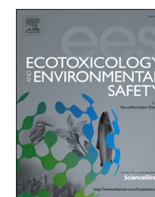
Ecotoxicology and Environmental Safety 127 (2016) 170–174

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



The protective effect of blueberry anthocyanins against perfluorooctanoic acid-induced disturbance in planarian (*Dugesia japonica*)

Zuoqing Yuan, Jianyong Zhang*, Changchao Tu, Zhijing Wang, Wenpeng Xin

School of Life Sciences, Shandong University of Technology, No. 12 Zhangzhou Road, Zibo, Shandong 255049, China

ARTICLE INFO

Article history:
Received 30 October 2015
Received in revised form
22 January 2016
Accepted 25 January 2016
Available online 4 February 2016

Keywords:
Dugesia japonica
Perfluorooctanoic acid (PFOA)
Anthocyanins
Mitochondria
Total antioxidant capacity
Enzyme activities

ABSTRACT

The influence of blueberry anthocyanins on perfluorooctanoic acid (PFOA)-induced stress response in planarian mitochondria was investigated. PFOA at 15 mg/L and anthocyanins at 10 or 20 mg/L were individually and simultaneously administered to planarians for up to 10 d. The results showed PFOA treatment induced an increase in mitochondrial permeability transition pore opening and a decrease antioxidant capacity and enzyme activities. In anthocyanin treated animals, the activity of succinate dehydrogenase, cytochrome oxidase and monoamine oxidase increased, but mitochondrial permeability transition pore opening decreased and total antioxidant capacity increased. An improvement in above-mentioned physiological and biochemical parameters was found in the combined PFOA and anthocyanin treated animals, in a dose-dependent manner. Anthocyanins attenuated the PFOA induced toxicity; antioxidant capacity and enzyme activities are involved in the protective mechanism of anthocyanins.

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1. Introduction

Dugesia japonica is common in East Asia and widely distributed in China. In view of the planarian regeneration capacity and chemical sensitivity, *D. japonica* are used as model organisms in the research fields of regenerative medicine, stem cell biology, neurological disease and toxicology (Abril et al., 2010; Newmark and Alvarado, 2002; Kitamura et al., 1998; Wu et al., 2014; Zhang et al., 2013). Perfluorooctanoic acid (PFOA) is one of the major fully fluorinated organic compounds found in a variety of environmental matrices and biological samples worldwide (Lau et al., 2007). However, the mechanisms of action, the affected metabolic pathways and their resulting toxicological effects were mainly examined in rodents, and only a few studies have been performed on aquatic organisms, despite the aquatic environment being an important site for PFOA deposit. Many PFOA studies demonstrated that long-term treatment with PFOA results in hepatotoxicity, developmental toxicity, immunotoxicity, hormonal effects and carcinogenic potency (Andersen et al., 2008). Previously, we found that the enzyme activities of superoxidedismutase (SOD) and catalase (CAT) were altered in PFOA-exposed planarian, with obvious increase of apoptosis in brain, eye and parenchyma region (Yuan et al., 2015). Additionally, PFOA caused oxidative stress and mitochondrial dysfunction in cells (Panaretakis et al., 2001), interfered with tissue metabolism by inducing mitochondrial permeability transition leading to inhibition of mitochondrial fatty acid β-oxidation (O'Brien and Wallace, 2004) and induced cell apoptosis via a p53-dependent mitochondrial pathway (Huang et al., 2013). These findings indicate that exposure to PFOA alters normal mitochondrial function or damages their structure.

Anthocyanins (ANT) are a water-soluble natural pigment that appears as red, purple, and blue in plants and belongs to the flavonoid parent class of molecules. ANT are phytonutrients that have phenolic groups in their structures and have been widely studied due to their antioxidant activities (Williamson and Clifford, 2010). Blueberries (*Vaccinium corymbosum L.*) are recognized as a good source of ANT. Human studies demonstrated that blueberry anthocyanins provide and activate cellular antioxidant protection, prevent cellular oxidative stress, and consequently protect against oxidant-induced inflammatory cell damage and cytotoxicity (Harborne and Wiliam, 2000; Zafra-Stone et al., 2007; Tan et al., 2014). To examine the potential protective role of blueberry anthocyanins to planarian, we administered the blueberry anthocyanins to planarian under PFOA stress. Mitochondrial permeability transition pore (MPTP) opening, total antioxidant capacity (TAC), succinate dehydrogenase (SDH), cytochrome oxidase (COX) and monoamine oxidase (MAO) activities were then examined and compared.

Although there are many studies investigating PFOA toxicity, little is known about the effect of PFOA on mitochondrial function

* Corresponding author.
E-mail address: zhangjy1977@126.com (J. Zhang).

http://dx.doi.org/10.1016/j.ecoenv.2016.01.019
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at the biochemical level in planarian. Additionally, the mechanisms of action associated with ANT are not well defined. Investigating the properties of this phytonutrient is of great significance, because it is a natural compound found in fruits and vegetables and is present in the human diet in many countries. The aim of this study was to assess the toxic effects of PFOA and the protective effects of blueberry anthocyanins, under continuous exposure, on the MPTP opening, TAC, SDH, COX and MAO activities in this freshwater planarian. The results of this study provide a mechanistic understanding of PFOA toxicity, and helpful information toward establishing *D. japonica* as a research model for studying the toxicity of other environmental contaminants.

2. Materials and methods

2.1. Animals and chemicals

The stock of *D. japonica* used in this study was obtained from a fountain in Quanhetou (Zibo, China) and acclimated in our laboratory for >2 weeks as described previously (Zhang et al., 2013). The animals were kept in autoclaved tap water at 20 °C. The water was aerated continuously and changed every 2 days until use. Size-selected intact planarians (>1 cm TL) were used for this study. Planarians were starved for 1 week to create a uniform metabolic status before starting the experiments. PFOA was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). ANT were extracted and purified from blueberries and were commercially available from Reain Biotechnology Co. (Xian, China).

2.2. Exposure experiments

Planarians 1.0–1.5 cm in length were used within 10 days for the experiments. Stock solutions of PFOA and blueberry anthocyanins were prepared on the day of the experiment and diluted to desired concentrations using autoclaved tap water. The planarians were continuously exposed to ANT and PFOA at different concentrations as follows: (1) control: incubated in autoclaved tap water; (2) PFOA-treatment: incubated in PFOA solution at 15 mg/L; (3) Anthocyanins treatment: incubated in solution containing ANT (10 mg/L); (4) PFOA+ANT treatment: incubated in solution containing PFOA (15 mg/L) and ANT (10 mg/L or 20 mg/L, respectively). All experiments at the different concentrations were repeated in triplicate and each experiment involved 100 planarians. Planarians were sampled on days 1, 3, 7 and 10, and used for the desired analysis.

2.3. Isolation of mitochondria

All of the procedures were carried out at 4 °C. 100 mg of the planarians undergoing the different treatments were washed with phosphate-buffered saline (PBS) buffer in 1.5 mL centrifugal tubes. Mitochondria were isolated from different treatments and different treatment days using a Mitochondria Isolation Kit (C3606; Beyotime Institute of Biotechnology, Shanghai, China). Isolation of mitochondria was repeated in triplicate and the isolated mitochondria were suspended in the storage solution from the Kit. Mitochondria solution (10 μL) was used for the determination of protein content by the Bradford method.

2.4. MPTP opening assay

The isolated mitochondria (0.1 mL) were pre-incubated in a Quartz cup containing 2.9 mL medium (pH 7.4) consisting of sucrose (0.25 mM), KCl (10 mM), MgCl (5 mM), KH_2PO4 (5 mM), Tris–HCl (10 mM), and succinate (10 mM) at 30 °C for 10 min. The MPTP opening in the mitochondria from different groups was measured by monitoring the absorbance at 520 nm (A520) as described (Zhao et al., 2010).

$$\text{MPTP opening } (\%) = \frac{Ac - At}{Ac}$$

where *Ac* represents the absorbance value of the control group and *At* is the absorbance value of treatment group.

2.5. Total antioxidant capacity assay

Planarians (20 mg) from different treatment groups and different treatment days were homogenized using a grinding rod in a centrifugal tube on ice. After centrifugation at 12,000 g for 10 min at 4 °C, the supernatant was collected and stored at −20 °C. Total antioxidant capacity of the planarians was measured using the Total Antioxidant Capacity Kit (S0116; Beyotime Institute of Biotechnology, Shanghai, China) according to manufacturer instructions using the ferric-reducing antioxidant power method (FRAP) (Benzie and Strain, 1999). For the FRAP assay, total antioxidant capacity is represented by different concentrations of $FeSO_4$ absorbance values. If the absorbance value of the planarian samples is same to the absorbance value of 1 mM $FeSO_4$, and the total antioxidant capacity of the tissue samples was 1 mM. Sample solutions (20 μL) were added directly to the 96-well microplate followed by 180 μL of working FRAP solution. The mixtures were incubated in the dark for 30 min at 37 °C and absorbance readings were recorded at 593 nm (A593). The standard curve was constructed using iron (II) sulfate solution (150–1500 μM). All measurements were performed in triplicate and the mean values were calculated.

2.6. Enzymes activity assays

The isolated mitochondria (10 μL) were used to determine the activities of SDH, COX and MAO. The activities of SDH, COX and MAO were measured using the Tissue SDH Assay Kit, COX Assay Kit and Tissue MAO Assay Kit (GenMed, Shanghai, China) according to manufacturer instructions, respectively. The enzyme activities were measured by ultraviolet-visible spectrophotometric (SP-756 Spectrophotometer; Shanghai Spectrum Instruments, Shanghai, China) analysis at 595 nm. Measurements were collected at 5-min intervals and normalized to protein content.

2.7. Statistical analysis

Data are presented as mean and standard deviation (SD). Statistical significance was determined by the analysis of variance using Statistical Package for the Social Sciences for Windows (SPSS, version 16.0; Chicago, IL, USA). Treatment groups were tested for differences from the control and PFOA groups using Dunnett's *t* test. Statistically significant differences were determined at $P<0.05$.

3. Results

3.1. Effect of PFOA and blueberry anthocyanins on MPTP opening

MPTP opening was investigated using the spectrophotometric method. Changes in the absorbance values were used to detect MPTP opening. As shown in Fig. 1, PFOA resulted in a significant increase in MPTP opening as compared to the control group ($P<0.01$). The MPTP opening slightly decreased in ANT treatment groups relative to the control. The treatment groups with exposure to PFOA and ANT exhibited rapidly decreasing absorbance values

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