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BRIEF COMMUNICATION

C-phycocyanin Mitigates Cognitive Impairment in Doxorubicin-Induced Chemobrain: Impact on Neuroinflammation, Oxidative Stress, and Brain Mitochondrial and Synaptic Alterations

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Abstract

Chemotherapy-induced cognitive impairment (CICI) is a common detrimental effect of cancer treatment, occurring in up to 75% of cancer patients. The widely utilized chemotherapeutic agent doxorubicin (DOX) has been implicated in cognitive decline, mostly via cytokine-induced neuroinflammatory and oxidative and mitochondrial damage to brain tissues. C-phycocyanin (CP) has previously been shown to have potent anti-inflammatory, antioxidant, and mitochondrial protective properties. Therefore, this present study was aimed to investigate the neuroprotective effects of CP against DOX-elicited cognitive impairment and explore the underlying mechanisms. CP treatment (50 mg/kg) significantly improved behavioral deficits in DOX-treated mice. Furthermore, CP suppressed DOX-induced neuroinflammation and oxidative stress, mitigated mitochondrial abnormalities, rescued dendritic spine loss, and increased synaptic density in the hippocampus of DOX-treated mice. Our results suggested that CP improves established DOX-induced cognitive deficits, which could be explained at least partly by inhibition of neuroinflammatory and oxidant stress and attenuation of mitochondrial and synaptic dysfunction.

Keywords C-phycocyanin · Chemotherapy · Cognitive · Mitochondria · Doxorubicin

Abbreviations

CICI	Chemotherapy-induced cognitive impairment
CP	C-phycocyanin
DOX	Doxorubicin
mPTP	Mitochondrial permeability transition pore
GSH	Glutathione
MM	Mitochondrial membrane potential
MDA	Malondialdehyde
MWM	Morris water maze
PSD95	Postsynaptic density protein 95
ROS	Reactive oxygen species
SOD	Superoxide dismutase

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Introduction

Advances in the efficacy of cancer treatment have contributed to a significantly reduced risk of recurrence and a sharp increase in patient survival rates. However, up to 75% of cancer patients have reported suffering from cognitive impairment during chemotherapy [1], sometimes lasting years after cessation of chemotherapy [2]. Chemotherapy-induced cognitive impairment (CICI), commonly referred to as "chemo brain," is manifested clinically as deficits in memory function and attention, decremented executive functioning, and slower processing speed. Though CICI poses a severe problem to public health, there are no specific therapeutic agents for the treatment of CICI.

Doxorubicin (DOX) is a highly potent anthracycline agent approved by the FDA and widely used in clinical for several types of malignancies. However, the clinical use of DOX is limited due to dose-dependent adverse severe reactions to

non-target tissues [3]. Cancer survivors undergoing DOX-based chemotherapy have been shown persistent cognitive dysfunction [4]. Moreover, experimental studies conducted in rodent models revealed DOX-evoked neurobehavioral alterations such as anxiety, depressive-like behavior, and cognitive deficit [5]. Although the mechanism underlying the DOX-induced chemo brain needs further studies, it has been confirmed that the disturbance of cytokines contributes to chemotherapy-related neurotoxicity [6, 7]. DOX promotes the generation of tumor necrosis factor-alpha (TNF-α), which can penetrate the brain and trigger glial activation and the release of inflammatory cytokines. The increased pro-inflammatory mediators and neuroinflammatory microenvironment following DOX stimulation induce oxidative damage [8]. DOX toxicity is related to mitochondrial structure and function impairment [9]. Reports also revealed the mitochondrial abnormalities in the brains of DOX-treated mice, including decreased respiratory capacity, disturbed redox state, increased mPTP opening susceptibility, and activated apoptotic signaling [10]. Consequently, these insulting events may be the reason for the DOX-induced brain toxicity and neuronal injury that eventually lead to the deterioration of cognitive function.

C-phycocyanin (CP) possesses extensive pharmacological effects such as antioxidant, anti-inflammatory, and mitochondrial protective properties [11]. CP is widely applied in the cosmetics, food, and pharmaceutical industries. Neuroprotective effects of CP have been observed in experimentally induced neurodegeneration models like Parkinson, Huntington, and Multiple Sclerosis [12–14]. Recently, CP has been reported to attenuate cisplatin-evoked nephrotoxicity via improving mitochondrial function and enhancing oxidative defense in mice [15]. However, little is known about the effects of CP on the CICI. Therefore, the present study is aimed to investigate the neuroprotective effects of CP against DOX-induced cognitive deficits in mice and explore the underlying mechanisms.

Materials and Methods

Group and Treatment

Male C57BL/6 mice were randomly assigned into four groups ($n = 12$): control (Control), control plus C-phycocyanin (Con + CP), DOX-induced chemobrain (DOX), and DOX-induced chemobrain plus C-phycocyanin (DOX + CP). Mice in the DOX group were treated with intraperitoneal injections of 2.5 mg/kg DOX every two days for a total of seven injections over a 2-week period. The dose of doxorubicin was chosen with reference to previous studies [16]. The DOX + CP mice were administrated daily intraperitoneal injections of CP (50 mg/kg; Sigma-Aldrich) for 3 weeks starting 1 week before the DOX challenge. On the day of DOX administration, CP was given 2 h prior to DOX injection. The Control group received an injection of DOX solvent every two days and a daily injection of CP solvent for 3 weeks. Con + CP group was treated with an injection of DOX solvent (saline) every 2 days and a daily injection of CP for 3 weeks. The selected dosage of CP was based on previous reports [17] and our preliminary pilot showing neuroprotective effects. The protocol was approved by the Animal Care Committee of Henan University of Science and Technology, China.

Morris Water Maze

The MWM test was conducted as described previously [18]. The apparatus consists of a white circular tank (100 cm in diameter) filled with warm water (22 ± 1 °C) and a platform (10 cm in diameter). The circular pool was divided into four equal quadrants, and the platform was placed 1.5 cm below the surface of the water at the midpoint of one quadrant. During the memory acquisition phase, each mouse was subjected to four training trials for 5 consecutive days. The time to find the hidden platform (escape latency) was recorded for each quadrant, and the average of four trials was determined as daily data. One day following the last acquisition trial, a probe test was performed to evaluate the memory retention. During the probe test, each mouse was allowed to swim freely for 60 s without the platform. The time spent in the target quadrant, the velocity, and the path length during the probe test were recorded. After the probe test, each mouse was trained on a visible platform for 2 days, wherein the platform is visible, to evaluate the visual acuity of the mice. The swimming behavior of all mice was monitored and analyzed by EthoVision XT 7.0 video tracking system (Noldus. Wageningen, Netherlands).

Assessment of Pro-inflammatory Cytokines Levels

Hippocampal homogenates in RIPA buffer were used for the determination of the level of TNF-α, IL-1β, and IL-6 by ELISA kits following the manufacturer's protocol (R&D Systems, Minneapolis, USA), and normalized to protein concentration.

Assessment of Oxidative Stress Biomarkers

The levels of MDA, reduced glutathione (GSH), and the SOD activity in the hippocampus were examined as described previously [19]. The values were presented as nanomoles (nmol) of MDA/mg of protein. The amount of reduced GSH was presented as nmol of GSH/mg of protein. The SOD activity was presented as units (U)/mg of protein. The levels of protein carbonyl and

8-hydroxy-2′-deoxyguanosine (8-OHdG) were analyzed by the commercial assay kits according to the recommended protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) [20].

Mitochondrial Function Analysis

The hippocampal mitochondria were isolated by using a standard procedure [21]. Mitochondrial enzyme Complex I, II, and IV activity, mitochondrial ATP, and mitochondrial ROS production were performed following our previously published method [22]. Briefly, Complex-I activity was assayed spectrophotometrically by measuring the oxidation of NADH to NAD^+ with subsequent reduction of cytochrome c in an assay mixture containing 0.2 M glycylglycine buffer pH 8.5, 6 mM NADH in 2 mM glycylglycine buffer, and 10.5 mM cytochrome C. The reaction started by the addition of mitochondrial sample, and the change in absorbance was recorded at 550 nm. Complex-II activity was assayed spectrophotometrically by measuring the oxidation of succinate by an artificial electron acceptor, potassium ferricyanide in an assay mixture containing 0.2 M phosphate buffer pH 7.8, 1% BSA, 0.6 M succinic acid, and 0.03 M potassium ferricyanide. The reaction started by the addition of mitochondrial sample, and the change in absorbance was recorded at 420 nm. Complex-IV activity was assayed by monitoring the oxidation of cytochrome c in a reaction mixture (0.3 mM reduced cytochrome c in 75 mM phosphate buffer). The reaction was initiated by the addition of solubilized mitochondrial sample, and the change in absorbance was recorded at 550 nm. Mitochondrial respiratory function was measured by using a Clark electrode as previously described [23, 24]. Briefly, isolated mitochondria (100 µg) were resuspended in mitochondrial respiration buffer. The addition of ADP triggered mitochondrial oxygen consumption. State III respiration was assessed by the addition of 200 µM ADP. State IV respiration was achieved by the addition of 1 µM oligomycin. Mitochondrial respiratory control ratio (RCR) was calculated as the ratio of State III respiration/State IV respiration.

Golgi Staining

Golgi staining was performed using an FD rapid Golgistain™ Kit. Briefly, freshly dissected brains were immersed into Golgi solution A + B at room temperature for 2 weeks in the dark. Next, brains were incubated with solution C at room temperature for 1 week in the dark. Coronal sections of 150 µm thickness containing the hippocampus were cut on a vibratome and stained following the manufacturer's instructions. CA1 pyramidal neurons (4–5 per mouse) that minimally overlapped adjacent neurons were selected, and the apical spines on tertiary dendrites were counted. Images were photographed using an Olympus BX5 microscope and quantified using Image-ProPlus version 6.0.

Histological Analysis

Histological staining was performed as described previously [25]. Paraffin-embedded brain sections were immersed in xylene and rehydrated by graded ethanol. For glial cell activation, the brain slices were incubated overnight at 4 ℃ with primary antibodies: anti-GFAP antibody (1:1000; Proteintech) and anti-Iba1 antibody (1:2000; Abcam). Then, sections were incubated with Horseradish Peroxidase-labelled secondary antibody and visualized by DAB. Images were acquired with the Olympus BX5 imaging system and quantified using Image-ProPlus 6.0 software.

For immunofluorescence staining, brain slices were incubated overnight at 4 °C with anti-synapsin-1 antibody (1:200; Cell Signaling Technology) and anti-PSD95 antibody (1:300; Millipore). Alexa Fluor 488- and Alexa Fluo594-labelled secondary antibodies were diluted in PBS and applied at room temperature for 60 min. Images were acquired under a ZEISS LSM 800 confocal microscope (Carl Zeiss, Germany), and the staining intensity was quantified using ZEN software.

Statistical Analysis

All data were expressed as the mean ± SEM. Statistical calculations were conducted using SPSS 13.0. One-way ANOVA with a Bonferroni post-hoc test for multiple comparisons assessed differences between multiple groups. Two-way ANOVA with repeated measures was used to analyze the group differences of escape latencies for the Morris water maze test. The significant difference was set at $p < 0.05$.

Results

CP Attenuates DOX-Induced Cognitive Impairment

The Morris water maze test was performed to assess the effects of CP on spatial learning and memory impairment induced by DOX. In the hidden platform (Fig. 1a), two-way ANOVA revealed a significant treatment effect on the escape latencies ($F (3, 44) = 16.05$, $p < 0.01$). The DOX group exhibited markedly longer escape latency than the controls, whereas co-administration with CP significantly decreased the escape latency. In the subsequent probe test, significant main effects of the group were found in the time spent in the target quadrant (Fig. 1b). The DOX group spent less time in the target quadrant than the controls, while DOX plus CP treatment exhibited a significant

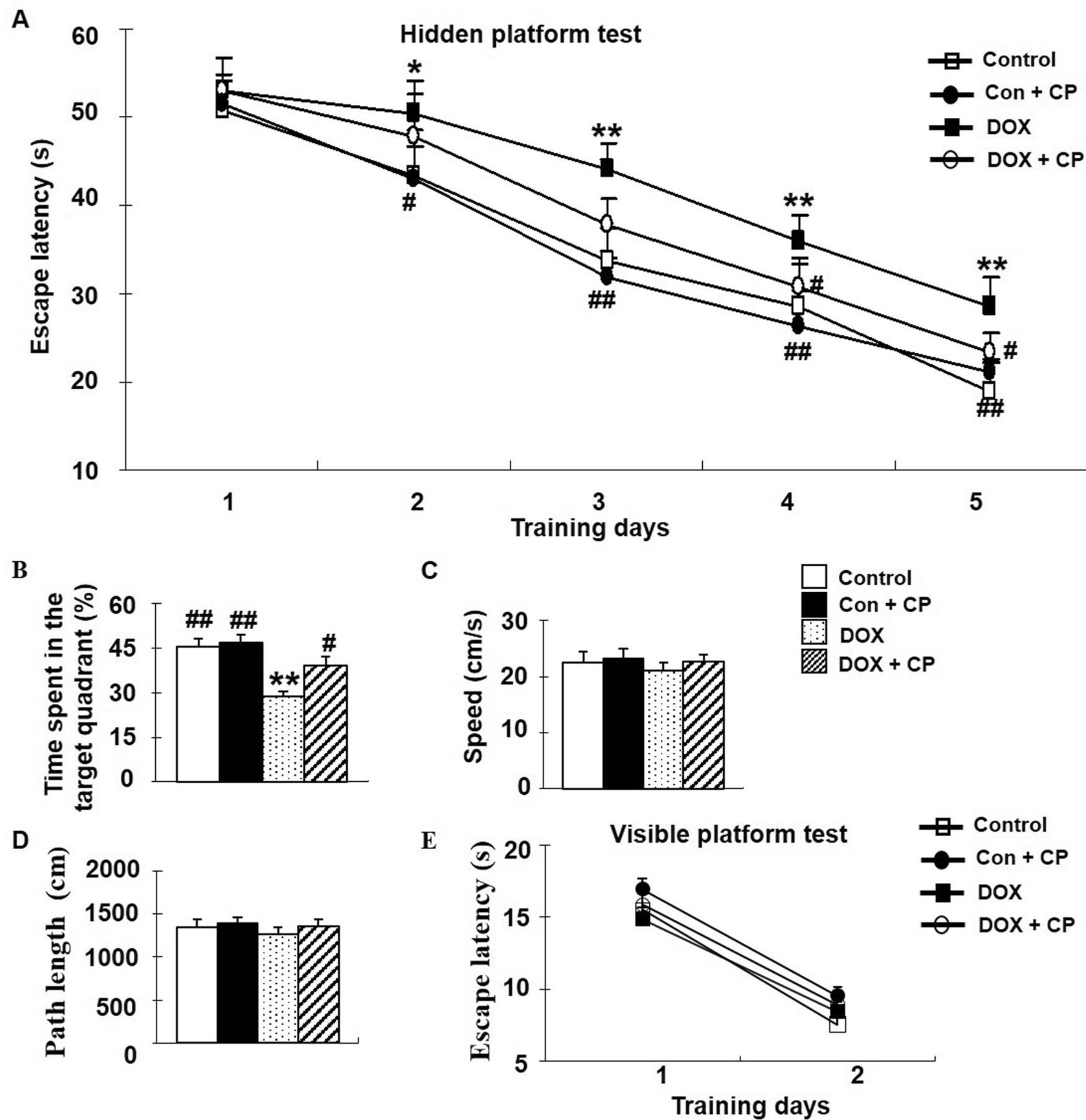

Fig. 1 CP improved DOX-induced cognitive impairment. **a** Escape latency during 5 days of hidden platform tests. **b** Time spent in the target quadrant in the probe test. **c** The speed in the probe test. **d** The total path length in the probe test. **e** Escape latency during the visible platform test. Values are expressed as mean ± SEM from 12 mice per group. $^{**}p<0.01$ compared to Control group; $^{\#\#}p<0.01$ compared to DOX group

increase. These results implied that CP attenuated the impaired spatial learning and memory task induced by DOX. There was no significant difference in the velocity (Fig. 1c) and the total path length (Fig. 1d) between the four groups in the probe trial. During the visible platform test, no significant difference was observed in the escape latencies between the four groups (Fig. 1e). These data further indicated that the beneficial effects of CP against DOX-elicited cognitive deficits were not linked to changes in non-cognitive parameters.

CP Prevents DOX-Induced Neuroinflammatory Response in the Hippocampus

Cytokines mediated neuroinflammation is considered to initiate an array of molecular and cellular cascades that lead to CICI. DOX stimulation induced an elevation of the pro-inflammatory cytokine (TNF-α, IL-1β, and IL-6) in the brains. Additionally, increased Iba1 and GFAP immunoreactivity were observed within the hippocampus of DOX-treated mice. Co-administration of CP with DOX

remarkably repressed neuroinflammatory response as shown by lowered levels of TNF-α by 38.6% ($p < 0.05$), IL-1β by 37.3% ($p < 0.05$), IL-6 by 40.0% ($p < 0.05$) (Fig. 2a), and reduced number of Iba1 (Fig. 2b) and GFAP (Fig. 2c) positive cells compared with those in the DOX-treated mice.

CP Reverses DOX-Induced Oxidative Stress in the Hippocampus

The levels of MDA, protein carbonyl, 8-OHdG, and GSH content, as well as SOD activity, were measured to examine the effects of CP on DOX-induced oxidative stress status in the hippocampus. Administration of DOX significantly induced dramatic elevation in the levels of MDA, protein carbonyl, and 8-OHdG, depletion of reduced GSH, and reduction of SOD activity compared to the control group. In contrast, co-administration of CP with DOX prominently restored the levels of MDA (Fig. 3a), protein carbonyl (Fig. 3b), 8-OHdG (Fig. 3c) and reduced GSH (Fig. 3d) as well as SOD activity (Fig. 3e).

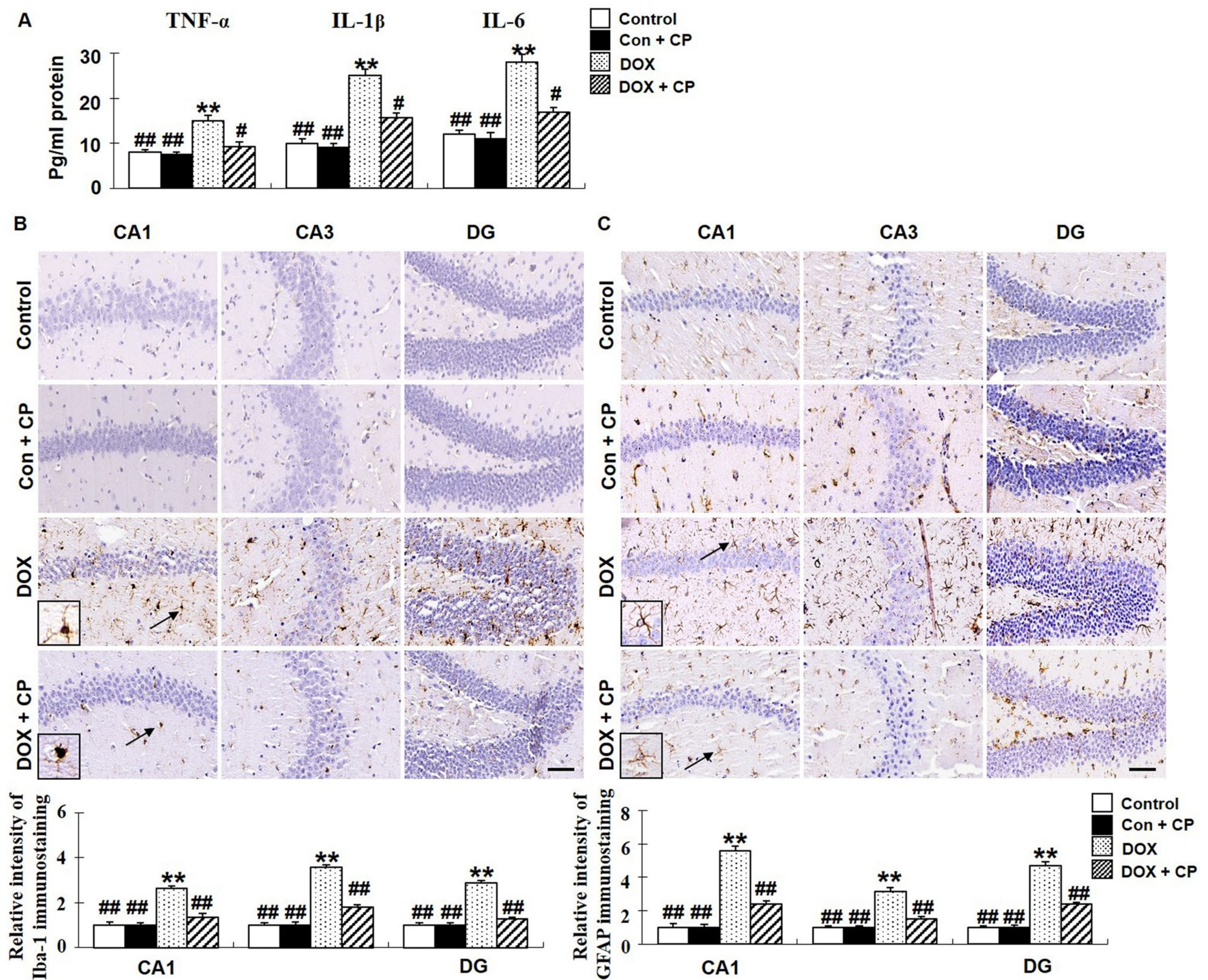

Fig. 2 CP suppressed neuroinflammatory response in the hippocampus of DOX-injected mice. **a** The levels of TNF-α, IL-1β, and IL-6 in the hippocampus of all of the groups. **b** Representative immunohistochemical images of microglia activation marker Iba-1 in the hippocampal CA1, CA3, and DG subregions of all of the groups (scale bars, 50 μm) and the quantitation of Iba1 staining intensity. Inserts indicate higher magnification of Iba1-positive cells from the CA1 area. **c** Representative immunohistochemical images of astrocyte activation marker GFAP in the hippocampal CA1, CA3, and DG subregions of all of the groups (scale bars, 50 μm) and the quantitation of GFAP staining intensity. Inserts indicate higher magnification of GFAP-positive cells from the CA1 area. Values are expressed as mean ± SEM from 3 mice per group. $^{**}p < 0.01$ compared to Control group; $^{\#\#}p < 0.01$ compared to DOX group

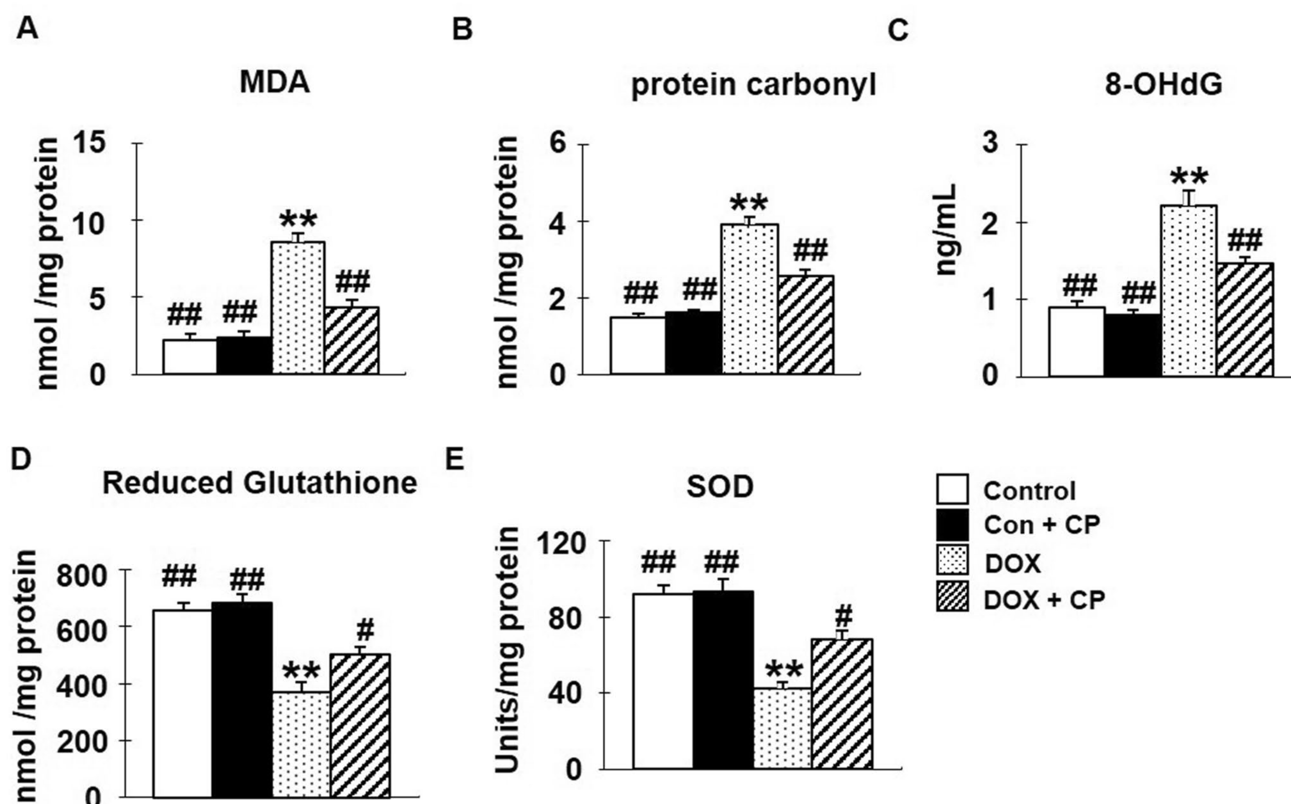

Fig. 3 CP prevented DOX-induced oxidative stress in the hippocampus of DOX-treated mice. **a** MDA levels. **b** Protein carbonyl. **c** 8-OHdG. **d** GSH level. **e** SOD activity. Values are expressed as mean ± SEM from 3 mice per group. $^{**}p < 0.01$ compared to Control group; $^{\#\#}p < 0.01$ compared to DOX group

CP Ameliorates DOX-Induced Mitochondrial Dysfunction in the Hippocampus

Brain mitochondrial dysfunction has been demonstrated in the brains of DOX-treated animal models and is presumed to contribute to CICI. The DOX group had an apparent mitochondrial defect, as indicated by impaired mitochondrial enzyme complex activity ($p < 0.01$), lowered RCR ($p < 0.01$), ATP deficiency ($p < 0.01$), and enhanced ROS production ($p < 0.01$) in the hippocampus than those found in the control group. In contrast, co-administration of CP with DOX significantly ameliorated hippocampus mitochondrial damage as exhibited by restored mitochondrial enzyme complex activity (Fig. 4a), elevated RCR (Fig. 4b), increased ATP production (Fig. 4c), and reduced ROS production (Fig. 4d).

CP Rescues Dendritic Spine and Synaptic Density in the CA1 Region of the Hippocampus in DOX-Treated Mice

Given the critical role of DOX-induced synaptic injury in the CICI, the effects of CP on dendritic spine and synaptic density against DOX neurotoxicity were explored. The DOX group showed a significant reduction in dendritic spine density compared with controls. In contrast, CP co-administration rescued the deficits in spine density ($p < 0.05$) (Fig. 5a, b). Next, we examined differences in synaptic density by double immunofluorescence staining of synapsin-1 and PSD95. The DOX group displayed a noticeable decrease in synaptic density compared with controls, whereas CP treatment partially restored the DOX-induced reduction in synaptic density ($p < 0.05$) (Fig. 5c, d).

Discussion

Clinical studies indicate that the increased pro-inflammatory cytokines occur in cancer patients who experienced CICI [26] and that the extent of the elevated inflammatory cytokines correlates with the severity of CICI [27]. The peripheral inflammatory cytokines such as TNF-α, IL-6, and IL-8 penetrate the brain and stimulate microglia and astrocytes to release more pro-inflammatory mediators and neurotoxic factors that lead to neuronal injury and neurodegeneration [7, 28]. Consistent with this, our results observed elevated pro-inflammatory levels (TNF-α, IL-1β, and IL-6)

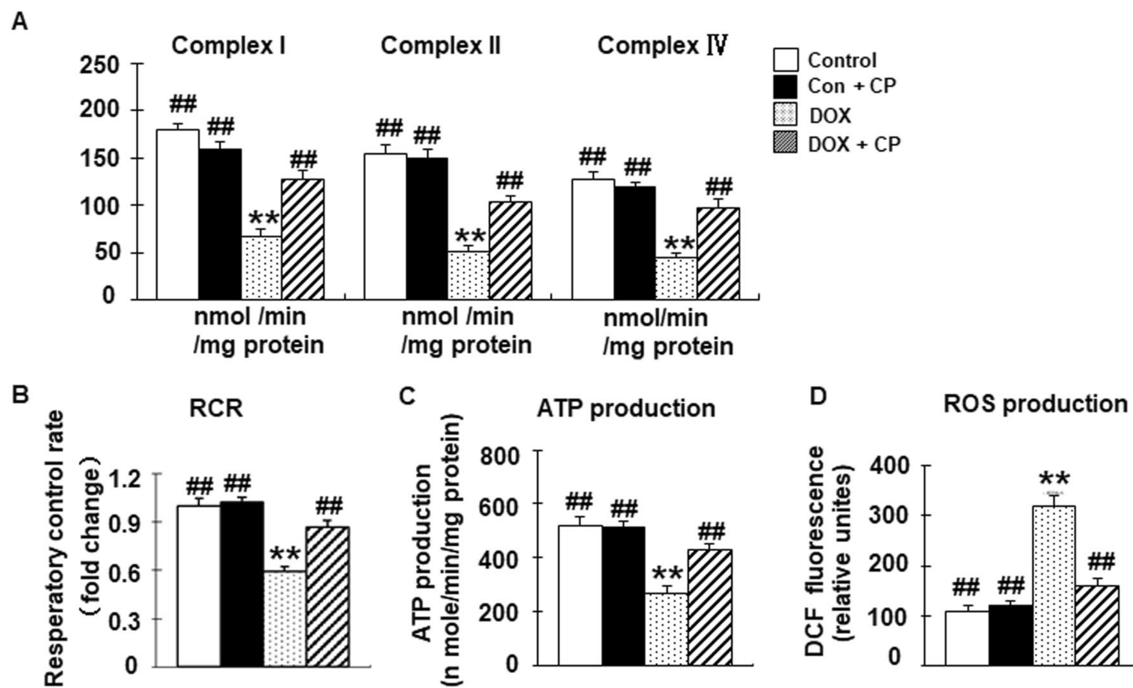

Fig. 4 CP mitigated DOX-elicited mitochondrial dysfunction in the hippocampus. Hippocampal mitochondria were separated and collected. **a** Mitochondrial enzyme complex I, II, and IV activity. **b** Mitochondrial RCR. **c** Mitochondrial ATP production. **d** ROS production. Values are expressed as mean ± SEM from 3 mice per group. $^{**}p<0.01$ compared to Control group; $^{\#\#}p<0.01$ compared to DOX group

and a marked increase in Iba1 and GFAP immunoreactivity in the brains of DOX-treated animals. Our findings suggest that the positive effects of CP against chemobrain might be through lowering chemotherapy-induced pro-inflammatory levels and inhibiting glial overactivation.

GFAP is exclusively expressed in astrocytes, which participates in synaptogenesis, controls the integrity of the blood-brain barrier (BBB), and maintains homeostasis in the central nervous system (CNS). Overactivation of astrocytes and upregulation of GFAP have been involved in brain injury and diseases in rodents. Pro-inflammatory cytokines, including IL-1, IL-6, and TNF-α, have been shown to trigger astrogliosis initiation and GFAP expression following the DOX challenge [28]. In turn, overactivated astrocytes are able to generate and secret a range of substances associated with disruptive BBB change, which may promote the penetration of pro-inflammatory cytokines and DOX itself into the CNS [29]. In this study, the immunohistochemical analysis of GFAP revealed that the GFAP expression in the DOX group was significantly elevated and that CP lowered the GFAP expression level. Therefore, it can be said that CP possesses protective effects on the CNS in DOX-treated mice. Oxidative stress has been implicated in the neurotoxicity of DOX and is one putative mechanism underlying DOX-induced cognitive impairment [30]. Despite DOX unable to pass the BBB, the peripheral TNF-α is generated in significant amounts as a result of Apo-A1 oxidative modification by DOX and penetrates the brain [6]. In the brain, TNF-α can produce markedly oxidative stress associated with reduced GSH levels in parallel with increased GSH peroxidase and reductase levels. Meanwhile, redox proteomics analysis revealed an altered antioxidant system and CNS oxidative stress induced by DOX via TNF-α generation [30]. In the present study, systemic DOX administration triggered oxidative damage as indicated by a significant elevation in the levels of MDA, protein carbonyl, and 8-OHdG, depletion of reduced GSH, and reduction of SOD activity. CP treatment significantly restored the antioxidant defense system and prevented oxidative damage in the hippocampus. The antioxidant potential of CP may be due to its direct scavenger of free radicals such as H_2O_2, singlet oxygen, nitric oxide, and peroxyl radicals [31].

There is growing evidence that chemotherapy-induced behavioral alterations are associated with neural mitochondrial dysfunction [32]. DOX-induced mitochondrial impairment has been well documented in the heart, kidney, and liver. The dysfunctional and pro-inflammatory state in the brain elicited by DOX promotes CNS oxidative stress [30]. Earlier studies have demonstrated that mitochondrial deficits occur by inducing oxidative stress via TNF-α elevation [33]. Accumulated Reactive oxygen species (ROS) in mitochondria can trigger the structural damage of the mitochondrial

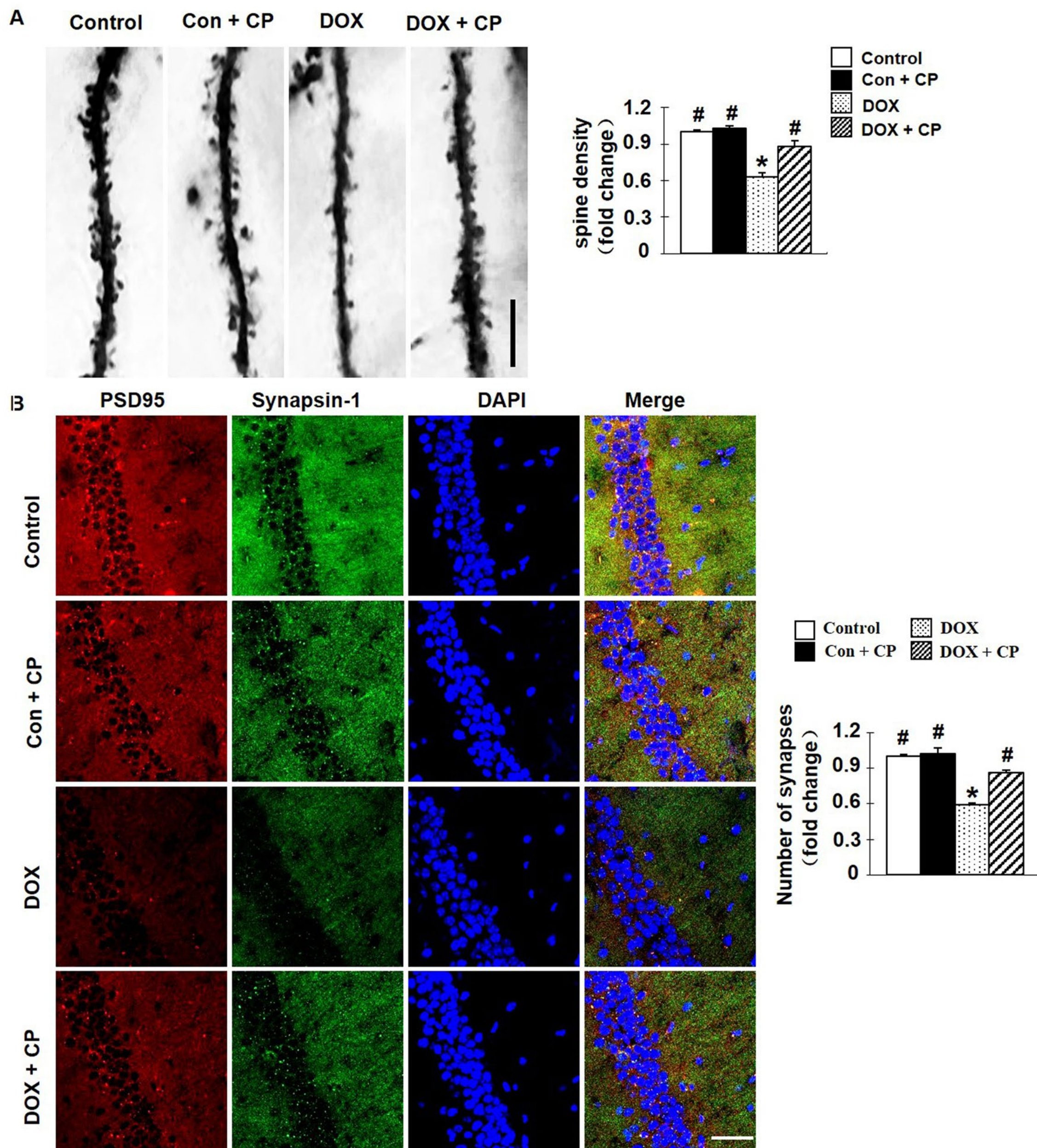

Fig. 5 CP rescued DOX-evoked synaptic injury in the CA1 region of the hippocampus. **a** Golgi staining images of dendritic spine density from hippocampal CA1 neurons (scale bars, 10 μm) and the quantification of dendritic spine density. **b** Representative confocal images of synapses stained with anti-sypnapsin-1 (green), anti-PSD95 (red), and DAPI (blue) (scale bars, 50 μm), and the quantification of synapses. Values are expressed as mean ± SEM from 3 mice per group. $^{**}p < 0.01$ compared to Control group; $^{\#\#}p < 0.01$ compared to DOX group

electron transfer chain (ETC) and subsequent ATP deficiency. Disrupted mitochondrial respiratory complex activities can produce more ROS. Therefore, mitochondria act as both targets and producers of ROS, which further potentiate oxidative damage. In our study, DOX impaired brain mitochondrial function, as evidenced by the decreased mitochondrial respiratory complex activities, the ATP deficiency, and the increased ROS in DOX-treated mice. CP treatment significantly mitigated brain mitochondrial dysfunction induced by DOX. Similar results have been found in CP's effects of mitochondrial protection [34].

Mitochondrial abnormalities are closely linked to synaptic dysfunction in chemobrain [35]. It is well-documented that the structural plasticity and number of dendritic spines are related to changes in synaptic function. Our results observed that decreased synaptic density and dendritic spine

loss were paralleled with mitochondrial dysfunction after DOX exposure. Notably, CP attenuated the DOX-induced mitochondrial abnormalities and restored synaptic dysfunction. The improvement of mitochondrial dysfunction in chemobrain seems feasible to maintain synaptic plasticity and mitigate cognitive impairment [36]. The beneficial effects of CP on cognition might profit from alleviating mitochondrial dysfunction.

Several limitations of this study for future research should be noted. First, we explored the preventive effects of CP against doxorubicin-induced cognitive deficits in this present study. Whether CP ameliorated the cognitive impairments following the doxorubicin challenge was undermined. Further investigations should focus on identifying the therapeutic effects of CP on chemobrain after doxorubicin treatment. Second, the combination chemotherapeutic regimen instead of single chemotherapeutic agents will be employed to establish a rodent model of chemobrain. Finally, this study was also carried out in "healthy" mice rather than tumorigenic mice. Recent studies implicated that tumor growth itself may aggravate the development of chemobrain [37]. Whether there exists a synergistic interaction between tumor cells and chemotherapy-related pathological factors is worthy of further investigations.

In conclusion, this study demonstrated that the nutraceutical CP could be a potential candidate for the treatment of CICI. The protective effect of CP might be related to its inhibition of neuroinflammation and oxidative stress and improved mitochondrial/synaptic dysfunction.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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