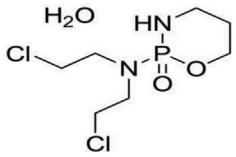

Fig. 1. Chemical structures of cyclophosphamide.

pathway mediated by the mitochondria [9,10]. There is a balance between oxidative stress and antioxidant defense system. An imbalance between oxidative stress and the antioxidant system may result in the production of lipid peroxides and free radicals. Reactive oxygen species (ROS) normally modulates cell apoptosis, proliferation, and inflammatory responses, all beneficial to cell survival. However, excessive accumulation of ROS can damage cellular components under specific conditions, such as DNA, lipids, and proteins, thereby interfering with mitochondria-induced apoptotic pathways [11,12]. Then, the damage caused by oxidative stress could result in mitochondrial outer membrane permeabilization, thus leading to the release of proapoptotic proteins and caspase activation [13]. Apoptosis pathways have been studied extensively, yet the signaling pathway of cyclophosphamide-induced apoptosis of the leukocyte of blunt snout bream remains poorly study.

Cyclophosphamide (CY) is a widely used anticancer drug that acts by alkylating mechanism (Fig. 1) [14]. Cyclophosphamide undergoes metabolic activation by hepatic enzymes and forms 4-hydroxy cyclophosphamide, which converts into two cytotoxic metabolites acrolein and phosphoramide mustard. These cytotoxic metabolites on enzymatic activation form covalent bonds with DNA and proteins, causing cell death [15]. Cyclophosphamide has strong immunosuppressive effect, which is often regarded as a positive control for the preparation of immunosuppressive model in the body's immunological toxicology. The immunosuppressive effect of CY has also received wide attention in the study of fish immunology. Intraperitoneal injection at the dose of 200 mg/kg CY in Asian catfish resulted in the decrease of nitroblue tetrazolium (NBT) and myeloperoxidase (MPO) in serum [16]. While in *Saurida elongate*, cyclophosphamide administration caused reduction in the blood cell production through bone marrow suppression leading to anaemia [17]. Although the toxicity of CY has been investigated in various fish species by means of measuring blood biochemical parameters and histopathological examination, the molecular and cellular mechanism of CY-induced apoptosis of the peripheral blood leukocyte (PBLs) of *M. amblycephala* remains poorly understood. Therefore, the present study aimed to explore the molecular and enzymatic levels of cyclophosphamide toxicity by studying its effect on oxidative stress, inflammatory response and apoptosis in the peripheral blood leukocyte of *M. amblycephala*. This research can establish a reasonable model of immunosuppressive model for aquatic animals, and to provide references for screening the optimization of disease resistance agents.

2. Materials and methods

2.1. Experimental fish

Blunt snout bream were obtained from Freshwater Fisheries Research Center of Chinese Academy of Fishery Science, Wuxi, China. Fish for in vitro study were about an average weight of 150 ± 5.15 g. Fish were reared in a recirculation system and hand-fed three times daily (8:00, 12:00 and 16:00) with commercial diets (32% crude protein, Wuxi Tongwei Feed Co., Ltd., China), which until apparent satiation on the basis of visual observation. A photoperiod was set at natural conditions, and water was maintained at 24–25 °C, pH 7.4–7.8, 6 mg/L dissolved oxygen and 0.05–0.08 mg/L NH_3 over the experimental period.

2.2. Collection of blood and isolation of PBLs

After 2 weeks for acclimation, the similar size 9 fish were randomly selected to collection of blood. The fish were starved for 24 h to enter a basic metabolic state and eliminate the dietary effect. The method for separation of leucocytes from peripheral blood was described by previous studies [18,19]. Peripheral blood was collected from the caudal vein after the fish had been anaesthetised with tricaine methane sulphonate at a concentration of 100 mg/L, and then removed it in the RPMI-1640 medium containing 10% fetal calf serum (FCS, Sijiqing Biological Engineering Materials Co. Ltd., Hangzhou, Zhejiang, PR China), penicillin (Gibco-Brl, France) (100 IU/mL), streptomycin (Gibco-Brl, France) (100 mg/mL) and heparin (Sanofi, Choay, France) (2000 IU/mL). The resulting blood suspension was layered carefully onto discontinuous percoll-II (3 mL)/percoll-I (4 mL) in 15 mL polypropylene conical tubes. The gradient was then centrifuged at 400 × *g* for 25 min at 4 °C with slow acceleration and reduced braking, and cells at three fractions interface were collected. PBLs were isolated from peripheral blood and washed twice (centrifugation 500 × *g*, 10 min, 4 °C) in RPMI-1640 medium. Feasible cells were counted by Trypan blue exclusion test using a hemocytometer [20], and the cell suspension was diluted in RPMI-1640 medium to obtain 1×10^6 cells/mL. Three replicates were separately performed for each treatment and control, and each replicate described below was performed using 3 samples from 3 flasks or plates, respectively.

2.3. Cyclophosphamide exposure

Cyclophosphamide was dissolved in 32 mg/mL of saline to treat as stock cyclophosphamide solution, and then diluted to the desired concentration by RPMI-1640 before use. In this experiment, PBLs were plated in 96 plates. Cells were treated with various cyclophosphamide concentrations (0.032, 0.32, 1, 1.6, 3.2 mg/mL) for 24 h. Control group was cultured in the same medium but without cyclophosphamide. Three replicates were performed for each treatment and the control, and each assay described below was performed using samples. Besides, all treatments were under atmospheric condition of 5% CO_2 at a temperature of 27 °C.

2.4. WST-1 cell viability assay

According to the method of Zhao et al. [19], cells were seeded in 6-well (1×10^6 cells/well) culture plates were incubated with cyclophosphamide for 24 h. PBLs viability was detected with a modified WST-1 cell viability assay by scanning the wells with a microplate reader (Bio-Rad model 3550, CA, USA) at 450 nm (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China).

2.5. LDH release assay

LDH is a soluble cytosolic enzyme present in most eukaryotic cells that is released into the culture medium upon cell death due to plasma membrane damage. The increase in the LDH activity in culture supernatant is proportional to the number of lysed cells [21,22]. Cells were seeded in 96-well (5×10^3 cells/well) culture plates were incubated with cyclophosphamide for 24 h. LDH were assayed according to the protocol described by Cui et al. [21].

2.6. Flow cytometric detection of apoptotic cells

Apoptotic cells were evaluated using Annexin V/FITC (Beyotime, Haimen, Jiangsu, PR China). In brief, Annexin V +/PI- cells were considered apoptotic, while Annexin V +/PI + cells were considered necrotic [23]. Cells were stained according to manufacturer's instructions and analyzed by flow cytometry (Cytomics FC 500, Beckman Coulter, USA). The cells were collected by centrifugation after

cyclophosphamide exposure and washed with PBS. The pellets were resuspended in the Annexin V-FITC staining reagent and fixed at 20–25 °C for 10 min. The cells were then washed and resuspended in the PI staining reagent. Staining was stable at 4 °C for 30 min [24]. Samples were then analyzed by flow cytometer.

2.7. ROS measurement

Changes in intracellular ROS levels were determined by measuring the oxidative conversion of cell permeable 2′, 7′dichlorofluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF) in a microplate reader (Fluoroskan Ascent FL, Thermo, USA). Cells in 6-well (1×10^6 cells/well) culture plates were incubated with cyclophosphamide for 24 h. The cells were then incubated with DCFH-DA at for 20 min at 37 °C and washed twice with serum-free medium. The distribution of DCF fluorescence in 20,000 cells was detected by fluorospectrophotometry at an excitation wavelength of 488 nm and an emission wavelength of 535 nm [21,25].

2.8. Mitochondrial membrane potential

Δψm was monitored using fluorescent Rhodamine 123 dye (Beyotime, Haimen, Jiangsu, PR China), which preferentially localizes to active mitochondria based on highly negative Δψm [26]. Rhodamine 123 (final concentration of 10 mM) was added to cells after cyclophosphamide treatment for 24 h. After 30 min at 37 °C, the cells were collected by pipetting, washed twice with PBS, and then analyzed by fluorospectrophotometry at an excitation wavelength of 488 nm and at emission wavelength of 535 nm.

2.9. MDA, SOD and CAT measurements

Cells were seeded in 6-well (1×10^6 cells/well) culture plates after cyclophosphamide exposure and were evaluated for SOD and CAT activity, MDA content according to manufacturer's instructions (Beyotime, Haimen, Jiangsu, China). SOD activity was determined using xanthine oxidase-derived superoxide and was then monitored at 450 nm [27]. One unit of SOD activity was defined as the quantity of SOD required for 50% inhibition. SOD activity was then normalized to on a protein basis. One unit of CAT activity was defined as the amount of enzyme that catalysed the decomposition of 1 μmol of H_2O_2 per min absorbance at 550 nm [28]. Lipid peroxidation was evaluated indirectly by measuring MDA formation from the breakdown of polyunsaturated fatty acids, the thiobarbituric acid reactive substances at 532 nm [29].

2.10. Real-time PCR analysis

The relative expression of Caspase-3, Caspase-9, TNF-α and IL-1β mRNA were determined using Real-time PCR analysis as described in our previous studies [19]. Briefly, total RNA extraction from the PBLs of juvenile blunt snout bream was performed by an RNAiso Plus kit (Dalian Takara Co. Ltd., China). Total RNA was incubated with RNase-free DNase (Dalian Takara Co. Ltd., China) to remove the contaminating genomic DNA. Quantity and quality of the RNA was assessed by OD 260/280 method and electrophoresis in 1.5% agarose gel. Primers (shown in Table 1) for each gene were designed using primer premier 5.0 based on the sequences obtained from this transcriptome sequencing library. All primers were synthesized by Shanghai Generay Biotechnology, Co., Ltd, China. Real-time quantitative PCR (RT-PCR) was performed with SYBR® Primix Ex TaqTM II (TliRNase Plus) Kit using ABI 7500 Real-time PCR System according to the manufacturer's protocol. The relative expression levels of the target genes were normalized to the housekeeping *M. amblycephala* gene β-actin [30], and further calculated using the $2^{-\Delta\Delta CT}$ method [31].

2.11. Heatmap analysis

The heatmap showed target genes expression of Caspase-3, Caspase-9, TNF-α and IL-1β, and conducted in R with the pheatmap package. The input data is a 6 by 4 symmetric Spearman correlation matrix. The rows and columns are reordered by dendrogram, because of the symmetry of the input data, rows and columns are rearranged in the same order.

2.12. Statistical analysis

All data were statistically analyzed using SPSS version 20 and then subjected to one-way analysis of variance (ANOVA), followed by Duncan's multiple comparisons. The results were expressed as mean ± standard error (X ± SEM), and $P < 0.05$ was considered statistically significant.

3. Results

3.1. The effect of cyclophosphamide on cell viability

Cell survival was assessed with the WST-1 cell viability assay as in Fig. 2. The amount of cell viability decreased in a dose-dependent manner with exposure to cyclophosphamide compared to the control cells ($P < 0.05$). Viability in cells treated with 0.32, 1, 1.6 and 3.2 mg/mL cyclophosphamide was significantly lower than that of the control group ($P < 0.05$).

3.2. Cytotoxicity of cyclophosphamide on cell

PBLs were exposed to different concentrations of cyclophosphamide for 24 h, and cytotoxicity was measured by LDH release (Fig. 3). Compared to the control cells, the amount of LDH release significantly increased in all treatment groups ($P < 0.05$). LDH release of cells treated with 3.2 mg/mLcyclophosphamide was significantly higher than that of other groups ($P < 0.05$).

3.3. Assessment of cyclophosphamide -induced apoptosis

We also examined the effect of cyclophosphamide on apoptosis and Δψm in the PBLs of blunt snout bream (Fig. 4). We found that the percentage of apoptotic cells in cells treated with 0.32, 1, 1.6, and 3.2 mg/mL cyclophosphamide were significantly higher compared to the control cells (Fig. 4A and B; $P < 0.05$). In addition, the percentage quantity apoptotic cells exposed to 0.32, 1, 1.6, and 3.2 mg/mL cyclophosphamide was significantly higher compared to those exposed to 0.032 mg/mL and the control group (Fig. 4B; $P < 0.05$). Δψm was much lower after 24 h of exposure to 0.32, 1, 1.6, and 3.2 mg/mlLcyclophosphamide than that of control cells (Fig. 4C; $P < 0.05$).

3.4. The effect of cyclophosphamide on ROS

We measured ROS production using the fluorescent dye DCFH-DA as in Fig. 5. ROS generation in the cells exposed to 0.032, 0.32, and 1 mg/mL concentration of cyclophosphamide were significantly higher than that of other groups ($P < 0.05$). Compared to control cells, the treatments with 1.6 and 3.2 mg/mLcyclophosphamide were not significantly different in the ROS ($P > 0.05$).

3.5. The effect of cyclophosphamide on antioxidant capacity

SOD activity in cells treated with 0.32, 1, 1.6, and 3.2 mg/mL cyclophosphamide was significantly lower than that of the control group (Fig. 6A, $P < 0.05$). CAT activity in respective cells treated with 0.032 or 3.2 mg/mL were significantly higher or lower ($P < 0.05$) than that of the control group (Fig. 6B, $P < 0.05$). Our findings reveal that MDA

Table 1
Primers for real time PCR analysis of Caspase-3, Caspase-9, TNF-α and IL-1β.

Genes	Primer sequences (5′→3′)	Product size (bp)	Amplification efficiency (%)	Sequence source
β-actin	(F) TCTGCTATGTGGCTCTTGACTTCG (R) CCTCTGGGCACCTGAACCTCT	132	92.07	AY170122.2
Caspase-3	(F) CCTGATCCAGGCTTGTCGAG (R) AGCCCAGAGGATGCATGAAC	137	104.89	KY006115.1
Caspase-9	(F) ACAGACCTGATCGCACTCCT (R) AGACAGATCCCACACGGACT	126	98.97	KM604705.1
TNF-α	(F) GTGGCCAGGGCAGAAGAAGA (R) CGCTCATCCACAGCCACATC	122	104.05	KP192119.1
IL-1β	(F) ACCAGCACGACCTTGCAGTG (R) CTGGGATGCATTCGGTTTGA	127	101.21	Song, 2018

Note: the primer sequence of was obtained from the reference of Song, 2018 [48].

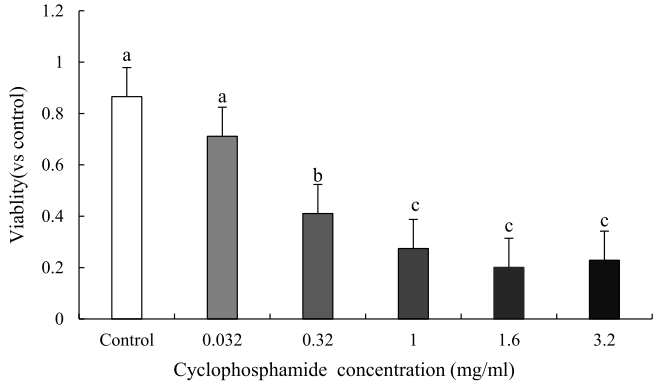

Fig. 2. The effects of cyclophosphamide on viability in PBLs of *M. amblycephalain*. Note: Data are expressed as means ± SEM ($n = 9$). Diverse little letters above the bars show significant differences ($P < 0.05$) in at different dosage groups in Duncan's multiple range test.

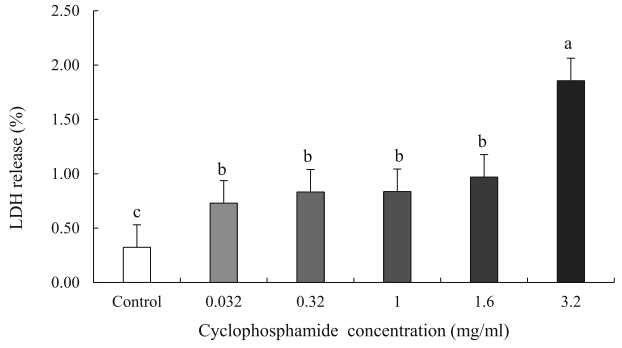

Fig. 3. The effects of cyclophosphamide on LDH release in PBLs of *M. amblycephalain*. Note: Data are expressed as means ± SEM ($n = 9$). Legends are the same as described in Fig. 2.

content in cells treated with 0.32, 1, 1.6, and 3.2 mg/mL cyclophosphamide were significantly higher ($P < 0.05$) than that of the control cells (Fig. 6C, $P < 0.05$).

3.6. Relative gene expression of Caspase-3, Caspase-9, TNF-α and IL-1β

The effects of cyclophosphamide on gene expression of Caspase-3, Caspase-9, TNF-α and IL-1β in PBLs are presented in Fig. 7 and Fig. 8. Compared to control cells, the treatments of 0.032 and 0.32 mg/mLcyclophosphamide significantly increased the gene expression of Caspase-3, while 0.32 mg/mLcyclophosphamide treatment group significantly increased gene expression of Caspase-9 ($P < 0.05$). In addition, the treatment of 0.032 mg/mL cyclophosphamide significantly increased the gene expression of TNF-α ($P < 0.05$). The treatment of 0.32 mg/mL cyclophosphamide significantly increased the gene expression of Il-1β ($P < 0.05$). In addition, the gene expression of TNF-α and Il-1β mRNA were reduced in the high concentration treatment group of 3.2 mg/mL cyclophosphamide ($P < 0.05$).

3.7. Cyclophosphamide PBLs relative gene expression heatmap

After clustering all treatments with single heatmap approach, the relationship among cyclophosphamide treatments and the four key genes (Caspase-3, Caspase-9, TNF-α and IL-1β) was detected by visualizing the pattern difference (Fig. 9). In this figure, the treatments were clustered in three main groups: 1.6 mg and 3.2 mg/mL represents the first group; Control and 1 mg/mL represents the second group and 0.032 mg and 0.32 mg/mL represents the third group. It is clearly shown that; low dosage exposure of cyclophosphamide (0.032 and 0.32 mg/mL) drastically up-regulated gene expressions of Caspase-3, Caspase-9, TNF-α and IL-1β.

To further explore the toxicity mechanisms of cyclophosphamide in PBL, their network analysis were performed (See Fig. 10). The low dose exposure of 0.32 mg/mL cyclophosphamide enhanced MDA and ROS, induced apoptosis and increased the expression levels of Caspase-3, Caspase-9 and IL-1β in PBL. Inversely, high dose exposure of 3.2 mg/mL cyclophosphamide inhibited antioxidant capacity, accompanying with the decreased the expression levels of Caspase-3, TNF-α and IL-1β and oxidative damage occurred in PBL.

4. Discussion

Cyclophosphamide is one of the most widely used alkylating agents in the treatment of various cancers and some autoimmune diseases [32]. The immunosuppressive effect of CY has also received wide attention in the study of fish immunology [16,17]. In the present study, the PBLs of blunt snout bream was used to study the cytotoxic effects of cyclophosphamide in vitro. The results of this study showed that decreased cell viability and increased LDH release in a dose-dependent manner. Geng. [33] also reported that cell viability was inhibited by exposure to high concentrations of cyclophosphamide. It showed that cyclophosphamide was toxic effect on PBLs of *M. amblycephalain* with a dose-dependent manner. The cellular damage caused by cyclophosphamide was further confirmed by apoptosis assays.

In our study, high dose cyclophosphamide-induced apoptosis in PBLs, which was consistent with findings that cyclophosphamide could trigger mouse hepatic cells apoptosis in vitro [34]. Drumond et al. [35] observed that germ cells of mouse exhibited apoptotic features after injecting 150 mg/kg cyclophosphamide for 4 days. These data emphasized that cyclophosphamide led to unavoidable apoptotic cell death. The signaling mechanisms that regulate apoptosis are very complicated. Generally there are two main apoptotic pathways, the mitochondrial pathway and the death receptor pathway. Mitochondrial changes are regarded as the central role in the apoptosis pathway in many experimental systems, include the enhanced production of oxygen radicals and loss of Δψm [10]. To understand the role of apoptosis in PBLs of

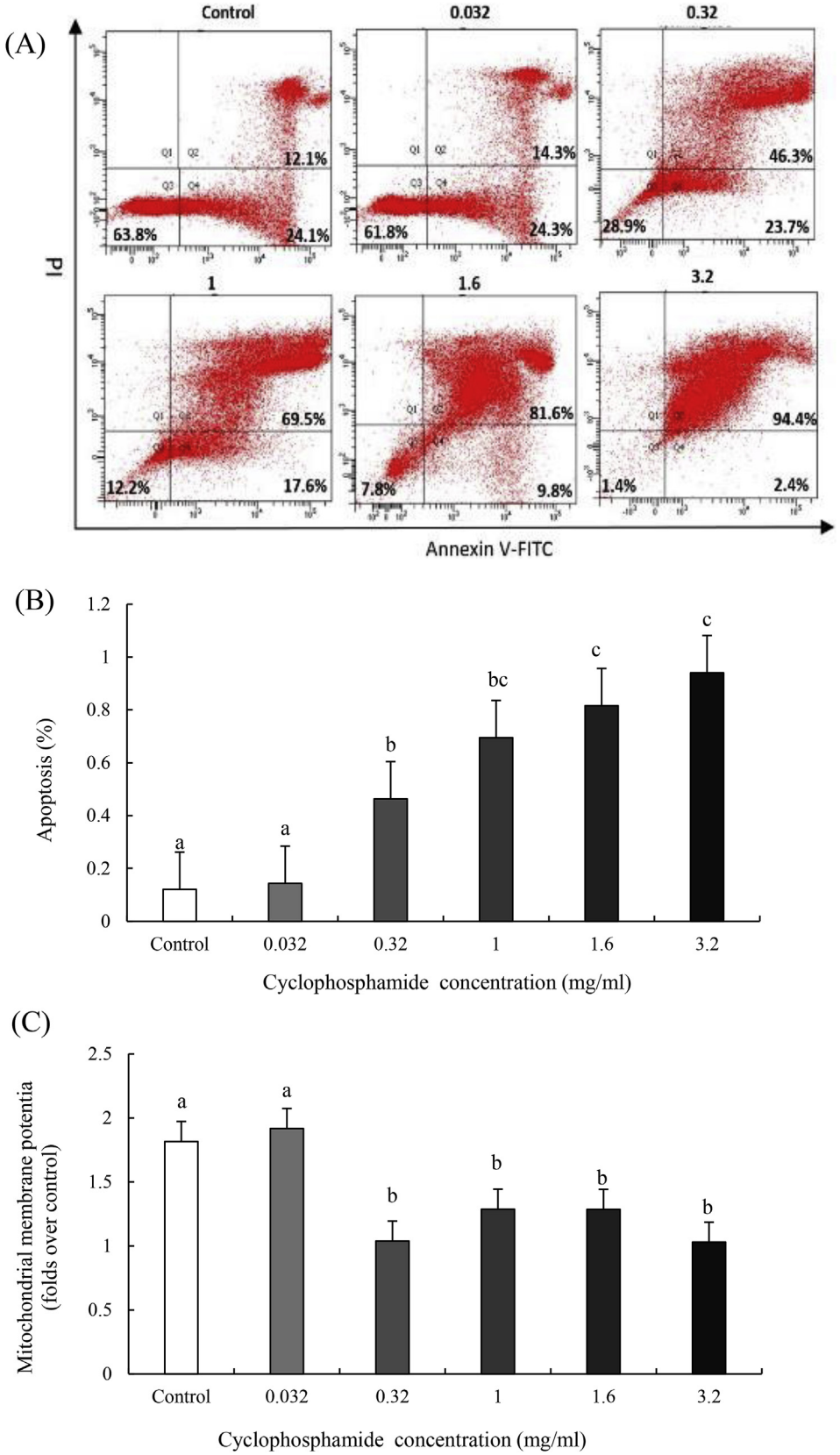

Fig. 4. The effects of cyclophosphamide on apoptosis in PBLs and quantitative analysis of apoptosis of *M. amblycephalain*. Note: Q1: death cells, Q2: terminal apoptosis, Q3: normal cells, Q4: early apoptotic. Data are expressed as means ± SEM (n = 9). The multiple range test of the data is the same as Fig. 2.

blunt snout bream following exposure to cyclophosphamide, we examined the changes of parameters such as ROS, SOD, CAT, MDA, Δψm in the PBLs in response to cyclophosphamide at different concentrations.

Δψm decreased in a dose-dependent manner in cyclophosphamide treated cells. Within 24 h of treatment, the Δψm declined to

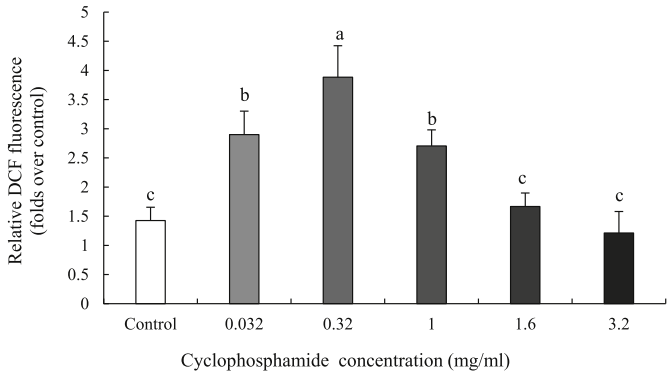

Fig. 5. The effects of cyclophosphamide on ROS in PBLs of *M. amblycephalain*. Note: Data are expressed as means ± SEM (n = 9). The multiple range test of the data is the same as Fig. 2.

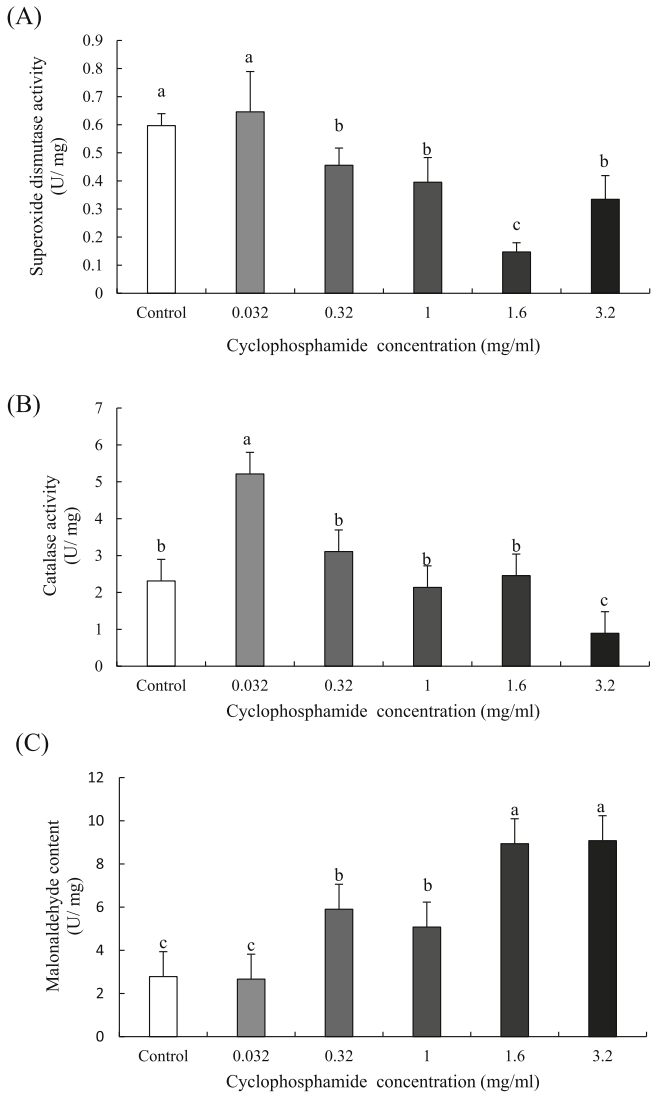

Fig. 6. The effects of cyclophosphamide on SOD (A), CAT (B) and MDA(C) in PBLs of *M. amblycephalain*. Note: Data are expressed as means ± SEM (n = 9). The multiple range test of the data is the same as Fig. 2.

approximately 60–75% of control levels, and this was apparently enhanced at cyclophosphamide concentrations of 0.32, 1, 1.6 and 3.2 mg/mL. This was consistent with previous studies that drug-induced apoptosis is accompanied by a fall in the Δψm [13,36–38].

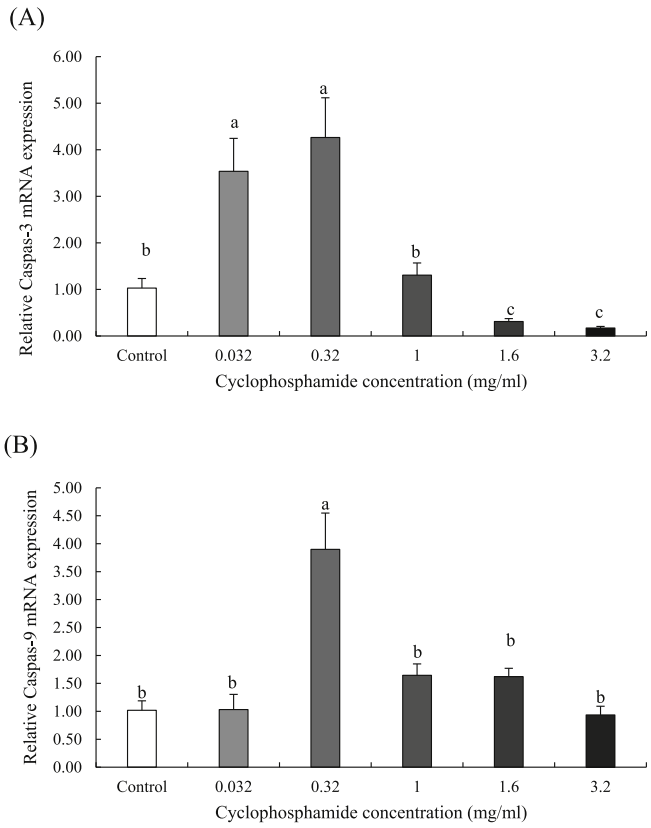

Fig. 7. The effects of cyclophosphamide on Caspase-3 (A) and Caspase-9 (B) in PBLs of *M. amblycephalain*. Note: Data are expressed as means ± SEM (n = 9). The multiple range test of the data is the same as Fig. 2.

Cyclophosphamide-induced pathogenesis is associated not only with apoptosis but with oxidative damage. The mechanisms driving apoptosis in cells under oxidative stress may involve high ROS levels, which directly inhibit caspase activity, disrupt intracellular Ca^{2+} homeostasis, and lead to ATP depletion [39]. We found that 0.32–1 mg/mL cyclophosphamide significantly increased the level of ROS in cells compared to the control group, which indicated that oxidative stress could play a role in apoptosis and necrosis induction by cyclophosphamide for PBLs of the blunt snout bream. However, when the cyclophosphamide concentration was more than 1.6 mg/mL, the ROS content gradually decreased with the increment of the cyclophosphamide concentration. This indicated that most of the cells became necrotic and the cells were no longer functioning properly [40]. Reviewed literature indicated that cyclophosphamide could induce mouse oocyte [32] and pre-pubertal spermatogonial cells [41] generates ROS and triggers mouse germ cells apoptosis [35]. As a result, our study indicated the ROS was probably responsible for cyclophosphamide-induced apoptosis, that it to say, oxidative stress likely played a role in cyclophosphamide-induced apoptosis in PBLs.

There is a balance between oxidative stress and antioxidant defense system for aquatic animals. An imbalance between lipid peroxides and the antioxidant system may result in cell dysfunction and the production of lipid peroxides and free radicals, leading to cell damage. ROS can rapidly be removed by enzymatic and non-enzymatic antioxidants, thereby maintaining a healthy pro-oxidant/antioxidant homeostasis [42]. The activation of endogenous antioxidant enzymes and non-enzymatic antioxidants, such as SOD and CAT, respectively, has a protective role against the damage of lipid peroxidation [43,44]. Meanwhile, the generation of excessive ROS results in lipid oxidation and produces a large amount of aldehydes, alcohols and hydrocarbon of which MDA is a substance with strong biotoxicity, which may hurt

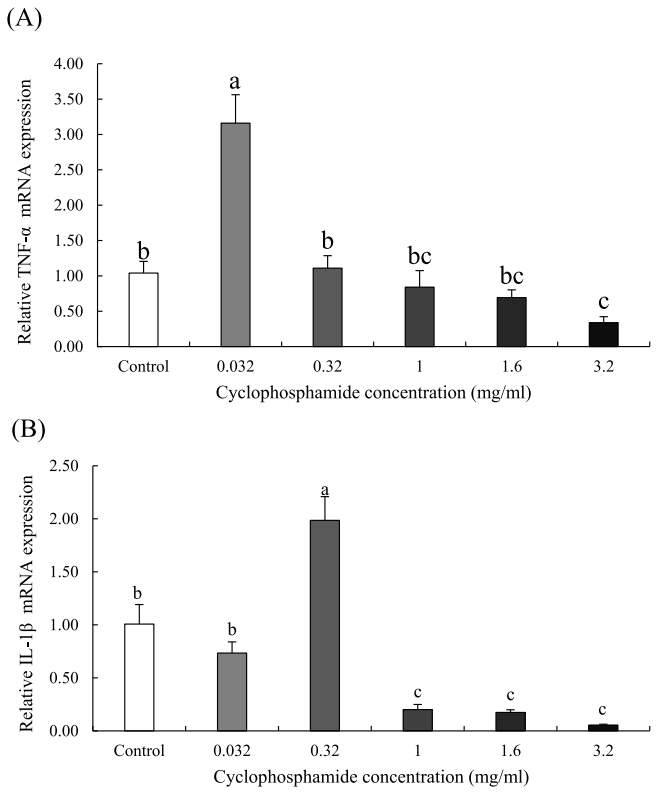

Fig. 8. The effects of cyclophosphamide on TNF-α (A) and IL-1β (B) mRNA expression in PBLs of *M. amblycephalain*. Note: Data are expressed as means ± SEM ($n = 9$). The multiple range test of the data is the same as Fig. 2.

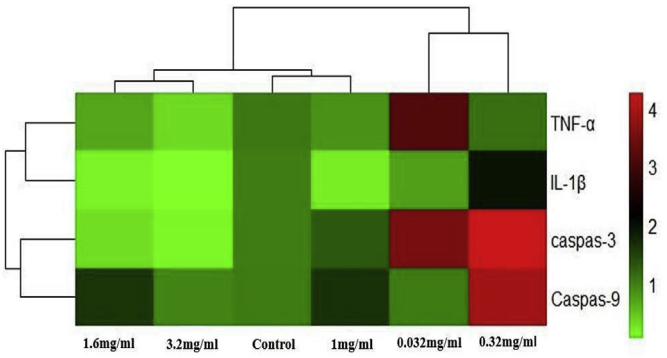

Fig. 9. Visualizing gene expression (Caspase-3, Caspae-9, TNF-α and IL-1β) on pairheatmap. Note: In this figure, the graphical presentation of data, numerical values are displayed by colors. The dendrogram for strain clustering is shown on the up and left sides of the heatmap. The width of the cluster merged from the two sides represent the distance of the two clusters. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

organisms [45]. In the present study, we found that 0.32, 1, 1.6 and 3.2 mg/mL cyclophosphamide significantly reduced SOD and enhanced MDA. This may indicate a deficiency in some enzymatic antioxidants or severe oxidative injury [46]. Therefore, the oxidative effect of higher concentrations of cyclophosphamide may be attributed to the loss of the affected cell's ability to maintain the activity of the radical-scavenging enzymes. In addition, the current study demonstrated that CAT significantly increased in the treatments of 0.032 mg/mL cyclophosphamide, which correlated to the increase of ROS in concentrations of 0.032, 0.32 or 1 mg/mL cyclophosphamide and it contributed to maintain the redox balance. However, ROS levels in the dose of 0.32 or 1 mg/mL cyclophosphamide might cause apoptosis.

Although apoptosis can be triggered by several different stimuli, apoptotic pathways were mainly classified into two groups: the intrinsic pathway and the extrinsic pathway [47]. The common event in the end point of both the intrinsic and extrinsic is the activation of a set of cysteine proteases (caspases) [48,49]. The intrinsic pathway is triggered by different extracellular or intracellular signals, such as oxidative stress, resulting in activation of the initiator caspase-9. Caspase-9, in turn, activates caspase-3, the major effector caspase, responsible for degradation of cellular substrates [50]. Caspase-3 a key apoptotic implementers in the caspase family, is located downstream of the apoptosis pathway and its activation indicates that the apoptosis enters an irreversible stage [51]. In this study, the expression of Caspase-3 and Caspase-9 genes were significantly increased with 0.32 mg/mL cyclophosphamide exposure. This suggests that white blood cells were apoptosis after stimulation by this concentration of cyclophosphamide. However, when cyclophosphamide concentration exceeded 1 mg/mL, the expression level of caspase-3 and caspase-9 mRNA was observed to significantly reduce, indicating that apoptosis and cell necrosis were coinstantaneous in the cell when higher cyclophosphamide concentration stimulated the cells.

It has been argued that inflammation is considered an important part of the immune response and mediated by cytokines [52]. And the expression levels of inflammation related genes could be induced or inhibited by chemicals or viruses [53,54]. In our findings as related to other researcher findings we observe that; IL-1β and TNF-1α are the two crucial pro-inflammatory cytokines that can lead to the activation of the inflammatory response by regulating the expression of other cytokines [55,56]. Further, we observed that the expression of TNF-α and IL-1β genes significantly increased after 0.032 or 0.32 mg/mL cyclophosphamide exposure compared to the control group. These findings indicated cyclophosphamide exposure caused an inflammatory response in PBLs of the blunt snout bream.

The effect of different concentrations of cyclophosphamide on the apoptosis-related factors of the PBLs of blunt snout bream were further understood when two concentration treatment groups including a lower and higher concentration i.e. 0.32 and 3.2 mg/mL respectively were studied. This exhibited the relationship between the related factors in the apoptosis process of white blood cells under cyclophosphamide exposure. As shown in Fig. 10. Defense systems within the cell begin to work when the cells were exposed to low concentrations of cyclophosphamide. When cells are harmful, they trigger the release of TNF-α and promote Caspase-9 together with IL-1β expression resulting in Caspase-9 activating Caspase-3 through cell membrane pathway. Meanwhile, the increment of IL-1β caused an inflammatory response which resulted in cell death. Cyclophosphamide could also cause apoptosis by destroying mitochondrial membrane potential. The antioxidant stress system in cells were also disorders when the cells were stimulated by higher concentration of cyclophosphamide that led to increased amounts of reactive oxygen species. In addition, a variety of molecular and cellular structures of the cell are affected including metabolic function abnormality, cell necrosis, resulting in a downward trend in all relevant indicators with the too high concentration of cyclophosphamide.

5. Conclusion

In conclusion, the present study demonstrated that cyclophosphamide exhibited cytotoxic effects in the peripheral blood leukocyte of blunt snout bream because of the induction of apoptosis. The concentration of 0.32 mg/mL cyclophosphamide produced ROS, which altered the subcellular redox equilibrium, reduced Δψm and subsequently triggered apoptosis in the peripheral blood leukocyte of blunt snout bream. In addition, it induced oxidative stress, activated TNF-α signaling and promoted peripheral blood leukocyte apoptosis, leading to cellular inflammation and necrosis. This finding provides a new

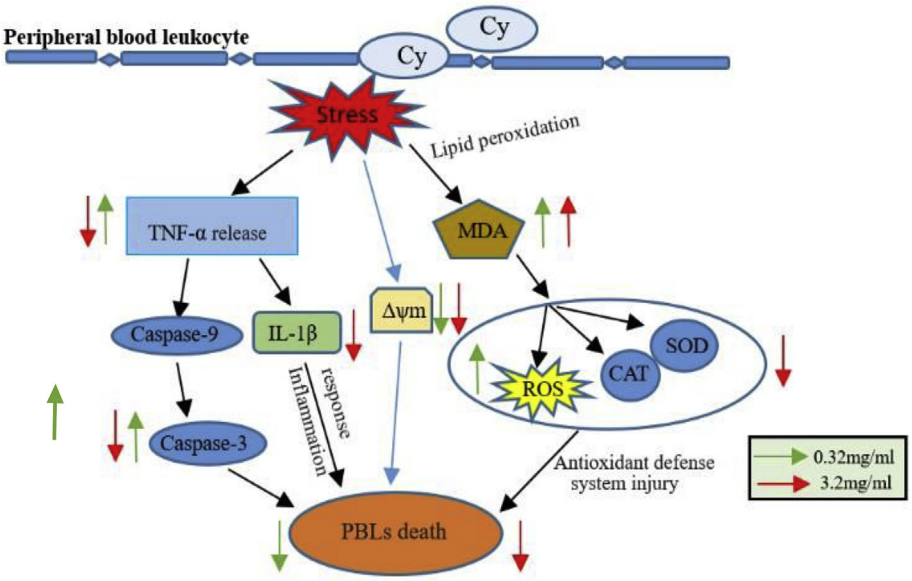

Fig. 10. Differential cytotoxic effects and apoptosis in PBLs exposed to two cyclophosphamide concentrations encountered in vitro. The green arrow is representative of the 0.32 mg/mL cyclophosphamide and the red arrow is 3.2 mg/mL cyclophosphamide exposure; The upward arrow is representative of cytotoxic effects and apoptosis activation and the downward arrow is inhibition of cytotoxic effects and apoptosis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

understanding of the cytotoxic effects on fish cells caused by cyclophosphamide.

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