Molecular Neurobiology (2019) 56:671–687
https://doi.org/10.1007/s12035-018-1101-1

Anthocyanins Improve Hippocampus-Dependent Memory Function and Prevent Neurodegeneration via JNK/Akt/GSK3β Signaling in LPS-Treated Adult Mice

Muhammad Sohail Khan[1] · Tahir Ali[1] · Min Woo Kim[1] · Myeung Hoon Jo[1] · Jong Il Chung[2] · Myeong Ok Kim[1]

Received: 11 January 2018 / Accepted: 29 April 2018 / Published online: 19 May 2018
© Springer Science+Business Media, LLC, part of Springer Nature 2018

Abstract

Microglia plays a critical role in the brain and protects neuronal cells from toxins. However, over-activation of microglia leads to deleterious effects. Lipopolysaccharide (LPS) has been reported to affect neuronal cells via activation of microglia as well as directly to initiate neuroinflammation. In the present study, we evaluated the anti-inflammatory and anti-oxidative effect of anthocyanins against LPS-induced neurotoxicity in an animal model and in cell cultures. Intraperitoneal injections of LPS (250 μg/kg/day for 1 week) induce ROS production and promote neuroinflammation and neurodegeneration which ultimately leads to memory impairment. However, anthocyanins treatment at a dose of 24 mg/kg/day for 2 weeks (1 week before and 1 week co-treated with LPS) prevented ROS production, inhibited neuroinflammation and neurodegeneration, and improved memory functions in LPS-treated mice. Both histological and immunoblot analysis indicated that anthocyanins reversed the activation of JNK, prevented neuroinflammation by lowering the levels of inflammatory markers (p-NF-kB, TNF-α, and IL-1β), and reduced neuronal apoptosis by reducing the expression of Bax, cytochrome c, cleaved caspase-3, and cleaved PARP-1, while increasing the level of survival proteins p-Akt, p-GSK3β, and anti-apoptotic Bcl-2 protein. Anthocyanins treatment increased the levels of memory-related pre- and post-synaptic proteins and improved the hippocampus-dependent memory in the LPS-treated mice. Overall, this data suggested that consumption of naturally derived anti-oxidant agent such as anthocyanins ameliorated several pathological events in the LPS-treated animal model and we believe that anthocyanins would be a safe therapeutic agent for slowing the inflammation-induced neurodegeneration in the brain against several diseases such as Alzheimer's disease and Parkinson's disease.

Keywords Anthocyanins · Lipopolysaccharide (LPS) · Oxidative stress · Neuroinflammation · Neurodegeneration · Memory impairment

Abbreviations

AD	Alzheimer's disease
Cyt.C	Cytochrome c
CNS	Central nervous system
DCFH-DA	2′7′-Dichlorodihydrofluorescein diacetate
DCF	2′7′-Dichlorofluorescei
DMEM	Dulbecco's modified Eagle medium
DAPI	4′, 6′-Diamidino-2-phenylindole
DG	Dentate gyrus
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FJB	Fluoro-jade B
HRP	Horseradish peroxidase
IL-1β	Interleukin-1β
I.P	Intraperitoneally
LPO	Lipid peroxidation
LPS	Lipopolysaccharide
MDA	Malondialdehyde
P-JNK	Phospho-c-Jun N-terminal kinase 1
MWM	Morris water maze
PARP-1	Poly (ADP-ribose) polymerase-1
PD	Parkinson's disease

✉ Myeong Ok Kim
mokim@gnu.ac.kr

[1] Division of Applied Life Science (BK 21), College of Natural Sciences, Gyeongsang National University, Jinju 52828, Republic of Korea

[2] Department of Agronomy, Research Institute of Life Science, Gyeongsang National University, Jinju 52828, Republic of Korea

ROS	Reactive oxygen species
S.D.	Standard deviation
SAPK	Stress-activated protein kinase
TRITC	Tetramethyl rhodamine isothiocyanate

Introduction

Inflammation is a protective mechanism in the body. However, prolonged inflammatory responses and chronic inflammation in the brain tissue lead to apoptosis and memory impairment [1]. Lipopolysaccharide (LPS) is a bacterial cell wall endotoxin and acts as potent inflammatory inducer which contributes to neuroinflammation and causes deleterious effects and neurodegeneration [2]. Previous studies reported that LPS treatment contributes to elevated reactive oxygen species (ROS) generation and cytokine production that eventually leads to neuronal cell death and learning and memory impairment [3–6]. LPS is a toxin that is generally used for ROS generation and inflammation inducer which has been reported to induce synaptic problem in animal models. The administration of systemic LPS triggers ROS, which mediates phosphorylated-nuclear factor kappa B (p-NF-kB), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) expression and disturbs the function of hippocampal neurons, ultimately causing neuronal apoptosis and memory impairments [7–11]. Oxidative stress is the critical hallmark of neurodegenerative diseases, as ROS generation involves oxidative modification of biomolecules such as lipids, protein, and nucleic acid which in turn deregulate the cellular function and eventually leads to neurodegeneration in the brain [8, 12–15]. Oxidative stress alters several stress protein kinases such as phospho-c-Jun N-terminal kinase 1 [p-JNK1] (T183/Y185) (p-JNK), which in turn plays an important role in the neuroinflammation and apoptotic neurodegenerations. Chronic oxidative stress and neuroinflammation contribute to the progression of various neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD) [13–18]. A broad epidemiological study suggested that most people in the world face neuronal and memory-related problems with unknown reasons; however, these memory problems might be modifiable by lifestyle changes, exercise, diet consciousness, and daily supplementation with natural foods [19, 20].

Flavonoids are naturally found in seeds and fruits of many plants and play a significant role in the prevention of ROS-mediated neuronal diseases such as AD because of its antioxidant and free radical scavenger's properties. Flavonoids have also been proven to be a good neuroprotective agent in various neuronal disorders [21–27]. Similarly, our group reported the neuroprotective effects of polyphenolic flavonoids such as anthocyanins which has been extracted from black beans. The anthocyanins induced potent neuroprotection both in vivo and in vitro studies against various central nervous system (CNS) insults. Furthermore, our group also reported that anthocyanins improved the learning and memory function in the aging, amyloid beta, and transgenic model of AD [27–34]. Anthocyanins are known to possess a very strong neuroprotective role; however, the mechanism through which anthocyanins improve ROS-mediated memory impairment and memory-related pre- and post-synaptic protein expression levels in LPS mouse models remains uncertain. In the present study, we examined that anthocyanins inhibits LPS-induced, ROS-mediated neuroinflammation, neuronal apoptosis, memory impairment and improved the behavior performance in the LPS-treated animals. We believe that natural anthocyanins supplementation would be a safe therapeutic agent against several oxidative stress and neuroinflammation-mediated neurodegenerative diseases such AD, PD, and other neurological disorders.

Materials and Methods

Chemicals

Anthocyanins extracted as previously we described [27–31]. The LPS, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), dimethyl sulfoxide (DMSO), and JNK inhibitor (SP600125) were purchased from Sigma Chemical Co (St. Louis, MO, USA).

Experimental Animals

Male C57BL/6N mice (aged 8 weeks 25–30 g) were purchased from Samtako Bio (Osan, Republic of Korea). The mice were acclimatized for 1 week in the university animal house under 12/12 light cycle at room temperature and humidity. The mice were provided with water and food ad libitum. All the experimental mice were carefully handled according to the animal ethical committee of the Gyeongsang National University, South Korea. The experimental procedures were approved by the animal ethics committee (Approval ID: 125) of the Gyeongsang National University, South Korea.

Experimental Animals Grouping and Treatment

The experimental animals were randomly selected (15–20 mice/group) and divided into the following three groups,

(i) Control (Cont.) group
(ii) LPS-treated group
(iii) LPS + Antho treated group

The (i) Cont. group received saline as a vehicle for 14 days; intraperitoneal injections (I.P.), (ii) LPS-treated group received 250 μg/kg/day for 7 days; I.P. as previous described

[3–6, 9–11], (iii) LPS + Antho treated group, (mice injected with LPS (250 μg/kg/day for 7 days; I.P.) + Antho (24 mg/kg/day for 14 days; I.P., 7 days before the LPS and 7 days co-treated with LPS).

The anthocyanins were dissolved in DMSO and the required volume was made in normal saline and injected I.P. to the mice, whereas LPS dissolved in normal saline and the same volume was injected I.P. to the mice.

ROS Assay (In Vivo)

ROS assay was performed to determine the elevated ROS level in the hippocampus (n = 15 mice per group) homogenates. The assay was performed as described earlier with modification [35], based on the conversion of DCFH-DA to 2,7-dichlorofluorescein (DCF). Briefly, the brain homogenate from all the treated group was diluted using Lock's buffer at a ratio of 1:20 (final concentration was adjusted to 2.5 mg tissue per 500 ml). The final mixture for the determination of ROS quantification in the homogenate was lock's Buffer (pH 7.4), brain homogenate (0.2 ml) and 10 ml DCFH-DA (5 mM). The mixture containing all the ingredients were covered and allowed for incubation at room temperature for 15 min. Following incubation, the obtained fluorescent product DCF was measured through microplate reader (excitation at 484 nm and emission at 530 nm). For the background, a parallel blank was used. The obtained results were expressed as pmol DCF formed/min/mg of protein in the tissue homogenate.

ROS Assay (In Vitro)

The ROS quantification in cell culture was performed following the same procedure as described in vivo. The in vitro assay for ROS determination was also based on the conversion of 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) to 2,7-dichlorofluorescein (DCF). The microglial BV2 cells (a kind gift from Dr. I. W. Choi, Inje University, Busan, Republic of Korea) were seeded in 75 cm^2 flasks (Thermo scientific, Nunc™ EasYFlask™ 75 cm^2 Nunclon™ Delta surface, thermofisher scientific A/S, Kamstrupvej 90.P.O.Box 280 DK-4000 Rosklide, Denmark) containing Dulbecco's modified Eagle medium (DMEM) (Gibco by life technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin) at 37 °C in humidified air containing 5% CO_2. After cells growing and counting, the cells were further subcultured in 35 mm Petri dishes (Thermo scientific, Nunc A/S, Kamstrupvej 90.P.O.Box 280 DK-4000 Rosklide, Denmark) in DMEM supplemented with 10% FBS and 1% antibiotics (penicillin-streptomycin) at 37 °C in humidified air containing 5% CO_2. After the cells reached 70–80% confluence, they were treated with LPS (1 μg/ml), LPS (1 μg/ml) + Antho (100 μg/ml) and LPS + SP600125 (20 μM). After treatment for 24 h, the cell lysate was analyzed for DCF products via microplate reader (excitation at 484 nm and emission at 530 nm). The obtained results were expressed as pmol DCF formed/min/mg of protein in the cell lysate.

Lipid Peroxidation (LPO) Assay (In Vivo)

The LPO assay was performed to evaluate the oxidative stress. The free malondialdehyde (MDA) level in the mouse hippocampal homogenates was assessed by using a LPO assay Kit (Biovision, USA catalog # K739-100). The LPO assay was performed according to the manufacturer's instructions. Briefly, the tissue from the mice brain was homogenized (300 μl of MDA lysis Buffer; 3 μl BHT) on ice followed by centrifugation (13,000×g, 10 min). Next, the protein was precipitated by homogenizing 10 mg sample in 150 μl dH2O + 3 μl BHT, adding 1 vol of 2 N perchloric acid, vortexed, and centrifuged to remove precipitated protein. The final volume of the 200 μl of the supernatant from each sample was transferred to 96-well plate. The absorbance was measured using a microplate reader at 532 nM. The MDA content was expressed as nmol/mg of protein in the tissue homogenate.

LPO Assay (In Vitro)

The in vitro LPO assay is performed to evaluate the oxidative stress in the BV2 cells. The LPO assay was performed using the commercially available kit (Biovision Incorporated; catalog # K739-100). The cells were grown and treated as described above in the ROS assay (in vitro) sections. The cell lysate was analyzed for MDA contents using microplate reader at 532 nM. The MDA content was expressed as nmol/mg of protein in the cell lysate.

Protein Extraction

After treatment and behavioral, all the animals were anesthetized, sacrificed (Fig. 1a) and the brain was carefully removed. The hippocampus was carefully dissected as previously described [36] and kept in liquid nitrogen for freezing and stored at 80 °C for further biochemical experimental work. Furthermore, homogenization was carried out in pre-prep extraction solution (iNtRON Biotechnology) and centrifuged at 13,000 rpm at 4 °C for 25 min. The supernatant of the centrifuged samples were collected and kept at 80 °C for further experiments.

Quantitative Analysis of the Proteins by Western Blotting

Western blot analysis was carried out according to the protocols with minor modifications [36, 37]. The optical densities

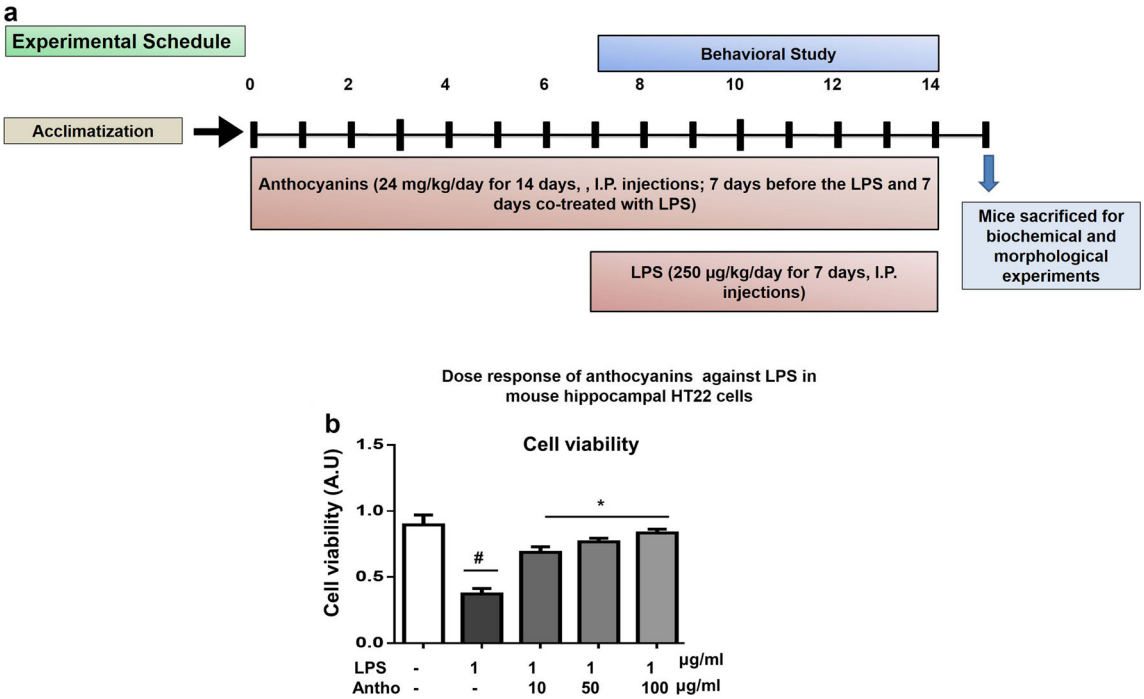


Fig. 1 a Showing experimental schedule for the drug treatment and behavioral analysis. The experimental animals were randomly divided into three groups, (i) Control (Cont. group) group received saline as a vehicle for 14 days; intraperitoneal injections (I.P.) (ii) LPS-treated group received 250 μg/kg/day for 7 days; I.P., (iii) LPS + Antho treated group, (mice injected with LPS (250 μg/kg/day for 7 days; I.P.) + Antho (24 mg/kg/day for 14 days; I.P., 7 days before the LPS and 7 days co-treated with LPS). b Anthocyanins improved HT22 cell viability against LPS, HT22 cells treated with different concentrations of anthocyanins against LPS (1 μg/ml). The MTT assay histogram indicating that anthocyanins at a dose of 100 μg/ml demonstrate more efficiency and representing adequate neuroprotective activity. The number of experiments = 3. Symbols for treatment groups and level of significance are mentioned in the data analysis section of the Materials and Methods

(O.D) of proteins were analyzed by Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, and USA). Then, equal amounts of proteins (20–30 μg) were electrophoresed using 4–12% BoltTM Mini Gels (Novex, Life Technologies). To reduce non-specific binding, the membranes were blocked in 5% (w/v) skim milk and incubated with primary antibodies (anti-caspase-3, anti-TNF-α, anti-IL-1β, anti-p-JNK, anti-p-NF-kB 65, anti-Bax, anti-Bcl-2, anti-Cytochrome c (Cyt. c), anti-PARP-1, anti-p-GSK3β (Ser 9), anti-p-Akt (Ser 473), anti-synaptosomal associated protein 25 (SNAP-25), anti-post-synaptic density protein 95 (PSD95), and anti-β-Actin from Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4 °C, followed by horseradish peroxidase-conjugated secondary antibodies for 1 h. The membranes were developed in dark using chemiluminescence system (Atto Corporation Tokyo, Japan). The protein analysis was made by using according to the manufacturer's protocols. Protein bands were scanned and were analyzed using computer-based Sigma Gel program, version 1.0 (SPSS, Chicago, IL, USA).

Morphological Analysis and Sample Preparation

After drug treatment and behavioral study, the mice were perfused transcardially with 4% ice-cold paraformaldehyde according to previous studies with minor modifications [37]. The brains were post-fixed for 72 h in 4% paraformaldehyde and then transferred to 20% sucrose for 72 h. After that, the brains were washed with PBS and frozen in O.C.T. compound (A.O, USA). After complete solidification of the blocks, 14-μm coronal sections of the hippocampus were cut using a CM 3050C cryostat (Leica, Germany). The sections were thaw-mounted on probe-on plus charged slides (Fisher, USA).

Immunofluorescence Staining

Immunofluorescence staining of the slides was performed according to previous studies with minor modifications [38, 39]. Shortly, the slides were dried overnight and then washed two times with 0.01 M PBS for 10 min, then blocked with blocking solution for 1 h (2% normal serum) according to the antibody treatment and 0.3% Triton X-100 in PBS. Then, incubation of primary antibodies (anti-caspase-3, anti-TNF-α, anti-p-JNK and anti-PARP-1, from Santa Cruz Biotechnology, Dallas, TX, USA) was carried out overnight at 4 °C with primary antibodies (Santa Cruz Biotechnology, USA). After incubation with primary antibodies, the sections were incubated for 2 h in the secondary antibodies TRITC/FITC-labelled antibodies (1:100) (Santa Cruz Biotechnology,

USA). After secondary antibodies incubation, the slides were washed twice for 5 min. Slides were mounted with 4′,6′-diamidino-2-phenylindole (DAPI) and Prolong Antifade Reagent (Molecular Probe, Eugene, OR, USA). Stained slides were examined using a confocal laser-scanning microscope (Flouview FV 1000, Olympus, Japan). The quantification of the immunofluorescence images was performed accordingly our recent published protocol [40].

Fluoro-Jade B (FJB) Staining

The FJB staining was performed for in vivo and in vitro study as previously performed and described with minor modifications [41]. Slides were kept overnight in order to dry, then washed twice for 5 min in 0.01 M PBS. After washing, the tissue slides were dipped in a solution of 1% sodium hydroxide and 80% ethanol for 5 min. Next, all slides were put in 70% alcohol and then in distilled water for 2 min each. Tissue slides were transferred to a solution of 0.06% potassium permanganate for 10 min, rinsed with distilled water, followed by immersion in a solution of 0.1% acetic acid and 0.01% FJB for at least 20 min. The slides were then washed with distilled water and allowed to dry for 10 min. Glass coverslips were mounted using DPX mounting medium and images were taken with confocal laser-scanning microscope (Flouview FV 1000, Olympus, Japan). Stained slides were analyzed using the computer-based, ImageJ program. The quantification of the immunohistofluorescence FJB images was performed accordingly our recent published protocol [40].

Cresyl Violet Staining

Cresyl violet (Nissl) staining is an authentic protocol for histological examination and determines the extent of neuronal cell death. The first step of this protocol is washing of the tissue slides with 14-μm sections of brain twice for 15 min in 0.01 M PBS and stained with a 0.5% Cresyl violet solution (containing few drops glacial acetic acids) for 10–15 min. Then, slides were washed with distilled water and dehydrated in graded ethanol (70, 95, and 100%), retained in xylene and coverslipped using the mounting medium (non-florescent). The slides were studied with a fluorescent light microscope. The quantification of the immunohistochemical images was performed accordingly our recent published protocol [40].

Morris Water Maze (MWM) Test

Behavior study was assessed for the mice (n = 15–20/group) using the MWM test. The MWM experimental apparatus consisted of a circular water tank (100 cm in diameter, 40 cm in height), containing water (23 ± 1 °C) at a depth of 15.5 cm, which was made opaque by adding white ink. A transparent escape platform (10 cm in diameter, 14.4 cm in height) was hidden 1 cm below the water surface and was placed at the midpoint of one quadrant. Each mouse received training for five consecutive days using a single hidden platform in one quadrant with three quadrants of rotational starting. Latency to escape from the water maze (finding the hidden escape platform) was calculated for each trial. Twenty-four hours after the 5th day, the probe test was performed for the evaluation of memory consolidation. The probe test was carried out by removing the platform and allowing each mouse to swim freely for the 60 s. The time that the mice spent on the target quadrant and the number of times the mouse crossed over the platform location (where the platform was located during hidden platform training) was measured. Time spent in the target quadrant was considered to represent the degree of memory consolidation. All data were recorded using video-tracking software (SMART, Panlab Harvard Apparatus; Bioscience Company, Holliston, MA, USA).

In Vitro Cell Culturing and Treatment for the Western Blotting and Confocal Microscopy

The mouse hippocampal HT22 neuronal cells a kind gift from Prof. Koh (Gyeongsang National University, S. Korea) and BV2 cells were seeded in 10% FBS and 1% antibiotics-supplemented with DMEM in a humidified 5% CO2 incubator at 37 °C. The BV2 cells were treated with LPS (1 μg/ml), LPS (1 μg/ml) + Antho (100 μg/ml) and LPS (1 μg/ml) + SP600125 (20μM) for 24 h. For microglia conditioned medium (MCM), the BV-2 cell line was cultured to above 75% confluence and was treated with LPS (1 μg/ml) dissolved in cell culture media. After 24 h media was aspirated and centrifuged to remove cells and debris. The supernatant was collected and transferred to HT22 cells (24 h) for further immunoblot and immunohistological analysis.

MTT Assay

The colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was used to measure the cell viability. The HT22 cells were seeded into 96-well plates (ThermoFisher Scientific, Rochester, NY) (1×10^5 cells/ well) in 150–200 μl of DMEM. After 70% confluences, the cells were exposed to LPS (1 μg/ml) and Antho at different three concentrations of 10 μg/ml, 50 μg/ml and 100 μg/ml for 24 h. However, the cells in the control group were exposed to the DMSO (0.01%). The assay was performed according to our previous described protocol [33]. Absorbance was then measured at 550–570 nm (L1) and 620–650 nm (L2) in a scanning microplate reader. The L2 absorbance measures cell debris and well imperfections. The corrected absorbance (A = L1–L2) of each well was used to calculate the absorbance for the viable cells.

Data and Statistical Analysis

Western blot bands were scanned and analyzed through densitometry using the Sigma Gel System (SPSS Inc., Chicago, IL). ImageJ software was used for immunohistological quantitative analysis. One-way analysis of variance (ANOVA) and Student's t test were used for comparisons among the control, LPS and LPS + Antho groups. All the values are mean ± S.E.M. All the values were analyzed through GraphPad Prism 5 software (Graph-Pad Software, In., and San Diego, CA). For all experiments, the data were expressed as the means ± SEM of three independent experiments. P values less than 0.05 were considered to be statistically significant. The symbols # showing significant differences from the control group, * showing significant differences from the LPS group. Similarly, for the in vitro symbols # showing significant differences from the control group * showing significant differences from the LPS or MCM group, whereas symbol Ø showing significant differences from the LPS or MCM group.

Results

Effect of Anthocyanins on Cell Viability Against LPS Treatment

Recently, few interesting studies (Leow-Dyke et al., 2012; Calvo-Rodriguez et al., 2017) reported that along with microglial cells, the neurons can also response to LPS and has a key role in the initiation of the inflammatory response to infection or brain injury [42, 43]. Therefore, in order to measure the cell viability, we performed MTT assay in vitro using mouse hippocampal HT22 neuronal cells. As per the established studies [42, 43] and our MTT results also indicated that LPS (1 μg/ml) induced detrimental effects on HT22 cells and significantly reduced the cell viability (Fig. 1b). To know the neuroprotective effects of anthocyanins, we designed dose-dependent studies and HT22 cells exposed to three different doses of anthocyanins (10, 50, and 100 μg/ml) while the dose of LPS remained constant (1 μg/ml). The MTT assay results showed that anthocyanins at a dose of 100 μg/ml is more effective and more significantly protects the HT22 cells against LPS-induced neurotoxicity (Fig. 1b).

Anthocyanins Ameliorates Activated Stress Kinase and Oxidative Stress both In Vivo and In Vitro

Several lines of investigation revealed that anthocyanins is a potent anti-oxidant agent and reduced the ROS burden [26–34]. It has been extensively cited that JNK is an important stress kinase which is expressed highly during intracellular ROS generation [16]; therefore, in this study, we investigated the p-JNK level through western blot and confocal microscopy. Our immunoblot and immunohistological results revealed that LPS treatment significantly increased the p-JNK expression level in the hippocampus of adult mouse brains. However, treatment with anthocyanins for 2 weeks significantly reduced the expressions level of p-JNK demonstrating the anti-oxidant properties of anthocyanins (Fig. 2a, b). The previous study demonstrated that neuronal cells exposed to LPS induced the over-activation of p-JNK pathway [42]. To investigate the microglia induces oxidative stress in neuronal cells, we used conditioned medium (LPS-treated BV2 microglia cells) in HT22 cells. Both immunoblot and immunohistological results indicated the increased expression level of p-JNK in HT22 cell line. It was interesting to find that anthocyanins treatment strongly alleviated the expression level of p-JNK in a similar way like SP600125, a specific JNK inhibitor (Fig. 2c, d). From this observation, we concluded that anthocyanins possess potent anti-oxidant properties against microglia-induced oxidative stress. Furthermore, we evaluated the ROS and LPO assay in mice that received LPS alone and the group treated with LPS and anthocyanins. Our ROS and LPO assay results demonstrated that LPS significantly increases oxidative stress in the mice hippocampus. Instead, anthocyanins overcome the burden of oxidative stress (Fig. 3a, c). In addition, various studies indicated that reactive microglia produces ROS and inflammatory cytokines [44]; therefore, we also performed ROS and LPO assays in BV2 cell line. Our in vitro results indicated the increased level of DCF and MDA, suggesting that LPS treatment increases oxidative stress in BV2 cells. However, anthocyanins treatment significantly reduced DCF and MDA in LPS-treated BV2 cells. We further observed that anthocyanins similarly overcome the oxidative stress burden in a same way as like SP600125 (Fig. 3b, d).

Anthocyanins Inhibited LPS-Induced Activation of Inflammatory Proteins in the Hippocampus of Adult Mouse and BV2 Microglia Cells

Several lines of investigation reported that LPS-stimulated microglia cells contribute to cytokine production which plays an important role in neuroinflammation-induced neurodegenerative disorders [3–11]. It has been known that LPS-exposed microglia triggers the activation of NF-kB which further initiate the transcription of various cytokine genes including TNF-α, IL-1β, and COX2 [44]. Previous mounting studies reported that anthocyanins treatment was effective against neuroinflammation [5, 27–32, 45, 46]. Likewise, in our immunoblot analysis, we also observed the inflammatory responses in LPS-treated mice hippocampus with the increased expression level of p-NFkB, TNF-α, and IL-1β which were significantly inhibited by anthocyanins treatment (Fig. 4a). Furthermore, using immunofluorescence technique, we confirmed the increased immunoreactivity of TNF-α in the LPS-treated mice hippocampus. However, anthocyanins treatment significantly reversed the effects and inhibited the increased

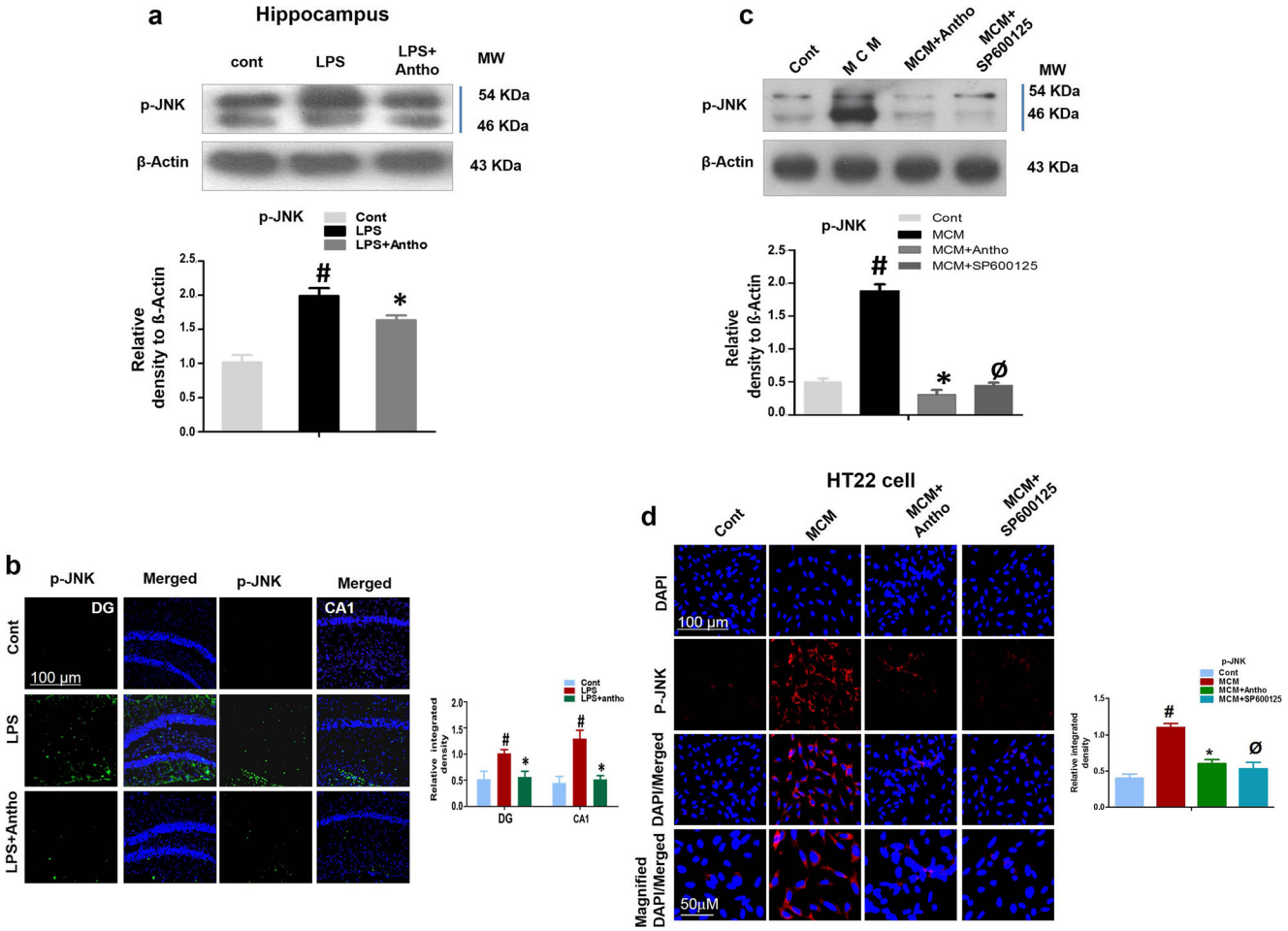


Fig. 2 Anthocyanins inhibited the LPS-induced activated stress kinase p-JNK in the hippocampus of adult mice and in HT22 cells exposed to LPS **a** Showing the western blot analysis of p-JNK in the hippocampus of adult mice. The bands were quantified using Sigma Gel software, and the differences are represented by a histogram. β-Actin was used as a loading control. The results present for n = 15 mice/group, and the number of experiments = 3. **b** Representative image of immunofluorescence staining of p-JNK (Green, FITC; Blue, DAPI) in the DG and CA1 region of the hippocampus. The results present for n = 5 mice/group, and the number of experiments = 3. Magnifications × 10. Scale bar = 100 µM. **c** Showing the western blot analysis of p-JNK in the protein obtained from the cell lysate of the HT22 cell treated with MCM, MCM + Antho, and MCM + SP600125. The bands were quantified using Sigma gel software and the differences are represented by a histogram. β-Actin was used as a loading control. The results present for n = 5/group, and the number of experiments = 3. **d** Representative image of immunofluorescence staining of p-JNK (Red, TRITC; Blue, DAPI) in the HT22 cells exposed to the MCM, MCM + Antho, and MCM + SP600125. The results present for n = 5/group, and the number of experiments = 3. Magnifications × 10. Scale bar = 100 and 50 µM respectively for the low magnification and magnified. Symbols for treatment groups and level of significance are mentioned in the data analysis section of the Materials and Methods

expression level of TNF-α (Fig. 4b), suggesting the possible inhibition of the cytokines production in LPS-treated mice brain. To know the inhibitory effect of JNK inhibitor and anthocyanins treatment in the LPS-exposed BV2 cells. Interestingly, our in vitro results elaborated that both anthocyanins and SP600125 significantly alleviated LPS-induced increase p-NFkB, TNF-α and IL-1β in microglial BV2 cells (Fig. 4c). Of note, these results validate that anthocyanins reduced the expression of inflammatory markers in a similar way like SP600125. From this observation, we concluded that anthocyanins might be inhibited the inflammatory markers via the downregulation of active JNK.

Anthocyanins Treatment Prevents LPS-Induced Neuronal Apoptosis in the Hippocampus of Adult Mice and in HT22 Cells Exposed to LPS and MCM

Previous studies reported that over-activation of microglia play a critical role in neuronal apoptosis, while anthocyanins protect the brain against apoptosis and neuronal damages [5, 27–34, 47]. It has been demonstrated that LPS induces mitochondrial ROS production that will initiate the mitochondrial apoptotic pathway, Cyt. C and caspases cascades including caspases-3 which consequently activates PARP-1, a well-known marker for DNA damage [48–50]. In the present study,

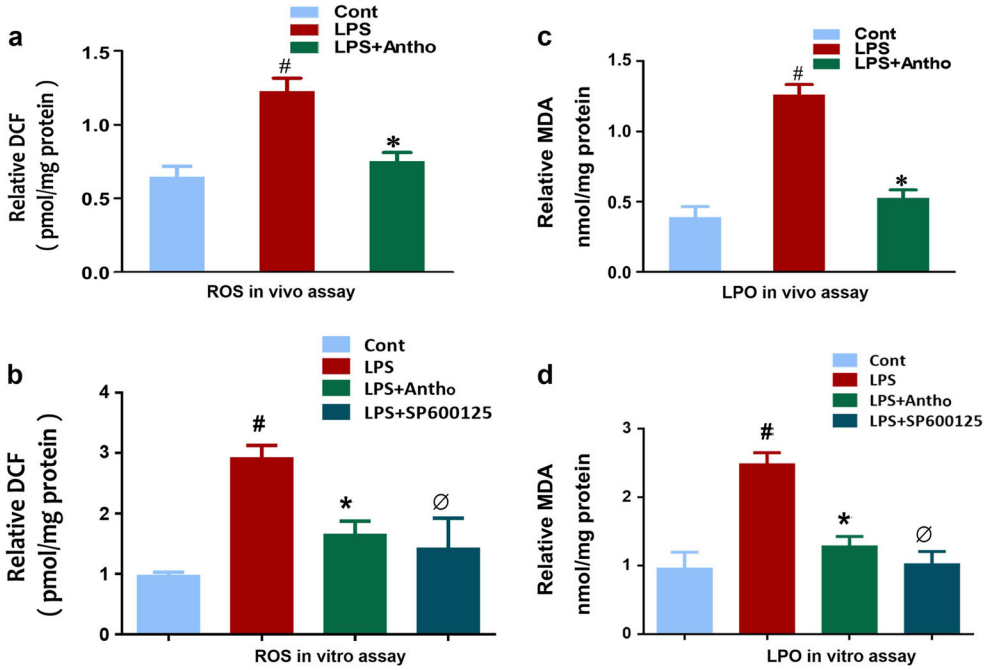


Fig. 3 Anthocyanins attenuate LPS-induced ROS accumulation and oxidative stress in the hippocampus of adult mice and in BV2 cells exposed to LPS. a Representative histogram showing a comparative DCF (ROS) level in the mice hippocampus. The results present for n = 15 mice/group, and the number of experiments = 3. b DCF (ROS) level in BV2 microglia cells. The results present for n = 5/group, and the number of experiments = 3. c MDA level in the mice hippocampus. The results present for n = 15 mice/group, and the number of experiments = 3. d MDA level in BV2 cells. The results present for n = 5/group, and the number of experiments = 3. Symbols for treatment groups and level of significance are mentioned in the data analysis section of the Materials and Methods

both immunoblotting and immunofluorescence analysis revealed the increased expression levels of apoptotic markers including Cyt. C, Bax, cleaved caspase-3, cleaved PARP-1 and reduced level of anti-apoptotic Bcl2 in the hippocampus of LPS-treated mice. However, anthocyanins treatment regulated the mitochondrial apoptotic pathway via reducing the level of Bax/Bcl2 ratio and prevents the apoptotic pathway through reducing the expression of Cyt. C, cleaved caspase-3 and cleaved PARP-1 (Fig. 5a–c). Furthermore, MCM was subjected to HT22 cell lines and after 24 h the HT22 cell was analyzed for immunoblotting and immunohistological analysis. These results indicated increase expression level of apoptotic markers including Bax, Cyt. C, cleaved caspase-3, cleaved PARP-1 and reduced level of anti-apoptotic Bcl2. It was interesting to show that anthocyanins treatment in MCM treated HT22 cell significantly regulated the mitochondrial apoptotic (Bax/Bcl2) pathway as well as reduced the overexpression of Cyt. C, cleaved caspase-3 and cleaved PARP-1, in a similar way like SP600125 (Fig. 5d, e), suggesting that anthocyanins treatment also is effective against microglia-mediated neuronal apoptosis. Further, the recent studies demonstrated that neuronal cells exposed to LPS-induced neuronal cell death [42, 43]. Based on these observations, we analyzed LPS effects on HT22 cells and examined nuclear apoptotic marker, e.g., cleaved PARP-1. Our immunoblot and immunofluorescence results indicated that exposure of the mouse hippocampal-derived HT22 cells to LPS increases the expression of cleaved PARP-1. However, both anthocyanins and SP600125 treatment to the LPS-exposed HT22 cells prevents the LPS effects and significantly reduced the cleaved PARP-1 expression (Fig. 5f, g), indicated and supported our hypothesis that anthocyanins prevent LPS-induced activated p-JNK-mediated neuroinflammation and neurodegeneration.

Anthocyanins Treatment Improves Survival Pathway and Ameliorates Neurodegeneration

Several studies have found that LPS provokes host defenses with marked inflammatory responses and disturb the activation of survival pathways such as Akt pathway and decrease the phosphorylation of Akt, while recently we reported that anthocyanins improved the survival pathway of p-Akt/p-GSK3β (Ser 9) [6–9, 34, 51]. Therefore, we investigated the expression levels of the p-Akt (Ser 473) and p-GSK3β (Ser 9) through western blot analysis in the hippocampus homogenate and in mouse hippocampal-derived HT22 neuronal cells. The results showed that LPS treatment decreases the expression level of p-Akt (Ser473), and p-GSK3β (Ser9) in mouse hippocampus when compared with control and anthocyanins groups (Fig. 6a). Similarly, we found the decreased expression level of p-Akt (Ser473), and p-GSK3β (Ser9) in HT22 cells exposed to LPS for 24 h. However, anthocyanins treatment restored these markers and significantly increased expression

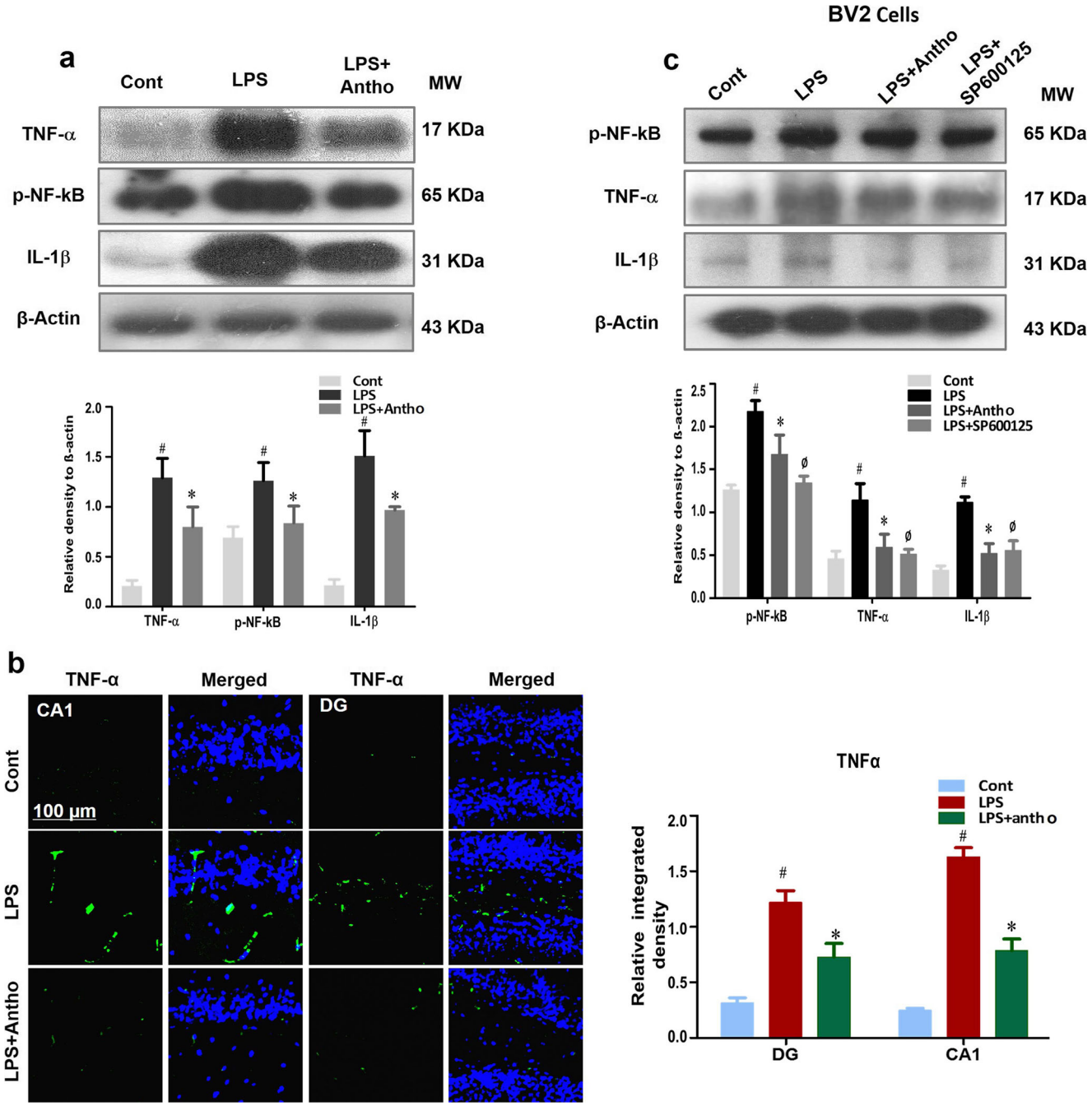
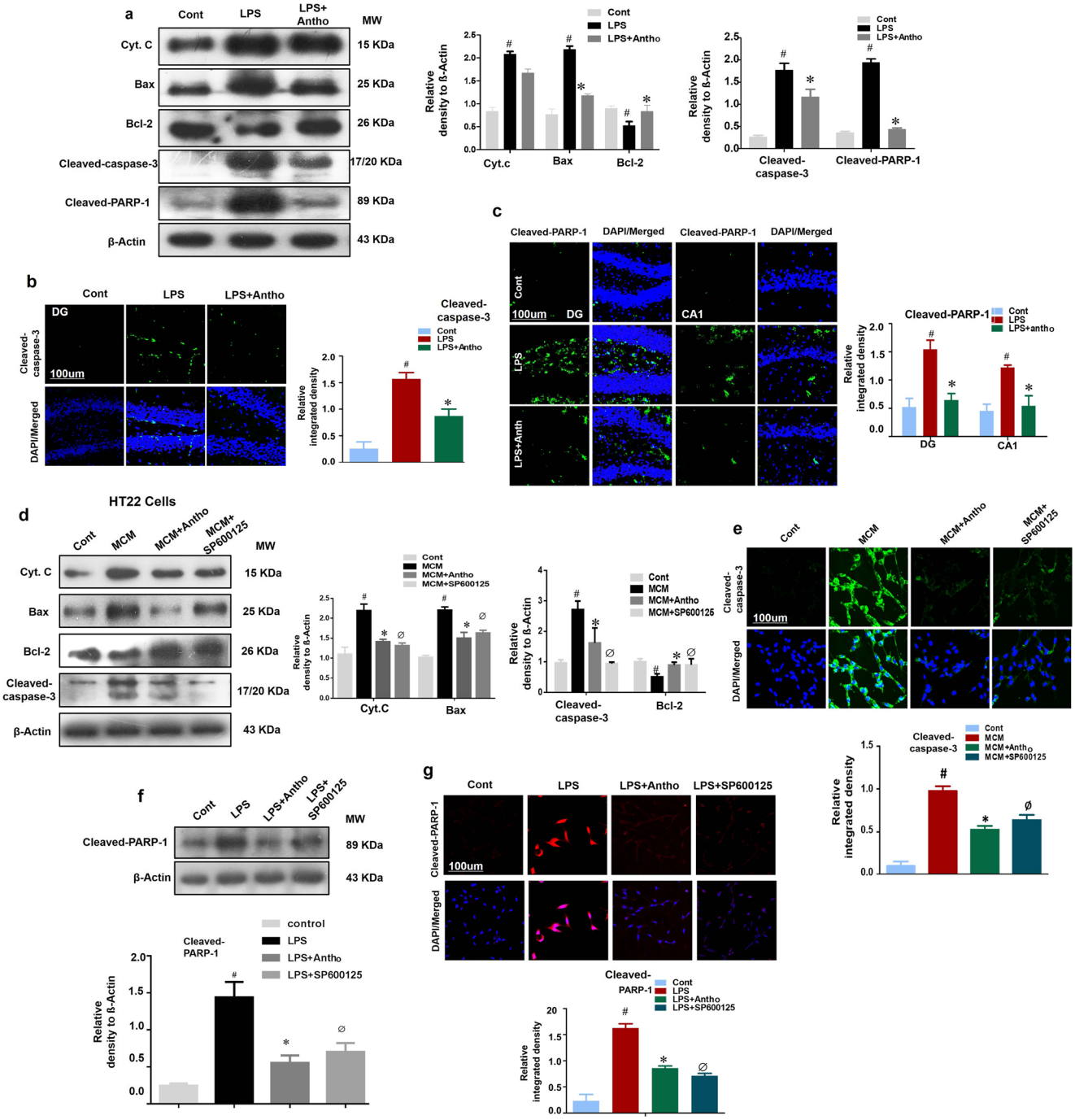


Fig. 4 Anthocyanins inhibited LPS-induced activation of inflammatory proteins in the hippocampus of adult mouse brains and in BV2 microglial cells. a The western blot analysis of TNF-α, IL-1β, and NF-$_k$B65 in the hippocampus of adult mice. The bands were quantified using Sigma Gel software, and the differences are represented by a histogram. β-Actin was used as a loading control. The results present for n = 15 mice/group, and the number of experiments = 3. b The immunofluorescence images represent the immunoreactivity of TNF-α (Green, FITC; Blue, DAPI) in the mice hippocampus. The results present for n = 5 mice/group, and the number of experiments = 3. Magnifications × 10. Scale bar = 100 μM. c The western blot analysis of TNF-α, IL-1β, and p-NF-$_k$B in the protein obtained from the cell lysate of the bv2 cells treated with LPS, LPS + Antho, and LPS + SP600125. The bands were quantified using Sigma gel software and the differences are represented by a histogram. β-Actin was used as a loading control. The results present for n = 5/group, and the number of experiments = 3. Symbols for treatment groups and level of significance are mentioned in the data analysis section of the Materials and Methods

level of p-Akt (Ser473), and p-GSK3β (Ser9) in HT22 neuronal cells (Fig. 6b). Additionally, FJB staining in the mice hippocampus and in HT22 cells was performed to analyze the extent of neurodegeneration. Our in vivo and in vitro results demonstrated that the numbers of dead neurons were more in LPS injected mice and in LPS-exposed HT22 cells when



compared with anthocyanins treated groups (Fig. 6c, d). Next, we performed Nissl staining to examine the neuronal survival effects of anthocyanins against LPS. The Nissl staining results showed that the number of survival neurons were less in LPS-treated mice when compared to the anthocyanins treated group (Fig. 6e). From the above observation as well as from our previous published papers [27, 32–34], we concluded that anthocyanins treatment not only reverses the apoptotic neurodegeneration but also restores the neuronal survival pathway.

Effect of Anthocyanins on Synaptic Dysfunction and Memory Functions in the LPS-Treated Mice

The ROS-mediated oxidative stress, neuroinflammation and neurodegeneration lead to synaptic deficits and memory impairment [6, 27, 32–34, 40, 52]. In order to determine the protective effects of anthocyanins on synaptic degeneration, we performed western blotting to analyze pre-synaptic protein SNAP25 and post-synaptic protein PSD-95. Western blot results showed that LPS treatment decreases the expression

Fig. 5 Anthocyanins treatment prevents LPS-induced neuronal apoptosis in the hippocampus of adult mice and in HT22 cells exposed to LPS and MCM. a The western blot analysis of Cyt.C, Bax, Bcl-2, cleaved caspase-3 and cleaved PARP-1 in the hippocampus of adult mice. The bands were quantified using Sigma Gel software, and the differences are represented by a histogram. β-Actin was used as a loading control. The results present for n = 15 mice/group, and the number of experiments = 3. b, c Represent the immunofluorescence analysis of cleaved caspase-3 (Green, FITC; Blue, DAPI) and cleaved PARP-1 (Green, FITC; Blue, DAPI) in the hippocampus. The results present for n = 5 mice/group, and the number of experiments = 3. Magnifications × 10. Scale bar = 100 μM. d Represents the western blot analysis of Cyt.C, Bax, Bcl-2, cleaved caspases-3 and cleaved PARP-1, in the protein obtained from the cell lysate of the HT22 cell treated with MCM, MCM + Antho, and MCM + SP600125. The bands were quantified using Sigma gel software and the differences are represented by a histogram. β-Actin was used as a loading control. The results present for n = 5/group, and the number of experiments = 3. e Represents the confocal microscopy images of the immunoreactivity of cleaved caspase-3 (Green, FITC; Blue, DAPI) in the HT22 cells exposed to the MCM, MCM plus anthocyanins, and MCM plus SP600125. The results present for n = 5/group, and the number of experiments = 3. Magnifications × 10. Scale bar = 50 μM. f Immunoblot represents the expression level of cleaved PARP-1 in the protein obtained from the cell lysate of the HT22 cell treated with LPS, LPS+ Antho, and LPS + SP600125. The bands were quantified using Sigma gel software and the differences are represented by a histogram. β-Actin was used as a loading control. The results present for n = 5/group, and the number of experiments = 3. g Represents the immunofluorescence analysis of cleaved PARP-1 (Red, TRITC; Blue, DAPI) in the HT22 cells exposed to LPS, LPS+ Antho, and LPS + SP600125. The results present for n = 5/group, and the number of experiments = 3. Magnifications × 10. Scale bar = 100 μM. Symbols for treatment groups and level of significance are mentioned in the data analysis section of the Materials and Methods

level of both pre- and post-synaptic proteins as compared to control group, while anthocyanins treatment (24 mg/kg/day for 14 days) significantly upregulates the expression level of SNAP25 and PSD-95 (Fig. 7a). Next, we performed the MWM test to examine the memory functions. The mice were allowed for 60 s to find the target in one quadrant of the tank. In our study, the LPS-treated mice showed increased escape latency time which indicates learning and memory problems. However, the treatment of anthocyanins reduced the latency time suggesting the possible memory improvements effects in LPS-treated mice (Fig. 7b). Next, the probe test was performed on day 6th and the hidden platform was removed. Our results showed less number of crossing and less time spent in the target quadrant in LPS-treated mice. However, the treatment of anthocyanins reversed the abnormalities and increased the number of crossing and time spent in the target quadrant (Fig. 7c–e). Overall, this data (Fig. 8) revealed that LPS treatment plays a critical role in oxidative stress and neuroinflammation-mediated synaptic/memory abnormalities while anthocyanins treatment regulated these abnormalities through reduction of oxidative stress, neuroinflammation and neurodegeneration which consequently prevents synaptic/memory impairment.

Discussion

The present research work was established to evaluate the neuroprotective capability of anthocyanins against LPS-induced ROS production, neuroinflammation, apoptotic neurodegeneration and memory impairment via the JNK/Akt/GSK3β signaling pathway in the adult mice hippocampus. A recent study provides evidence that oxidation process is critically involved in the pathogenesis of several neurodegenerative diseases including AD [18]. Several lines of investigation demonstrated that LPS-evoked macrophages contribute to ROS generation which ultimately results in protein modification, alteration in cell function, and exaggerated systemic inflammatory response [17, 18, 53]. Similarly, in the present study, we observed that LPS (250 μg/kg/day for 7 days) results in elevated oxidative stress which further evokes inflammatory responses and exhibits deleterious effects on neuronal cells and eventually modulates pre- and post-synaptic proteins (SNAP25 and PSD-95) level in the mice brain. The previous study reported that release of ROS and pro-inflammatory mediators from microglia cells disrupt the hippocampal neurons and impair their function resulting in hippocampal-related memory deficits [6, 26]. Other studies demonstrated that LPS is a potent inducer of ROS which mediates and activates pro-inflammatory cytokines and apoptotic signals that consequently leads to the hippocampal neuronal loss which ultimately results in memory dysfunction [3–7]. One of the important stress-activated kinases, JNK is activated in response to excessive ROS production that ultimately participates in neurodegeneration via multiple mechanisms [17]. Several lines of investigation elucidated that activated JNK is critically involved in neuronal apoptosis and induces inflammatory mediators which ultimately results in cell death [3, 32, 35, 39]. In the present study, we demonstrated that systemic administration of LPS increases the expression level of p-JNK in the mouse hippocampus as well as in MCM-incubated HT22 neuronal cells. However, our results clearly indicated the reduced expression level of p-JNK upon treatment with anthocyanins. Our immunofluorescence results were consistent with our immunoblot results indicating the increased immunoreactivity of p-JNK in LPS-treated mice and in MCM-stimulated HT22 cells which was significantly downregulated by anthocyanins. Many studies have shown that caspase-3 (executor of apoptosis) and PARP-1 (DNA damage marker) are the downstream targets of p-JNK. Furthermore, it has been also explored that JNK activation mediates neuroinflammation and neuronal apoptosis which in turn causes memory dysfunction [3–7, 54–56]. In this study, we observed that LPS-exposed mice show increased Bax/Bcl2, Cyt. C, cleaved caspase-3 and cleaved PARP-1 expression in the hippocampus region

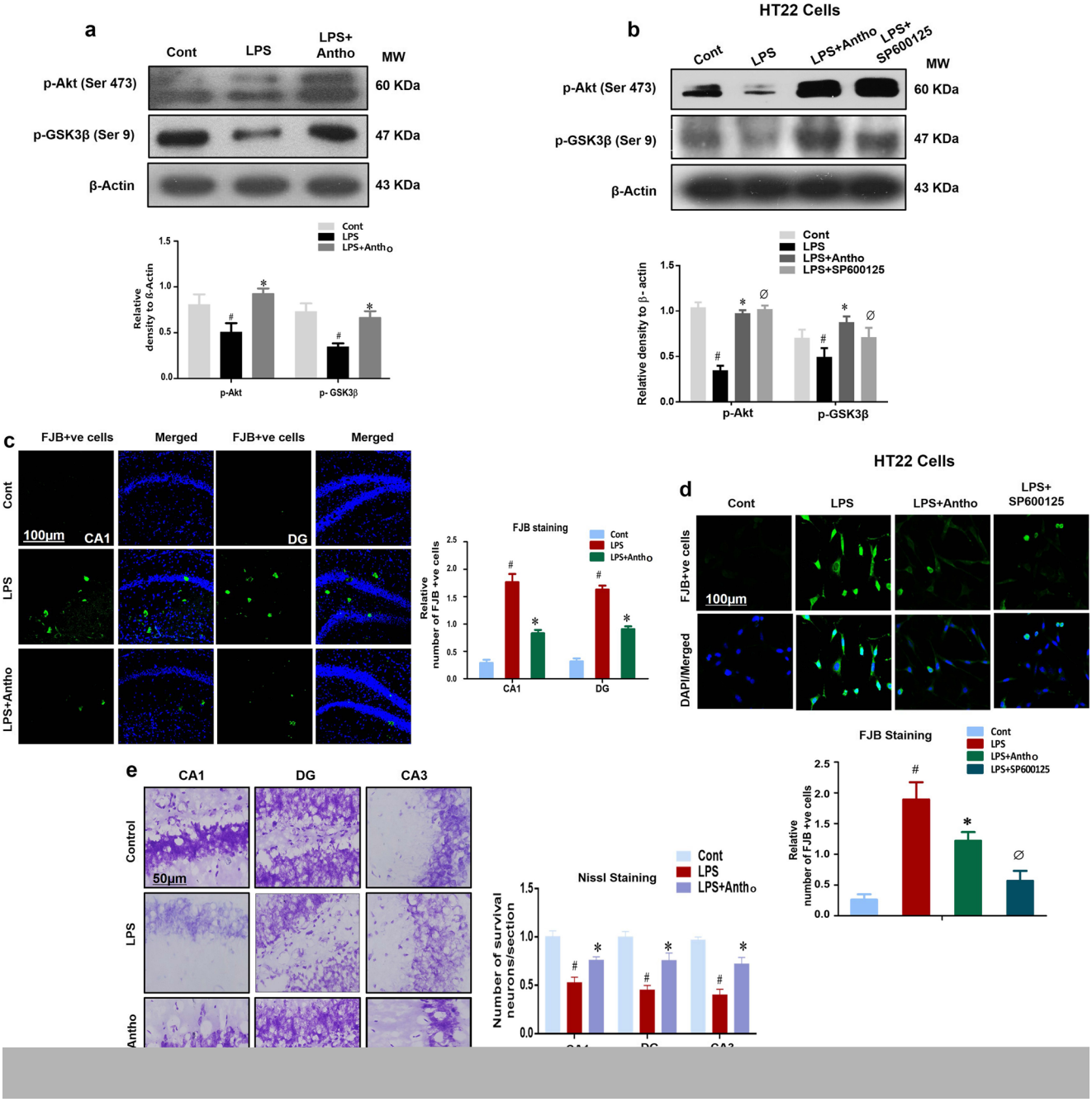


Fig. 6 Anthocyanins treatment improves survival pathway and ameliorates neuronal neurodegeneration. a The western blot analysis of p-Akt and p-GSK3β in the hippocampus of adult mice. The bands were quantified using Sigma Gel software, and the differences are represented by a histogram. β-Actin was used as a loading control. The results present for n = 15 mice/group, and the number of experiments = 3. b The western blot analysis of p-Akt and p-GSK3β in the protein obtained from the cell lysate of HT22 cells treated with LPS, LPS+ Antho, and LPS + SP600125. The bands were quantified using Sigma gel software and the differences are represented by a histogram. β-Actin was used as a loading control. The results presents for n = 5/group, and the number of experiments = 3. c Representative images and histograms showing FJB staining (Green, FITC; Blue, DAPI) in the CA1 and DG regions of the hippocampus in the adult mice. The results present for n = 5 mice/group, and the number of experiments = 3. Magnifications × 10. Scale bar = 100 μM. d Representative images and histograms showing FJB staining (Green, FITC; Blue, DAPI) in the HT22 cells exposed to the LPS, LPS+ Antho, and LPS + SP600125. The result presents for n = 5/group, and the number of experiments = 3. Magnifications × 10. Scale bar = 100 μM. e Histological analysis represents the Nissl staining in the CA1, CA3, and DG regions of the hippocampus in the adult mice. The results present for n = 5 mice/group, and the number of experiments = 3. Magnifications × 20. Scale bar = 50 μM. Symbols for treatment groups and level of significance are mentioned in the data analysis section of the Materials and Methods