The protective effect of C-phycocyanin on paraquat-induced acute lung injury in rats

Yingxin Sun\textsuperscript{a,b}, Juan Zhang\textsuperscript{c}, Yongjian Yan\textsuperscript{c}, Mingfeng Chi\textsuperscript{d}, Wenwen Chen\textsuperscript{c}, Peng Sun\textsuperscript{e}, Song Qin\textsuperscript{a,f,*}

\textsuperscript{a} Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
\textsuperscript{b} Department of Postgraduate, Shandong University of Traditional Chinese Medicine, Jinan 250014, China
\textsuperscript{c} Shandong Academy of Occupational Health and Occupational Disease, Jinan 250062, China
\textsuperscript{d} The Athletes Rehabilitation Centre of ShanDong Province, Jinan 250014, China
\textsuperscript{e} Department of Pharmaceutical Analysis, Luye Pharmaceutical Limited Company, Yantai 264003, China
\textsuperscript{f} Laboratory of Biological Resources, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, 17 Chunhui Road, Laishan District, Yantai 264003, Shandong Province, China

\textbf{A R T I C L E   I N F O}

Article history:
Received 3 November 2010
Received in revised form 16 April 2011
Accepted 27 April 2011
Available online 5 May 2011

Keywords:
C-phycocyanin
Paraquat
Acute lung injury
Lipid peroxidation
Inflammation

\textbf{A B S T R A C T}

To investigate the potential protective effect of C-phycocyanin (PC) on paraquat (PQ)-induced acute lung injury, rats were divided into control, PQ-treated and PQ + PC-treated groups. Rats in PQ-treated group were orally administered with 50 mg/kg PQ, and rats in PQ + PC-treated group were intraperitoneally injected with 50 mg/kg PC after administration of PQ. At 8, 24, 48 and 72 h after treatments, GSH-Px and SOD activities, MDA levels in plasma and BALF, HYP, NF-\(\kappa\)B, I\(\kappa\)B-\(\alpha\) and TNF-\(\alpha\) contents in lung tissues were measured. The pathological changes in lung were observed. After treatment with PC, the levels of MDA and the relative contents of NF-\(\kappa\)B and TNF-\(\alpha\) were significantly decreased, the activities of GSH-Px and SOD and the relative contents of I\(\kappa\)B-\(\alpha\) were significantly increased. The degree of rat lung damage was obviously reduced in PQ + PC-treated group. The results suggested that PC treatment significantly attenuated PQ-induced acute lung injury.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Paraquat (PQ; 1,1′-dimethyl-4,4′-bi-pyridinium chloride) is one of the most widely used herbicide in the world and a highly toxic compound for humans and animals (Suntres, 2002; Parvez and Raisuddin, 2006; Neves \textit{et al.}, 2010). PQ poisoning is more frequently fatal than poisoning caused by other pesticides. Reports showed that PQ poisoning accounted for only 0.34% of pesticides poisoning cases, but PQ poisoning had the highest mortality rate (50–90%), accounting for 13% of all fatal cases (Klein-Schwartz and Smith, 1997; Lock and Wilks, 2001).

Severe PQ poisoning is characterized by multiple-organ failure, involving mainly the lung, kidney, and liver. The lung is the major target organ in PQ poisoning characterized by edema, hemorrhage, interstitial inflammation, and proliferation of bronchial epithelial cells (Berisha \textit{et al.}, 1994; Venkatesan, 2000), and respiratory failure from lung injury is

\textit{Abbreviations}: PQ, paraquat; PC, C-phycocyanin; GSH-Px, glutathione peroxidase; HYP, hydroxyproline; MDA, maleic dialdehyde; SOD, superoxide dismutase.

\textsuperscript{*} Corresponding author. Tel.: +86 5352109018; fax: +86 5352109000.
E-mail address: qinsongqd@126.com (S. Qin).
1382-6689/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
the most common cause of death. The main mechanism of PQ's toxic effects is redox reaction by reactive oxygen species, and lipid peroxidation of cellular membranes is a significant pathway (Yasaka et al., 1986). In addition to redox reaction, inflammatory reaction has been reported as a main mechanism of tissue injury (Orito et al., 2004). Several drugs have been investigated against PQ-induced lung toxicity, but the specific antidote has not been currently founded yet (Bateman, 1987; Suntres, 2002; Dinis-Oliveira et al., 2008).

C-phycocyanin (PC), a biliprotein pigment and an important constituent of the blue-green alga Spirulina platensis, contains phycocyanobilin, an open-chain tetrapyrrole chromophore that is covalently attached to the apoprotein and plays a major role in some of its important biological properties (Lissi et al., 2000). PC has been shown to possess significant antioxidant, radical-scavenging, anti-inflammatory, hepatoprotective, radical scavenging, antiarthritic, neuroprotective and anti-tumor effects (Romay et al., 2003; Li et al., 2005; Cheng et al., 2007; Sathysaikumar et al., 2007). Therefore, in the present study an attempt was made to investigate whether PC has the protective effect on PQ-induced acute lung injury in a rodent model of rats. The results showed that PC treatment significantly attenuated PQ-induced acute lung injury.

2. Materials and methods

2.1. Materials

Male Wistar rats (weighting 180–200 g, SPF grade) were purchased from the Laboratory Animal Research Center of Shandong University of Traditional Chinese Medicine (Jinan, Shandong, China). Analytical Grade PC (Purity: A620/A280 ≥ 4) was provided by Yantai Institute of Coastal Zone Research (Yantai, Shandong, China). PQ was provided by Shandong Kexin Biochemical Co. (Jinan, Shandong, China). All other chemicals were of analytical grade, and procured from local commercial sources.

2.2. Animals treatments

After 3 days of acclimatization, the rats were randomly divided into three groups. The rats in control group (n = 24) were treated with the saline solution and sacrificed at 8 h (n = 6, control 8 h group), 24 h (n = 6, control 24 h group), 48 h (n = 6, control 48 h group) and 72 h (n = 6, control 72 h group). The rats in PQ-treated group (n = 24) were orally given aqueous solution of PQ (50 mg/kg) by gastric gavage and sacrificed at 8 h (n = 6, PQ 8 h group), 24 h (n = 6, PQ 24 h group), 48 h (n = 6, PQ 48 h group) and 72 h (n = 6, PQ 72 h group). The rats in PQ + PC-treated group (n = 24) were immediately intraperitoneally injected with 50 mg/kg PC after administration of PQ, then were sacrificed at 8 h (n = 6, PQ + PC 8 h group), 24 h (n = 6, PQ + PC 24 h group), 48 h (n = 6, PQ + PC 48 h group) and 72 h (n = 6, PQ + PC 72 h group). Throughout the study period, each animal was observed carefully for clinical signs of toxicity related to PQ. Handling of animals strictly followed the ethical guidelines set forth by the European Community guidelines (EEC Directive of 1986; 86/609/EEC).

2.3. Sample collection

At 8, 24, 48 and 72 h after treatments, 6 rats were randomly selected in each group and deeply anesthetized with an intraperitoneal injection of pentobarbital sodium (35 mg/kg). Blood samples were taken from the jugular vein of the rats, and centrifuged at 4000 rpm for 15 min. The supernatant was collected for the measurement of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities, maleic dialdehyde (MDA) levels.

After blood samples collected, immediately thoracotomy was sterilely performed to obtain bronchoalveolar lavage fluid (BALF). The BALF was collected from the right lung. The bronchus of the left lung was clamped with forceps, and then, the right bronchus was cannulated. Subsequently, 5 ml cold saline 4°C was instilled and aspirated. This was repeated three times and about 3 ml BALF was obtained and centrifuged at 3000 rpm for 15 min. The supernatant was harvested for the measurement of SOD, GSH-Px and MDA. Right lung was harvested for the contents of hydroxyproline (HYP) and tumor necrosis factor-α (TNF-α) in lung homogenate measurement. The inferior lobe of rat left lung at 72 h was cut into small pieces (1 mm3) and fixed in 5% glutaraldehyde for ultra-morphological examination, and the remaining inferior lobe of left lung was fixed in 10% neutral buffered formalin solution before immunohistochemical and histological analysis.

2.4. Measurement of biomarkers of oxidative stress

The levels of MDA were determined as an indicator of lipid peroxidation. The activities of GSH-Px and SOD and the levels of MDA both in plasma and BALF of rats were determined using the assay Kits (Nanjing Jiancheng Corp., China), according to the manufacturer's recommendations. The units for MDA levels, GSH-Px and SOD activities were expressed as nmol/ml, U and U/ml, respectively.

2.5. HYP assay of lung tissue

The HYP contents of lung tissues were determined and the data were expressed as ng/g wet lung tissue. The 100 mg frozen lung tissue from control, PQ-treated and PQ + PC-treated rats was thoroughly homogenized in distilled water and determined using an assay Kit (Nanjing Jiancheng Corp., China), according to the manufacturer's recommendations.

2.6. Measurements of NF-κB, IκB-α and TNF-α

Nuclear factor kappa-B (NF-κB), IκB-α (an inhibitor of NF-κB) and TNF-α were measured by immunohistochemistry combined with semiquantitative analysis. In brief, after dewaxing of sections, endogenous peroxidase activity was quenched with 3% H2O2, and cross-reactivity was blocked with normal serum. The tissues were incubated overnight at 4°C with primary antibodies according to introduction. Localization of the primary antibodies was achieved by subsequent use of a biotinylated anti-primary antibody, an avidin–biotin complex conjugated with horseradish peroxidase and 3’/5’-diaminobenzidine (Vectasitain Elite Kit, America). Normal
Fig. 1 – The content of HYP in lung tissue in control, PQ-treated and PQ + PC-treated group. *Compared with control group, **p < 0.01, ▲ compared with PQ-treated group, ▲▲p < 0.01.

Immunoreactivity was semiquantified within 5 random fields at 200× magnification with a Leica DM4000 B system and Leica QWin V3 software was applied to evaluate grey scale intensity variations in order to determine the relative contents of NF-κB, IκB-α and TNF-α in lung. In computing, a grayscale or greyscale digital image is an image in which the value of each pixel is a single sample. Displayed images of this sort are typically composed of shades of grey, varying from black at the weakest intensity to white at the strongest. The grey scale intensity and protein expression have inverse relation.

2.7. Statistical analysis

All data were expressed as the mean ± standard deviation (S.D.). The data were analyzed by one-way ANOVA using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Statistical probability of p < 0.05 was considered significant.

3. Results

3.1. Rats phenotypes

During the whole experiment, no deaths were observed. But the clinical signs including polypnea, blausucht, crouch, less activity, diarrhea and anorexia were observed in some of the rats exposed to PQ. However, these symptoms were greatly improved after treatment with PC.

3.2. Effect of PC on HYP content in lung

As shown in Fig. 1, compared with control group, PQ significantly increased HYP concentration in lung tissue at 8h, 24h, 48h and 72h (p < 0.01). After treatment with PC, HYP was significantly decreased (p < 0.05, p < 0.01), compared with PQ-treated group.

Fig. 2 – The activity of GSH-Px in plasma (A) and BLFA (B) in control, PQ-treated and PQ + PC-treated group. *Compared with control group, *p < 0.05, **p < 0.01, ▲ compared with PQ-treated group, ▲▲p < 0.01.

After administration of PQ, the activity of GSH-Px in plasma and BALF was significantly decreased at 8h, 24h, 48h and 72h (p < 0.05, p < 0.01), compared with control group. But the activity of GSH-Px in plasma and BALF, especially in BALF, was markedly increased after treatment with PC with the time prolonged (p < 0.05, p < 0.01) (Fig. 2).

3.4. Effect of PC on SOD activity in plasma and BALF

After administration of PQ, the activity of SOD in plasma and BALF was significantly decreased at 8h, 24h, 48h and 72h (p < 0.05, p < 0.01), compared with control group. After treatment with PC, the activity of SOD in plasma and BALF was increased at different degrees with the time prolonged (p < 0.05, p < 0.01), compared with PQ-treated group (Fig. 3).
3.5. Effect of PC on MDA level in plasma and BALF

After administration of PQ, the levels of lipid peroxidation marker MDA in plasma and BALF were significantly increased at 8 h, 24 h, 48 h and 72 h ($p < 0.05$, $p < 0.01$), compared with the control group. After treatment with PC, the levels of MDA were decreased at different degrees, statistical differences were found at different times ($p < 0.05$, $p < 0.01$) except for the level of MDA in plasma at 8 h, compared with PQ-treated group (Fig. 4).

3.6. Effect of PC on the relative contents of NF-κB, IκB-α and TNF-α in lung

Fig. 5 showed that the relative contents of NF-κB and TNF-α in lung of PQ-treated rats at 72 h were significantly increased ($p < 0.01$) and IκB-α in lung was remarkably decreased ($p < 0.01$), compared with the control group. After treatment with PC, NF-κB and TNF-α in lung were remarkably inhibited ($p < 0.01$) and IκB-α in lung was increased ($p < 0.05$).

3.7. Effect of PC on histological changes in lung

PQ-induced lung structural changes, ultrastructural alterations and the alleviative effects of PC on pathological changes of PQ-induced lung injury are depicted in Figs. 6 and 7. Histological changes were assessed with hematoxylin and eosin (HE). Animals from control group showed a normal pulmonary structure at light microscopy and electron microscopy, without evidences of cellular infiltrations, alveolar collapse or collagen accumulation. Compared with control group, the lung tissue of PQ-treated group of lung injury showed marked alterations, such as intense vascular congestion, hemorrhage and inflammatory cell infiltration, mainly characterized by a diffuse alveoli collapse with an increased thickness of its walls. In PQ + PC-treated group, although the changes mentioned above also could be observed referred alterations were drastically attenuated compared with PQ-treated group, particularly inflammation, hemorrhage, and the amount of accumulation of collagenous fiber. Comparing to PQ-treated...
group, the vascular congestion and the alveolar collapse were not as noticeable in PQ + PC-treated animals. Furthermore, despite the existence of several pneumocytes and interstitial edema, the exuberance of those signals was drastically attenuated in PQ + PC-treated animals.

4. Discussion

The main target organs involved in PQ toxicity are the lung. The lungs are preferentially targeted because of PQ's rapid uptake and accumulation in this organ through polyamine transporters (Forman et al., 1982). Severe PQ poisoning produces adult respiratory distress syndrome, pulmonary hypertension, edema and progressive lung fibrosis. Free radicals are known to play a crucial role in PQ-induced lung toxicity (Tampo et al., 1999; Minakata et al., 2000; Kim et al., 2010). It has been reported that the toxicity is related to redox cycling of an iron–PQ complex, which in turn catalyzes the formation of ROS with the ultimate progression of lipid peroxidation (Tsukamoto et al., 2002; Gil et al., 2007). In this study, PC reduced the acute lung injury induced by PQ intoxication, and the MDA concentration, as a marker of lipid peroxidation, was lower in the PQ + PC-treated group than in the PQ-treated group. The PQ-induced biochemical changes as indicated by significant decrease of SOD and GSH-Px activities, increase of MDA levels in blood plasma and BALF and increase of HYP contents in lung tissue were alleviated by PC. In these results, we could infer that PC reduced acute lung injury by attenuation of free radicals-induced lipid peroxidation.

Acute lung injury, as a kind of inflammatory syndrome clinically characterized as increase in blood vessel permeability, can be induced by various dangerous factors outside pulmonary system with complicated pathogenesis. One of them is NF-κB, a kind of eukaryon cell transcription factor, which promotes and regulates the gene transcription of some inflammatory mediators (TNF-α, IL-8, iNOS, COX2, etc.). In non-stimulated cells, NF-κB dimers exist in the cytoplasm in inactive forms due to the binding of an inhibitory protein called IκB (Siebenlist et al., 2005). Many inflammatory conditions, including bacterial and viral infections, can rapidly activate the NF-κB signaling pathway. Activation of NF-κB is induced by the phosphorylation, ubiquitination and degradation of IκB. Then, NF-κB dimers translocate to the nucleus

Fig. 5 – The relative contents of NF-κB, IκB-α and TNF-α in rat lung at 72 h. *Compared with control group, **p < 0.01, * compared with PQ-treated group, *p < 0.05, **p < 0.01.

Fig. 6 – The lung histopathology of rats in control (A), PQ-treated (B) and PQ + PC-treated group (C). At 72 h after treatments, rats were sacrificed and their left lungs were paraffin embedded and stained with hematoxylin and eosin. The magnification of all of the images is 200×.
where they stimulate the transcription of hundreds of genes that participate in inflammatory responses (Mizutani et al., 2010). NF-kB plays an important role in lung injury of PQ poisoned rats (Tong et al., 2007).

The pulmonary toxicity caused by PQ is assumed to have a connection with the activation of neutrophils (Hybertson et al., 1995). Furthermore, various inflammatory mediators including TNF-α have been expected to be increased in the lung during PQ toxicity (Dinis-Oliveira et al., 2006a). It is known that TNF-α triggers the synthesis of leukotrienes and prostaglandin E (E₂) which then stimulate the infiltration of polymorphonuclear leukocytes into the lungs and cause lung injury (Dinis-Oliveira et al., 2006b).

Recent reports showed that PC could significantly inhibit the LPS-induced nitrite production and iNOS protein expression accompanied by an attenuation of TNF-α formation (Cheng et al., 2007). Pre- or post-treatment with PC significantly attenuated carrageenan-induced inflammatory nociception and the induction of iNOS and COX-2 accompanied by an inhibition of the formation of TNF-α, E₂ and myeloperoxidase activity (Shih et al., 2009). PC also reduced the levels of TNF-α in the blood serum of mice treated with endotoxin and it showed neuroprotective effects in rat cerebellar granule cell cultures and in kainate-induced brain injury in rats (Romay et al., 2003). In our study, PC significantly inhibit NF-kB and TNF-α formation. We suggested that the anti-inflammatory effect of PC may be ascribed to an inhibition of NF-kB-mediated cytotoxicity.

We also have observed the characteristic PQ-induced pathological alterations including alveolar edema, hemorrhage, inflammatory cell infiltration, a diffuse alveoli collapse with an increased thickness of its walls, the swollen-type II alveolar epithelial cells, and deformed mitochondria by electron-microscopy and the development of an extensive fibrosis in lung after PQ exposure. In this study, treatment with PC was very effective in the preventing oxidative damage induced by PQ, which are characterized by the reversal of PQ-induced tissue damages.

In our study, we used only a single dosage of PC. It would be more valuable if we study either low dose or high dose of PC. However, the results in the present study clearly demonstrated that PC significantly increased SOD and GSHP-x activities, decreased MDA and HYP levels, and reduced accumulation of NF-κB and TNF-α in PQ-treated rats. These findings suggested that PC might exert its protective effects on PQ-induced damage by alleviating the earlier inflammation damage via PQ-induced oxidative stress in rat lung. Future studies are warranted to further investigate the underlying mechanisms involved in this complicated process.

**Conflicts of interest**

All the authors declare that there are no conflicts of interest.

**Acknowledgment**

This work was supported by the science and technology funds from Shandong Education Department (J07YD09).
REFERENCES


