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The protective effects of vitamin C on apoptosis, DNA damage and proteome of pufferfish (*Takifugu obscurus*) under low temperature stress

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ABSTRACT

The aim of this study was to investigate the protective effects of vitamin C on apoptosis, DNA damage and proteome of pufferfish under low temperature stress. Six diets were formulated to contain 2.60, 48.90, 95.50, 189.83, 382.40, 779.53 mg/kg vitamin C. After 8-week feeding trial, fish were exposed to low temperature challenge. The results showed that pufferfish receiving vitamin C diet exhibited a significant decrease in ROS production (48.9–189.83 mg/kg vitamin C diet groups), cytoplasmic free-Ca^{2+} concentration (48.9–779.53 mg/kg vitamin C diet groups), apoptotic cell ratio (95.5–779.53 mg/kg vitamin C diet groups) and DNA damage (189.83–779.53 mg/kg vitamin C diet groups) under low temperature stress in comparison with those of control. We also investigated the alteration in protein expression under low temperature stress by a comparative proteomic analysis. The results demonstrated that 24 protein spots showed significantly differential expression in the cold-stress-treated group compared with those of the control group, and 5 protein spots were successfully identified. Furthermore, comparative proteomic analysis revealed that vitamin C could increase expressed proteins related to energy metabolism, immune responses and cytoskeleton. These findings would be helpful to understand the protective effects of vitamin C against cold stress.

1. Introduction

In aquaculture, fish often suffers from various ambient stressors such as temperature change, environmental pollutants, invasion of bacteria and viruses, leading to an immunosuppressive effect in the teleostean immune system (Magnadottir, 2010). Water temperature is one of an important environmental factors in aquaculture farming, while the change in water temperature may significantly influence the survival, physiological functions in the teleost fish (Bowden, 2008; Lee et al., 2014).

Previous studies indicate that low temperature can induce the oxidative stress in the aquatic organisms (Qiu et al., 2011; Fan et al., 2013). Reactive oxygen species (ROS) is generally regarded as a part of normal metabolism in animal cells. The stress-induced ROS production plays a pivotal role in several major cellular events, including cell growth, apoptosis and signal transduction pathways. Although cells have developed various defensive systems including enzymatic defense system and naturally antioxidant system to counteract the adverse effect of oxidative stress and balance the intracellular redox status, overproduction of ROS can damage important biomolecules, such as proteins, lipids and DNA, and then initiate a cascade of events involved in impaired cellular functions (Liu et al., 2014; Luo et al., 2015).

Vitamin C, a water-soluble vitamin, is an essential nutrient for all animals, playing a key role in the regulation of growth, reproduction and the immune response to stressors (Ming et al., 2012; Wan et al., 2014). Vitamin C is also a powerful antioxidant, which can prevent lipid peroxidation by reducing the generation of free radical levels in lipids (Padayatty and Levine, 2001). In addition, vitamin C is potentially beneficial to teleost by increasing the immune response to ambient stressors, thus reducing the stress-induced oxidative damage to tissues (Lin and Shiau, 2005; Shahkara et al., 2015).

Proteomics, focusing on the whole set of proteins, is an useful tool to study the dynamic of protein accumulation, regulation and interaction in response to various events. To date, comparative proteomics has been widely used in the immune response of aquatic animals to bacterial infection and environmental stress (Chongsatja et al., 2007; Chen et al., 2009; Jiang et al., 2009). However, few studies have evaluated the comparative proteomics in fish exposed to low temperature stress.

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The river pufferfish (*Takifugu obscurus*), widely distributed in the Sea of Japan, the East China Sea and the Yellow Sea, is an anadromous fish. Therefore, this species provides a good opportunity for the study of stress responses (Kim et al., 2010). In recently years, the wild resource of pufferfish population sharply decreased, which is due to the water pollution and overfishing. With the success of artificial propagation, farmed pufferfish has become increasingly popular in China. However, pufferfish farming suffers from serious disease problems induced by environmental stress. In this study, we investigated the effects of vitamin C on ROS production, cytoplasmic free-Ca^{2+}, apoptosis and DNA damage of pufferfish under low temperature stress. Furthermore, differentially expressed proteins of pufferfish fed with vitamin C diet were investigated through a proteomic approach. Our results would be helpful to understand the regulation mechanism of vitamin C against cold stress in pufferfish.

2. Materials and methods

2.1. Experimental diet

The formulation and proximate composition of the basal diet were shown in Table 1. The ingredients were purchased from the institute of animal science, Guangdong academy of agriculture science (Guangdong, China). The $_{L}$-ascorbyl-2-monpolyphosphate (35% ascorbic acid equivalent, DSM, Netherland) was selected as the source of vitamin C. Six diets were formulated to contain 2.60 (the control diet), 48.90, 95.50, 189.83, 382.40, 779.53 mg/kg vitamin C, respectively. All ingredients were ground into fine power through a 60 mm mesh. They were thoroughly mixed until homogenous in a Hobart-type mixer, then lipid and water were added and thoroughly mixed. Each mixture was pelleted (2 mm diameter) by a laboratory pellet machine (Institute of Chemical Engineering, South China University of Technology, Guangzhou, China). After air-drying, all samples were sealed in plastic bags and frozen stored (−20 °C).

Table 1
Formulation of the basal diet.

Ingredients	%
Fish meal	38.00
Soybean meal	18.00
Peanut meal	16.00
Yeast meal	2.00
Mixed oil[a]	4.00
Wheat meal	13.50
$Ca(H_2PO_4)_2$	2.00
Choline chloride	0.50
immunopotentiator	2.00
Cellulose	2.00
Mineral premix[b]	1.00
Vitamin premix (vitamin C free)[c]	1.00
Proximate composition	
Crude protein	41.94
Crude lipid	8.94
Ash	6.16

[a] 1:1 mixture of fish oil and Soybean oil.

[b] Mineral premix (g $kg^{-1}$of mixture): $MgSO_4{\cdot}7H_2O$, 80.0; $NaH_2PO_4{\cdot}2H_2O$, 370.0; KCl, 130.0; $FeSO_4{\cdot}7H_2O$, 40.0; $ZnSO_4{\cdot}7H_2O$, 20.0; Ca-lactate, 356.5; $CuSO_4$, 0.2; $AlCl_3{\cdot}6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4{\cdot}H_2O$, 2.0; $CoCl_2{\cdot}6H_2O$, 1.0.

[c] Vitamin premix (g $kg^{-1}$of mixture): myoinositol, 40.0; niacin, 36.0; DL-α-tocopheryl acetate, 20.0; thiamin hydrochloride, 4.0; riboflavin, 9.0; pyridoxine hydrochloride, 4.0; $_{D}$-pantothenic acid hemicalcium salt, 14.5; D-biotin, 0.3; folic acid, 0.8; menadione,0.2; retinyl acetate, 1.0; cholecalciferol, 0.05; cyanocobalamin, 0.01.

2.2. Experimental animals

Pufferfish were obtained from a fish farm in Panyu (Guangdong, China). Prior to the experiment, the experimental fish were acclimatized for two weeks at the laboratory conditions. The basal diets were fed to all fish during the experimental period. At the beginning of the experiment, 360 fish averaging at 10.3 ± 0.21 g (mean ± SD) were weighed and randomly distributed into 18 recirculating water tanks (500-L) with 20 individuals per tank. Each experimental diet was randomly assigned to three tanks. Each tank was provided with a continuous flow of water (3 L min^{-1}) and continuous aeration through air stones to maintain dissolved oxygen at or near saturation. Fish were fed twice daily (08:00 h and 17:00 h) at a rate of 4–6% wet body weight. The feeding trial lasted for 8 weeks. Water quality parameters were monitored by water quality analyze. During the experimental period, water temperature ranged from 25 to 28 °C, pH was 7.5–7.8, dissolved oxygen was no less than 6.0 mg L^{-1}, and ammonia nitrogen was lower than 0.05 mg L^{-1}.

2.3. Low temperature challenge experiment

After 8-week feeding trial, 30 fish were sampled from each group and subjected to low temperature challenge experiment. The Artificial Climate Chamber (temperature range 5–50 °C, RXZ-500D, Ningbo Dongnan Instrument Limited Company, China) was used to control the water temperature that decreased from 25 °C to 13 °C at a rate of 1 °C /1 h. Six fish from each group were sampled under different temperature treatment (25 °C, 21 °C, 17 °C, and 13 °C, respectively), and each treatment was maintained for 1 h. The temperature treatment of 25 °C was used as the control treatment. The pufferfish was anesthetized with MS-222 (tricaine methanesulfonate, Sigma, USA) at a concentration of 100 mg L^{-1} for 2 min before collecting tissue samples, and then the samples were immediately frozen in liquid nitrogen and preserved in −80 °C until use.

2.4. Determination of respiratory burst activity

The blood samples from the pufferfish were withdrawn from each fish by using 1 mL syringe. The measurement of respiratory burst activity was performed by using 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma) method, as described by Xian et al. (2009). A volume of 200 μL blood cells suspension was diluted with anticoagulant solution to obtain a final concentration of 1×10^6 cells/mL. DCFH-DA was set at a final concentration of 10 μM for 30 min in the dark at room temperature. Then the fluorescence of the cell suspensions was analyzed using the flow cytometer (Becton–Dickinson FACSCalibur).

2.5. Cytoplasmic free-Ca^{2+} concentration

Cytoplasmic free-Ca^{2+} concentration were examined using previously described methods (Xian et al., 2010). A volume of 200 μL blood cells suspension was diluted with anticoagulant solution to obtain a final concentration of 1×10^6 cells/mL, and then incubated with 10 μM fluo-3/AM for 30 min in the dark. Then the fluorescence of the cell suspensions was analyzed by flow cytometer.

2.6. Apoptotic cell ratio

The apoptosis of blood cells were determined by flow cytometry using an apoptosis detection kit (Invitrogen) following the manufacturer's instructions. The collected blood cells were diluted with anticoagulant solution to obtain a final concentration of 1×10^6 cells. The pellets were resuspended by Annexin V-FITC binding buffer, and then incubated with Annexin V-FITC in dark at 20–25 °C for 10 min. Samples were then analyzed by flow cytometer.