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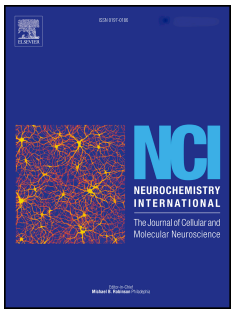

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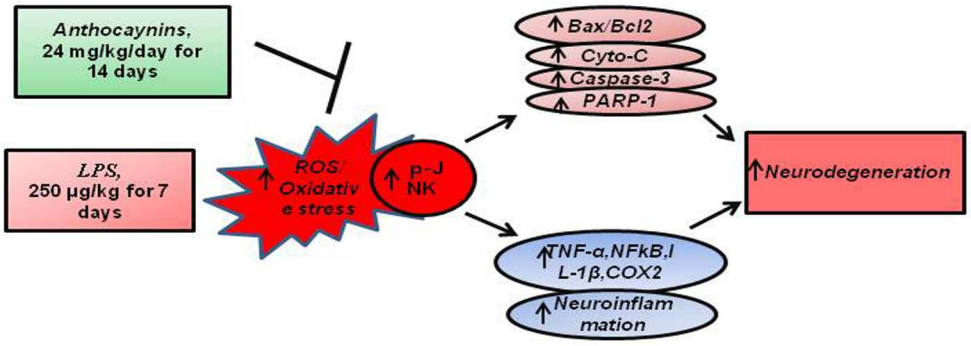

Anthocyanins protect against LPS-induced oxidative stress-mediated neuroinflammation and neurodegeneration in the adult mouse cortex

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Running Title: Anthocyanins attenuates LPS-Induced Neurotoxicity.

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Abstract

Several studies provide evidence that reactive oxygen species (ROS) are key mediators of various neurological disorders. Anthocyanins are polyphenolic compounds and are well known for their anti-oxidant and neuroprotective effects. In this study, we investigated the neuroprotective effects of anthocyanins (extracted from black soybean) against lipopolysaccharide (LPS)-induced ROS-mediated neuroinflammation and neurodegeneration in the adult mouse cortex. Intraperitoneal injection of LPS (250 μg/kg) for 7 days triggers elevated ROS and oxidative stress, which induces neuroinflammation and neurodegeneration in the adult mouse cortex. Treatment with 24 mg/kg/day of anthocyanins for 14 days in LPS-injected mice (7 days before and 7 days co-treated with LPS) attenuated elevated ROS and oxidative stress compared to mice that received LPS-injection alone. The immunoblotting results showed that anthocyanins reduced the level of the oxidative stress kinase phospho-c-Jun N-terminal Kinase 1 (p-JNK). The immunoblotting and morphological results showed that anthocyanins treatment significantly reduced LPS-induced-ROS-mediated neuroinflammation through inhibition of various inflammatory mediators, such as IL-1β, TNF-α and the transcription factor NF-$_k$B. Anthocyanins treatment also reduced activated astrocytes and microglia in the cortex of LPS-injected mice, as indicated by reductions in GFAP and Iba-1, respectively. Anthocyanins also prevent overexpression of various apoptotic markers, i.e., Bax, cytosolic cytochrome C, cleaved caspase-3 and PARP-1. Immunohistochemical fluoro-jade B (FJB) and Nissl staining indicated that anthocyanins prevent LPS-induced neurodegeneration in the mouse cortex. Our results suggest that dietary flavonoids, such as anthocyanins, have antioxidant and neuroprotective activities that could be beneficial to various neurological disorders.

Key Words: Reactive oxygen species (ROS); Lipopolysaccharide (LPS); Anthocyanins; Oxidative stress; Neuroinflammation and Neurodegeneration.

1. Introduction

In brain inflammation, the innate immune system initially protects against central nervous system (CNS) insults. Therefore, the temporary upregulation of inflammatory responses in the CNS is natural and has no detrimental effect on neuronal cells. However, long-term upregulation of inflammatory processes may cause neurodegeneration (Serhan et al., 2007; Delegge and Smoke 2008). Elevated reactive oxygen species (ROS) are a key mediator in the etiology of neurological disorders (Reynolds et al., 2007). It has been shown that systemic administration of lipopolysaccharide (LPS), an endotoxin that is a potent inducer of inflammation, triggers the activation of microglia that later release pro-inflammatory mediators, such as cytokines and ROS. These mediators are involved in neuroinflammation and neurodegeneration (Gibertini et al., Dantzer et al., 1998; Kobayashi et al., 2002; Ozato et al., 2002; Hayley et al., 2002; Liu et al. 2002; Ulloa et al., 2005; Block et al., 2007; Qin et al., 2007). ROS from various cellular reactions are known to cause damage to DNA, lipids and proteins (Thannickal et al., 2000). Several studies proposed that elevation of ROS and increased oxidative stress is associated with various types of nervous system damage i.e., neuro-inflammation and certain neurodegenerative disorders, such as (AD) (Olanow 1993; Castegna et al., 2002; Ali et al., 2015; Ullah et al., 2015).

The increased prevalence of neurological disorders and the lack of specific therapies for neurological disorders suggest that changes in life style, such as exercise and dietary supplements, might be beneficial in preventing neurological diseases. Notably, plant-derived polyphenolic flavonoids have been acknowledged as having bioactivity against various neurological disorders (Williamson et al., 2005; Willis et al., 2009; 2010; Williams et al., 2012; Vauzour et al., 2015). Among the important flavonoids are anthocyanins, which are found in various fruits, vegetables and beverages. Anthocyanins are water soluble and give

blue, red and purple colors to different plant tissues, particularly flowers and fruits, and protect the body from various toxins (Sun et al., 1999; Benvenuti et al., 2004). Many studies have shown that anthocyanins have strong antioxidant properties (Scalbert et al., 2000; He et al., 2010). Additionally, it has also been reported that anthocyanins reduce the level of inflammatory mediators in inflammatory disease models (Tsuda et al., 2002; Rosi et al., 2003). Previously, our group investigated the neuroprotective effect of black bean anthocyanins against various CNS-insults, both *in vitro* and *in vivo* (Ullah et al., 2013; Badshah et al., 2015). Recently, Rehman et al., 2016 found that anthocyanins reverse oxidative stress and neuroinflammation in D-galactose-treated rats (Rehman et al., 2016). However, the effects of black bean anthocyanins against LPS-induced oxidative stress are still not known. In this study, we hypothesized that anthocyanins intraperitoneally (I.P) administered at a dose of 24 mg/kg for 14 days prevent LPS-induced-oxidative stress, neuroinflammation and neurodegeneration in the adult mouse cortex.

2. Materials & Methods

2.1.1 Chemicals

Anthocyanins used in the current study were extracted from Korean black soybeans as previously described (Badshah et al., 2015). LPS and 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma Chemical Co (St. Louis, MO, USA).

2.1.2. Mice used in the experiments

Eight-week-old wild-type male C57BL/6N mice (25–30 g) were purchased from Samtako Bio (Osan, Korea). Mice were habituated for one week in the university animal house under a 12-h/12-h light/dark cycle at a temperate of 23°C with 60% humidity. All animals were provided with food and water ad libitum. Attempts were made to minimize the number of mice used and their suffering. All experimental procedures were carried out according to the animal ethics committee of the Division of Applied Life Sciences, Department of Biology at Gyeongsang National University, South Korea.

2.1.3. Mice grouping and their treatments

Mice were randomly divided into the following three groups: (i) Control (C) saline injected as a vehicle for 14 days; (ii) saline injected for 7 days, following LPS injection of the mice at a dose of 250 µg/kg for an additional 7 days; and (iii) mice injected with LPS (250 µg/kg) for 7 days and anthocyanins 24 mg/kg for 14 days (7 days prior to LPS and 7 days co-treated with LPS (LPS+Antho).

Anthocyanins were dissolved in dimethyl sulfoxide (DMSO), and the administered volume was given in normal saline. Anthocyanins were intraperitoneally (I.P.) administered to the mice at a dose of 24 mg/kg of body weight/day for 14 days, and similarly, LPS was dissolved in saline and administered I.P. at a dose of 250 µg/kg) for 7 days.

2.1.4. *In vivo* reactive oxygen species (ROS) assay

The ROS assay based on the conversion of 2′ 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to 2′ 7′-dichlorofluorescein (DCF) was performed as described previously (Ali et al., 2015).

2.1.5. *In vivo* lipid peroxidation (LPO) assay

The LPO assay, an indicator of oxidative stress and measure of the LPO biomarker malondialdehyde (MDA), was performed according to the manufacturer's instructions (catalog # K739-100) from Biovision Incorporated, A 95035 USA.

2.1.6. Protein extraction from mouse brain

For Western blot (Immunoblot) analysis, brains were rapidly isolated, and cortex tissue was properly separated, frozen in liquid nitrogen vapours, and stored at −80°C. The cortex tissue was homogenized in 0.2 M PBS with phosphatase and protease inhibitors and then centrifuged at 10,000 g for 25 min at 4°C. After centrifugation the supernatants were collected and kept at −80°C until further processing.

2.1.7. Assessment of cytosolic/mitochondrial cytochrome C

Mitochondrial and cytosolic fractions of cortex homogenates were separated using a Mitochondria/Cytosol Fractionation Kit according to the manufacturer's instructions (catalog # K256-25) Biovision Incorporated, A 95035 USA.

2.1.8. Western blots analysis

Western blot was performed according to the previously described protocol (Shah et al., 2016). Briefly, quantification of the proteins for Western blot analysis was assessed by a BioRad protein assay kit, BioRad Laboratories, CA, and USA. After protein quantification, equal amounts of protein (20-30 µg) were electrophoresed using 4-12% BoltTM Mini Gels (Novex, Life Technologies). Then, membranes were blocked in 5 % (w/v) skim milk to reduce non-specific binding and incubated with primary antibodies overnight at 4ºC at a 1:1000 dilution. After reaction with a horseradish peroxidase-conjugated secondary antibody as appropriate, the proteins were detected using an ECL detection reagent according to the manufacturer's instructions (Amersham Pharmecia Biotech, Uppsala, Sweden). X-ray films were scanned, and the optical densities of the bands were analyzed through densitometry using the computer-based Sigma Gel program, version 1.0 (SPSS, Chicago, IL, USA).

2.1.9. Primary antibodies used in the Western blotting

The primary antibodies used in this study were anti-caspase-3 (SC-7148), anti-TNF-α (52746), anti-IL-1β (SC-7884), anti-Bcl2 (SC-492), anti-Bax (SC-493), anti-p-JNK (SC-6254), anti-p-NF-kB 65 (101749), anti-Iba-1(SC-98468), anti-Cytochrome C (sc-8385), anti-PARP-1(SC-8007) and anti-β-Actin (SC-47778) from Santa Cruz, Biotechnology, CA, USA.

2.1.10. Tissue sample preparation for morphological analysis

After completion of drug treatments, mice were perfused transcardially with 4% ice-cold paraformaldehyde, and the brains were post-fixed for 48-72 hr in 4% paraformaldehyde and transferred to 20% sucrose for 72 hr. Brains were frozen in an O.C.T compound (A.O, USA), and 14-µm coronal sections of the cortex were cut using a CM 3050C cryostat (Leica, Germany). The sections were thaw-mounted on probe-on plus charged slides (Fisher, USA).

2.2. 1.Immunofluorescence staining

Immunofluorescence staining was performed according to the previously described protocol with a few changes (Ahmad et al., 2016; Ali et al., 2015a; 2015b; Badshah et al., 2016). In brief, tissue slides were air dried overnight; after air drying the tissue slides were washed twice in 0.01 M PBS for 10 minutes, followed by incubation for 1 hr in blocking solution containing 2% normal serum according to the antibody treatment and 0.3% Triton X-100 in PBS. After blocking, the slides were incubated overnight at 4^{o}C in primary antibodies (rabbit polyclonal COX2 (sc-7951), mouse TNF-α, goat-Cyt.C, mouse-GFAP (SC-6170), mouse-p-NF-$_{K}$B from Santa Cruz Biotechnology, USA, and 8-oxoguanine (MA3560) from Millipore, USA). The antibodies were diluted 1:100 in blocking solution and incubated overnight. After incubation with primary antibodies, the sections were incubated for 2 hr in secondary TRITC/FITC-labeled antibodies (1:50) (Santa Cruz Biotechnology, USA). After secondary antibody incubation the slides were washed twice for 5 minutes. 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).

2.2.2. Immunohistochemical fluoro-jade B (FJB) staining

Immunohistochemical FJB staining was performed as previously described (Badshah et al., 2014; Ali et al., 2015) with minor modifications. After overnight air-drying, the tissue slides were washed twice for 5 minutes in 0.01 M PBS. Following washing, the tissue slides were immersed in a solution of 1% sodium hydroxide and 80% ethanol for 5 min. Then, they were immersed in 70% alcohol and 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 and then immersed in a solution of 0.1% acetic acid and 0.01% FJB for at least 20 minutes. The slides were washed with distilled water and allowed to dry for 10 min. Glass cover slips were mounted using a DPX non-fluorescent mounting medium, and images were obtained with a confocal laser scanning microscope (Flouview FV 1000, Olympus, Japan). Stained slides were analyzed using the Image J program.

2.2.3. Immunohistochemical Cresyl violet staining

Cresyl violet (Nissl) staining was used for the histological examination to evaluate the extent of neuronal cell death. Tissue slides of 14-µm sections were washed twice for 15 min in 0.01 M PBS and stained with a 0.5% cresyl violet solution (containing a few drops of glacial acetic acid) for 10-15 minutes. Then, sections were washed with distilled water and dehydrated in a graded ethanol series (70%, 95% and 100%), placed in xylene, covered with a coverslip using mounting medium and finally examined with a fluorescent light microscope. The results were evaluated with the Image J program.

2.2.4. Statistical analysis

Western blot bands were scanned and analyzed via densitometry using the Sigma Gel System (SPSS, Inc., Chicago, IL). Then, the density values were expressed as the means ± standard error mean (SEM). Image-J software was used for immunohistological quantitative analysis. One-way analysis of variance (ANOVA) followed by a two-tailed independent Student's t-test were used for comparisons of the LPS-injected groups and the control saline-injected group, as well as the LPS-injected group alone and LPS+Anthocyanins-treated groups. Calculations and graphs were made using Prism 5 software (Graph-Pad Software, Inc., and San Diego, CA). P values less than 0.05 were considered to be statistically significant. An asterisk (*) indicates that a result was significantly different from the saline-injected control group, while a hash tag (#) indicates that a result was significantly different from the LPS-injected group.*$P < 0.05$., # $P < 0.05$.

3. Results

3.1. Anthocyanins overcome LPS-induced ROS elevation and oxidative stress in the cortex of adult mice

Exposure to LPS induces mitochondrial ROS and oxidative stress, which increases the expression level of pro-inflammatory mediators and plays a central role in various neurodegenerative disorders (Block et al., 2007; Park et al., 2013). Here, we performed ROS and LPO assays to assess the antioxidant properties of anthocyanins against-LPS-induced ROS elevation. Our results showed that anthocyanins (24 mg/kg for 14 days) treatment significantly reduced the increased level of ROS and MDA (a marker of oxidative stress) compared with the LPS treated group (Fig. 1A&B). For further confirmation, we also performed immunofluorescence staining to analyze the expression level of 8-oxoguanine

(8-OxoG), an oxidative stress marker primarily expressed in the brain of AD and PD patients (Iida et al., 2002; Hamilton et al., 2012). Besides, the ROS and LPO assays, our immunofluorescence results also showed that anthocyanins can reduce the expression level of 8-OxoG in the adult mouse cortex (Fig. 1C).

3.2. Anthocyanins reduced the expression of p-JNK protein in the cortex of LPS-injected mice

JNK or C-Jun N-terminal kinase is a well-known stress-activated kinase that is usually overexpressed under elevated ROS conditions (Manning et al., 2003). Its activation causes neuroinflammation and neurodegeneration (Cao et al., 2004; Ullah et al., 2015). Therefore, we examined the activation of JNK through its phosphorylation (p-JNK) level by Western blotting. Our results showed that anthocyanins reduced the activated p-JNK level in LPS treated mice (Fig. 2).

3.3. Anthocyanins prevent activated astrocytes and microglia in the cortex of LPS-injected mice

The systemic administration of LPS leads to activated microglia and astrocytes (Badshah et al., 2015a). Here, our Western blots and confocal microscopy results indicated that anthocyanins treatment significantly reduced LPS-induced glial fibrillary acidic protein (GFAP) from activated astrocytes and ionized calcium-binding adapter molecule 1(Iba-1) from activated microglia in the cortex of adult mice (Fig. 3 A & B).

3.4. Anthocyanins decreased LPS-induced NF-$_k$B activation in the cortex of adult mice

The mammalian NF-k_B family has five members and is implicated in the transcription of various inflammatory genes and in the response to oxidative stress-induced DNA damage (Hayden et al., 2004). In our study, we also investigated the expression level of p-NF-$_K$B 65 through Western blot and confocal microscopy. Similarly, our results showed that LPS treatment for 7 days increased the expression and immunofluorescence reactivity of p-NF-$_k$B65, while anthocyanins treatment for 14 days significantly reversed and reduced its expression level as well as immunofluorescence reactivity in the cortex of LPS-injected mice (Fig. 4 A & B).

3.5. Anthocyanins reduced LPS-induced expression of inflammatory mediators in the cortex of adult mice

Research has shown that both inflammation and reactive gliosis are implicated in the pathogenesis of important neurodegenerative diseases, such as AD and Parkinson's disease (PD) (Nguyen et al., 2002). It has also been reported that exposure to LPS activates microglia cells, which in turn release pro-inflammatory mediators to enhance neuro-inflammation and neurodegeneration (Hauss-Wegrzyniak et al., 1998; Barger et al., 2001; Rossi et al., 2003; Rosi et al., 2004a; 2004b). Here, we also investigated the levels of important inflammatory markers, such as TNF-α, IL-1β and COX-2. Our Western blot results show that LPS treatment significantly increased the level of TNF-α and IL-1β, whereas anthocyanins prevented LPS effects and significantly reduced their expression (Fig. 5A). The immunofluorescence results of TNF-α and COX-2 also show that anthocyanins reduced the

immunofluorescence reactivity of TNF-α and COX-2 in the cortex of LPS-treated mice (Fig. 5B & C).

3.6. Anthocyanins inhibit apoptosis and neurodegeneration in the cortex of LPS-injected mice

It is known that Bax and Bcl-2 are cytoplasmic proteins that take part in the regulation of apoptosis. These proteins are involved in mitochondrial membrane disruption during the apoptotic process. Bax is pro-apoptotic and increases the permeability of mitochondrial membranes, whereas Bcl-2 is an anti-apoptotic protein (Mattson 2000). Activation of Bax and its oligomeric form with BaK on the mitochondrial membrane enhances the permeability of mitochondrial membrane transition pores which later results in the release of cytochrome C (Cyt.C) into the cytoplasm, ultimately leading to cell death (Garcia et al., 1992; Vander-Heiden et al., 1999). Therefore, we used Western blotting to study the effect of anthocyanins against LPS on the expression level of Bax and Bcl-2. Our results showed that LPS treatment significantly increased Bax oligomeric form compared to controls, whereas anthocyanins reduced Bax oligomeric form in the cortex of LPS-treated mice (Fig. 6A). Similarly, anthocyanins also increased anti-apoptotic Bcl2 in the cortex of LPS-treated mice (Fig. 6A). In addition, Western blotting was performed to assess the mitochondrial and cytosolic fractions of the Cyt.C protein levels in the treated groups. Our results showed that LPS treatment significantly decreased the level of mitochondrial Cyt.C compared with the control group, while administration of anthocyanins resulted in an increase in the expression of mitochondrial Cyt.C protein in the cortex of LPS-treated mice (Fig. 6B). Consequently, in the cytosolic fraction, LPS treatment increased the level of Cyt.C, while anthocyanins treatment

reversed the LPS-induced expression of cytosolic Cyt.C protein in the cortex of LPS-treated mice (Fig. 6B). We also observed an increased immunofluorescence reactivity of Cyt.C in the cortex of LPS-treated mice, which was reversed by the anthocyanins treatment. These results showed that anthocyanins inhibit the mitochondrial apoptotic pathway *in vivo* and have an antiapoptotic effect against LPS-induced neuroapoptosis (Fig. 6 A, B & C).

Several studies have confirmed that caspases are essential mediators as well as executors of apoptosis (Li et al., 1997; Thornberry et al., 1998). After the release of Cyt.C into the cytoplasm, it activates the caspase protein cascade, including caspase-9 and caspase-3 which finally induces neuronal cell death. To assess LPS-induced apoptosis in the mouse cortex, we examined the cleaved caspase-3 protein marker using a Western blot. Our result showed that LPS treatment significantly increased the expression level of cleaved caspase-3, whereas anthocyanins reversed their induction and significantly reduced the cleaved caspase-3 level in in the cortex of LPS-treated mice (Fig. 6A). Moreover, we also examined the level of elevated poly (ADP-ribose) polymerase 1 (PARP-1). It is well known that elevated cleaved PARP-1 is responsible for DNA damage and also plays a key role in excitotoxicity and neuronal death (Ali et al., 2015; Zhang et al., 1994; Mandir et al., 2000; Chaitanya et al., 2010). Our, Western blot results showed that LPS treatment significantly increased the expression of cleaved PARP-1, whereas anthocyanins at a dose of 24 mg/kg significantly reduced the cleaved PARP-1 expression in the cortex of LPS-treated mice (Fig. 6A). Next to determine the extent of neurodegeneration, we performed FJB staining, an immunohistochemicalfluorescence technique that only stains degenerating neurons (Schmued et al., 2000). The results showed that the number of FJB-positive neurons was greater in the cortex region of LPS-injected mice compared to the control group. However, anthocyanins

treatment significantly attenuated the number of FJB-positive neuronal cells in the cortex of LPS-injected mice (Fig. 6D).

Furthermore, we performed Nissl staining to examine neuronal loss. Our result showed that treatment with LPS significantly reduced the number of surviving neuronal cells, whereas anthocyanins at a dose of 24 mg/kg maintained the number of surviving neuronal cells and prevented LPS-induced neurodegeneration in the cortex of adult mice (Fig. 6E).

4. Discussion

In this study, we investigated the anti-oxidant and neuroprotective effects of anthocyanins against LPS-induced ROS-mediated neuroinflammation and neurodegeneration in the adult mouse brain cortex. Anthocyanins are polyphenolic flavonoids that found in various flowers, fruits and vegetables and have potent antioxidant and anti-inflammatory activities (Tsuda et al., 2002; Rossi et al., 2003; Rehman et al., 2016). Numerous studies have reported on the anti-atherosclerotic, anti-hypertensive and anti-carcinogenic activity of anthocyanins extracted from plants (Mortensen 2006; Kwon et al., 2007).

Recently, several studies have reported that treatment of LPS both *in vivo* and *in vitro* causes loss of neurons as well as activates microglia. This subsequently produces a massive amount of pro-inflammatory cytokines, prostanoids, ROS and nitric oxide (NO) that later cause neuronal damage (Boje et al., 1992; Merrill et al., 1992; Banati et al., 1993; Bronstein et al., 1995; Minghetti et al., 1998; Gonzalez-Scarano et al., 1999; Liu et al., 2001; 2002; Ling et al.,2002; Wang et al., 2004).

LPS has been implicated as a potent inducer of inflammatory responses, which in turn causes the release of ROS from mitochondria and NO and other cell mediators from monocytes and macrophages (Gibertini et al., Dantzer et al., 1998; Kobayashi et al., 2002; Ozato et al., 2002; Hayley et al., 2002; Liu et al. 2002; Ulloa et al., 2005; Qin et al., 2007). Elevated levels of ROS are responsible for producing various pathological events, such as peroxidation of lipids, DNA and proteins (Tucsek et al., 2011). Anthocyanins exist in various fruits, vegetables, and beans as well as other plant parts and have strong antioxidant properties (Mortensen 2006; Kwon et al., 2007). Similarly, in the present study we also found that cortex tissues of LPS-treated mice showed significantly increased levels of ROS and oxidative stress (elevated MDA and 8-oxoguanine). This could be reversed in LPS-treated mice by treatment with anthocyanins at a dose of 24 mg/kg for 14 days.

Several CNS insults, such as ethanol, D-galactose and LPS, induce the overactivation of p-JNK, an important stress kinase and a key mediator in the activation of microglia and astrocytes as well as various inflammatory mediators (Ali et al., 2015a; Badshah et al., 2015a). Furthermore, activation of p-JNK is implicated in the promotion of apoptosis and neurological disorders (Walton et al., 1998; Kim et al., 2004; Waetzig et al., 2005; Pablos et al., 2006). Here we observed that LPS treatment for 7 days increased the expression of p-JNK, which was overcome and significantly attenuated by anthocyanins treatments in the LPS-injected mouse brain cortex.

Systemic administration of LPS is a potent neurotoxic agent and causes neuro-inflammation in animal models (Shaw et al., 2005; Qin et al., 2007; Lee et al., 2008). Evidence from both *in vivo* and *in vitro* studies indicates that activation of glial cells (microglia and astrocytes) leads to the increased expression of inflammatory mediators, while different polyphenolic flavonoids, such as curcumin, inhibit reactive gliosis (Zhang et al.,

2010;Badshah et al., 2015a; Cho et al., 2015; Czapski et al., 2016). Our systemic administration of LPS for 7 days also triggered the activation of microglia and astrocytes in the mouse cortex. Anthocyanins administration for 14 days prevented the activation of microglia and astrocytes in the cortex of the LPS-treated mice. Previously, both *in vitro* and *in vivo* studies showed that LPS activated various inflammatory markers, such as TNF-α, IL-1β and COX-2 (Zhang et al., 2010; Badshah et al., 2015a; Cho et al., 2015; Czapski et al., 2016). Similarly our systemic administration of LPS also induced NF-$_K$B transcription factors and other inflammatory markers, such as TNF-α, IL-1β and COX-2, which might trigger neuroinflammation in the LPS-treated mouse brain cortex (Badshah et al., 2015a; Cho et al., 2015; Czapski et al., 2016). Treatment with anthocyanins reduced the expression level of TNF-α and IL-1β and the immunoreactivity of COX-2 in the LPS-treated adult mouse brain cortex.

It has been reported that activated p-JNK and other inflammatory mediators play a key role in the overexpression of downstream apoptotic markers, such as Bax, cytochrome-C, cleaved caspase-3 and PARP-1 level (Ali et al., 2015a; Badshah et al., 2015a; Ullah et al., 2015). Badshah et al., 2015a also showed that systemic administration of LPS induced these apoptotic markers in the adult mouse brain (Badshah et al., 2015a). In our study we also observed that systemic LPS administration significantly boosted the expression level of apoptotic markers i.e., Bax, cytosolic Cyt.C, cleaved caspase-3 and PARP-1 level, while administration of anthocyanins (24 mg/kg for 14 days) reversed the toxic effects of LPS and markedly alleviated apoptosis by reducing the expression levels of Bax, cytosolic Cyt.C, cleaved caspase-3 and PARP-1 (Badshah et al., 2015a). Along with the above apoptotic markers, our immunohistochemical staining with Nissl and FJB also indicated that daily anthocyanins administration for 14 days reduced the LPS-induced neurodegeneration in the

cortex of adult mice. All of our immunoblots and immunohistochemical results indicate that anthocyanins are potent anti-oxidant flavonoids and neuroprotective agents that prevent LPS-induced apoptotic neurodegeneration in the cortex of adult mice. Figure 7 shows a schematic of the neuroprotective effect of anthocyanins (24 mg/kg for 14 days) versus LPS-injected mice.

In conclusion, our results demonstrate that black bean anthocyanins inhibit LPS-induced ROS elevation and oxidative stress mediated neuroinflammation as well as neurodegeneration in the adult mouse cortex. Recently, Rendeiro et al. reviewed the direct and indirect mechanisms of the beneficial effects of flavonoids and their derivatives in the brains of humans and animal models (Rendeiro et al., 2015). Our results also suggest that the daily intake of anthocyanins existing in various fruits and beans may be beneficial for humans and protect against various CNS-insult-induced neurological disorders.

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Competing Financial Interests

The authors declare no competing financial interests.

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Figure legends

Fig. 1. Anthocyanins overcome LPS-induced ROS elevation and oxidative stress in the cortex of adult mice. (A & B) Representative ROS and LPO assay graphs showing comparative ROS and MDA levels, respectively, in the cortex of adult mice (5 mice/group). Number of experiments=3. (C) A representative image of immunofluorescence staining of 8-Oxoguanine in the cortex region of mice. The data are presented relative to the control. Magnified 10x. Scales bar = 100 µm.* significantly different from the saline-injected; # significantly different from LPS-injected. Significance = *P < 0.05, #P < 0.05.

Fig. 2. Anthocyanins reduced the expression of p-JNK protein in the cortex of LPS-injected mice. Western blot band of p-JNK was quantified using Sigma Gel software, and the differences are represented by a histogram. β-actin was run as a loading control. The density values are expressed in arbitrary units (A.U) as the means ± SEM for the respective indicated protein extracted from the cortex of mice (7 mice/group). * Significantly different from the saline-injected; # significantly different from LPS-injected. Significance = *P < 0.05, #P < 0.05.

Fig. 3. Anthocyanins prevent activated astrocytes and microglia in the cortex of LPS-injected mice. (A) Western blot band of Iba-1 was quantified using Sigma Gel software, and the differences are represented by a histogram. β-actin was run as a loading control. The density values are expressed in arbitrary units (A.U) as the means ± SEM for the respective indicated protein extracted from the cortex of mice (7 mice/group). (B) Immunofluorescence reactivity of GFAP in the experimental mice (5 mice/group). The data are presented relative to the control. Magnified 10x. Scales bar = 100 µm.* significantly different from the saline-injected; # significantly different from LPS-injected. Significance = *P < 0.05, #P < 0.05.

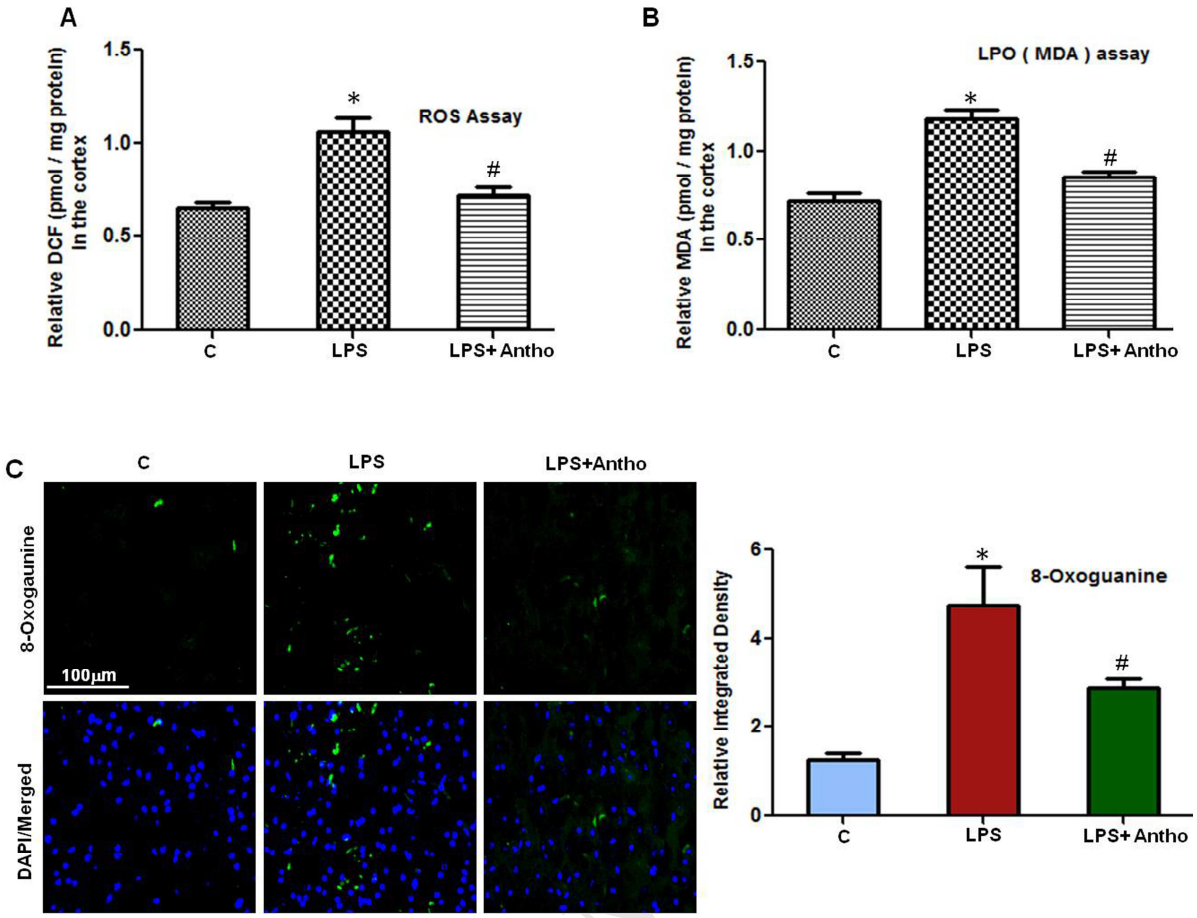
Fig. 4. Anthocyanins decreased LPS-induced NF-$_k$B activation in the cortex of adult mice. (A) The Western blot bands of NF-$_k$B were quantified using Sigma Gel software, and the differences are represented by a histogram. β-actin was run as a loading control. The density values are expressed in arbitrary units (A.U) as the means ± SEM for the respective indicated protein extracted from the cortex of mice (7 mice/group). (B) Immunofluorescence reactivity of the NF-$_k$B in the experimental mice (5 mice/group). The data are presented relative to the control. Magnified 10x. Scales bar = 100 µm.* significantly different from the saline-injected; # significantly different from LPS-injected. Significance = *P < 0.05, #P < 0.05.

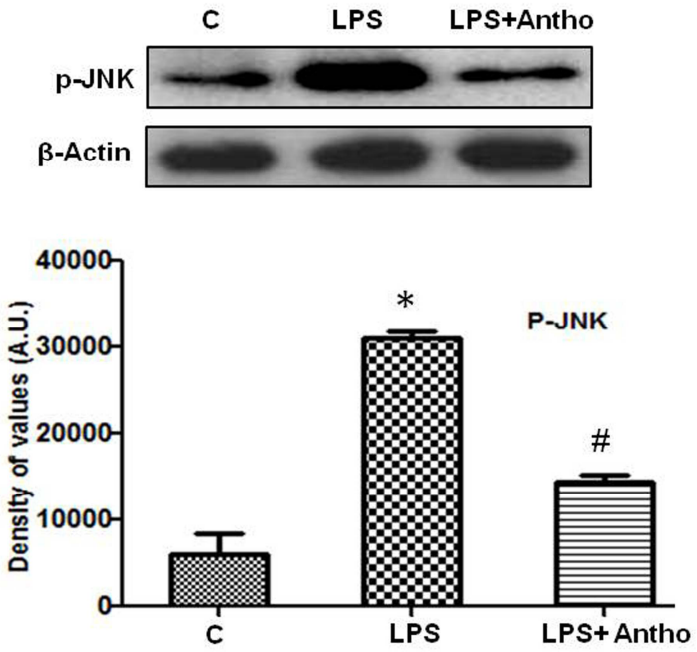
Fig. 5. Anthocyanins reduced LPS-induced expression of inflammatory mediators in the cortex of adult mice. (A) The western blot analysis of IL-1β and TNF-α in the cortex of mice. The bands were quantified using Sigma Gel software, and the differences are represented by a histogram. β-Actin was used as a loading control. The density values are expressed in arbitrary units (A.U) as the means ± SEM for the respective protein extracted from the cortex of mice (7 mice/group). (B & C) Immunofluorescence reactivity of TNF-α and COX-2 in the

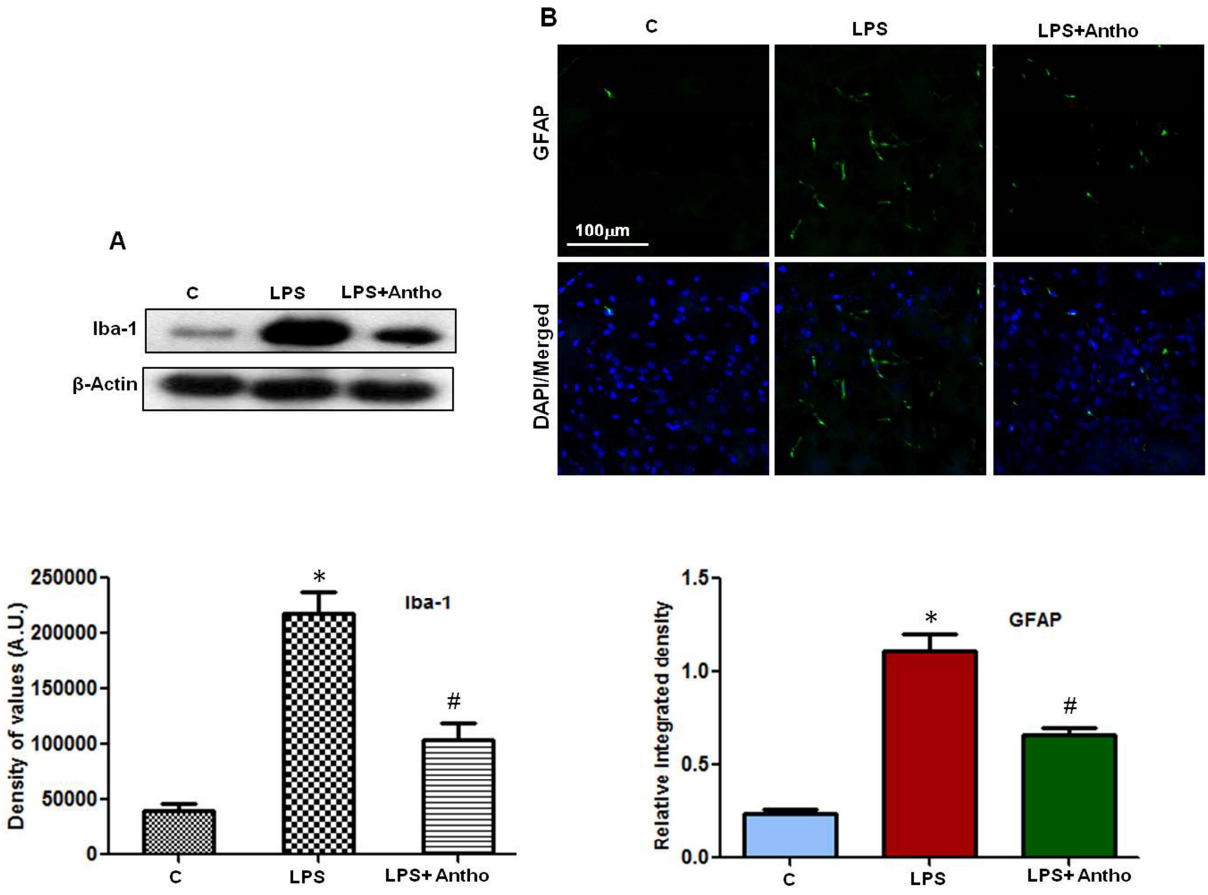
experimental mice (5 mice/group) respectively. The data are presented relative to the control. Magnified 10x. Scales bar = 100 µm.* significantly different from the saline-injected; # significantly different from LPS-injected. Significance = *P < 0.05, #P < 0.05.

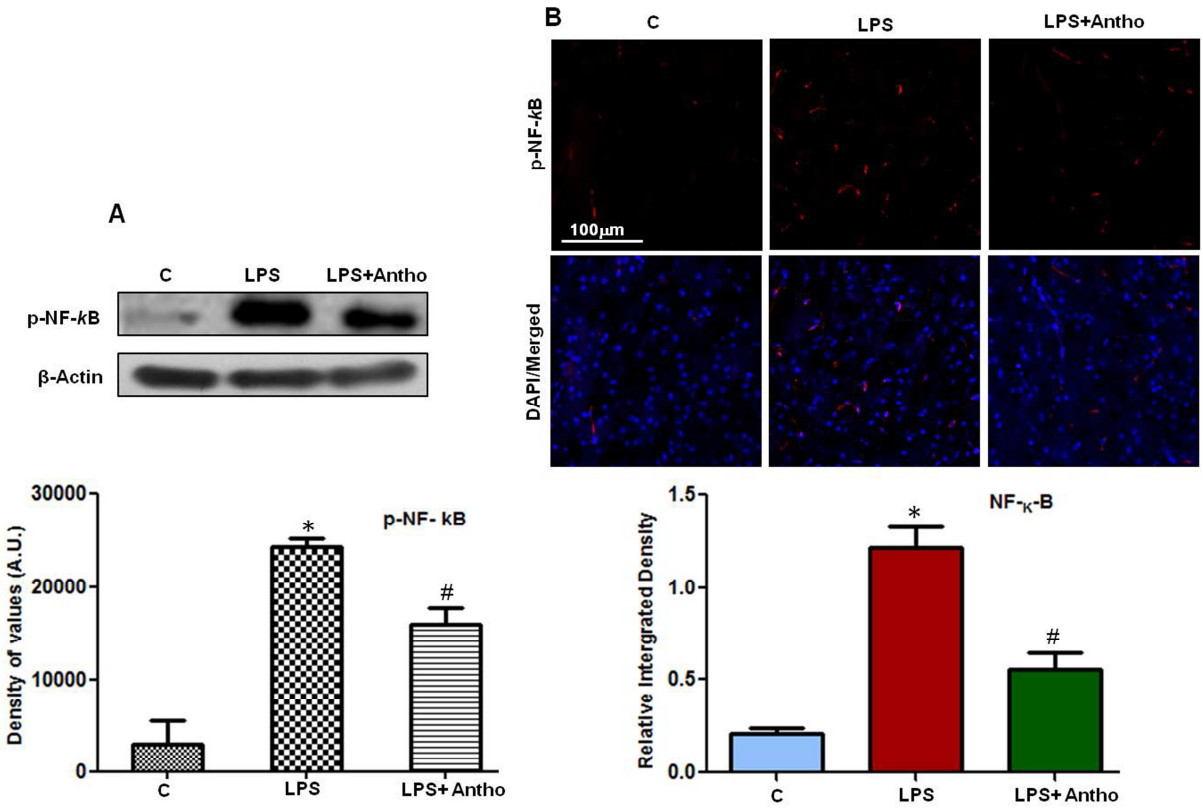
Fig. 6. Anthocyanins inhibit apoptosis and neurodegeneration in the cortex of LPS-injected mice. (A) Western blot analysis of the mouse cortex using Bcl2, Bax, cleaved caspase-3 and PARP-1 antibodies. The bands were quantified using Sigma Gel software, and the differences are represented by a histogram. β-actin was used as a loading control. The density values are expressed in arbitrary units (A.U) as the means ± SEM for the respective indicated cortex proteins extracted from the cortex of mice (7 mice/group). (B) Western blot analysis of the mitochondrial Cyt. C and cytosolic Cyt.C using Cyt.C antibody. 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 density values are expressed in arbitrary units (A.U) as the means ± SEM for the respective indicated cortex proteins extracted from the cortex of mice (7 mice/group). (C) Immunofluorescence reactivity of Cyt.C in the experimental mice (5 mice/group). The data are presented relative to the control. Magnified 10x. Scales bar = 100 µm. (D). Images of FJB staining in the cortex region of the mouse brain. The data are presented relative to the control. Magnification 40x. Scale bar = 50 µm. (E). Photomicrograph of Nissl staining in the cortex region of the mouse brain. The data are presented relative to the control. Scale bar = 100 µm.* significantly different from the saline-injected; # significantly different from LPS-injected. Significance = *P < 0.05, #P < 0.05.

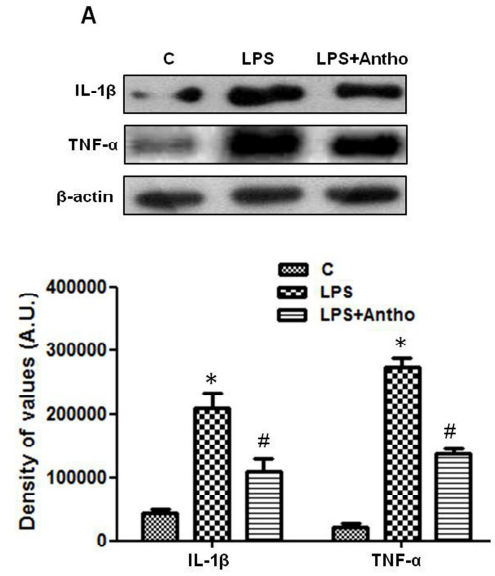
Fig. 7. The proposed pathway of neuroprotection of anthocyanins treatment (24 mg/kg/day i.p., 14 days) against LPS-injected (250 µg/kg day i.p, 7days) mice.







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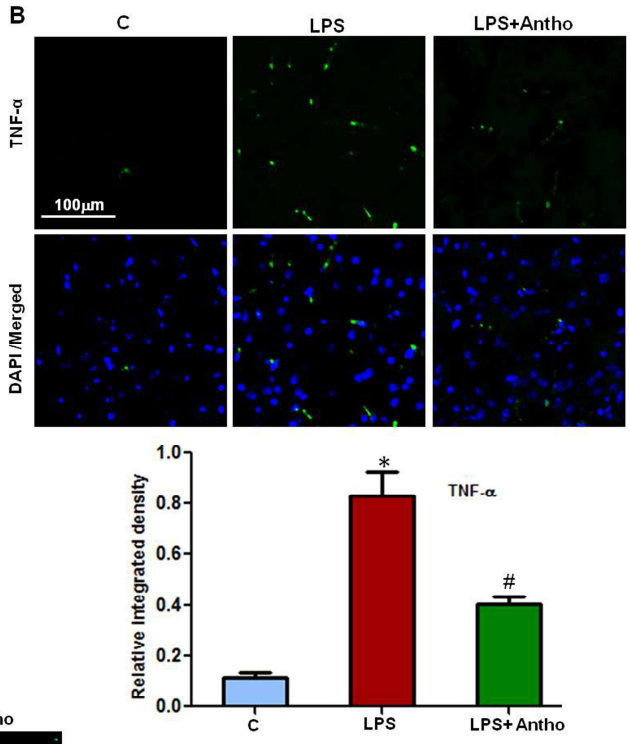
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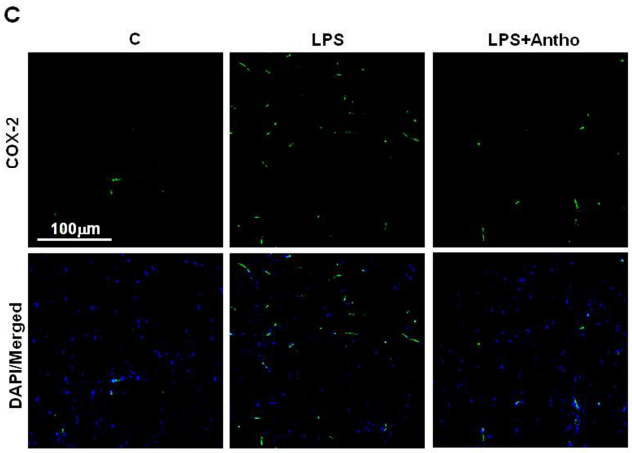
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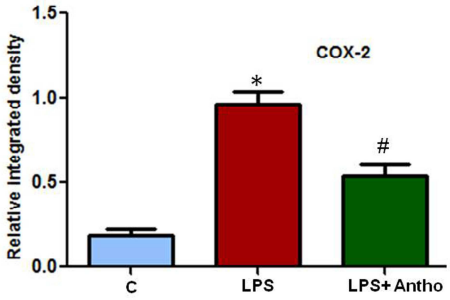
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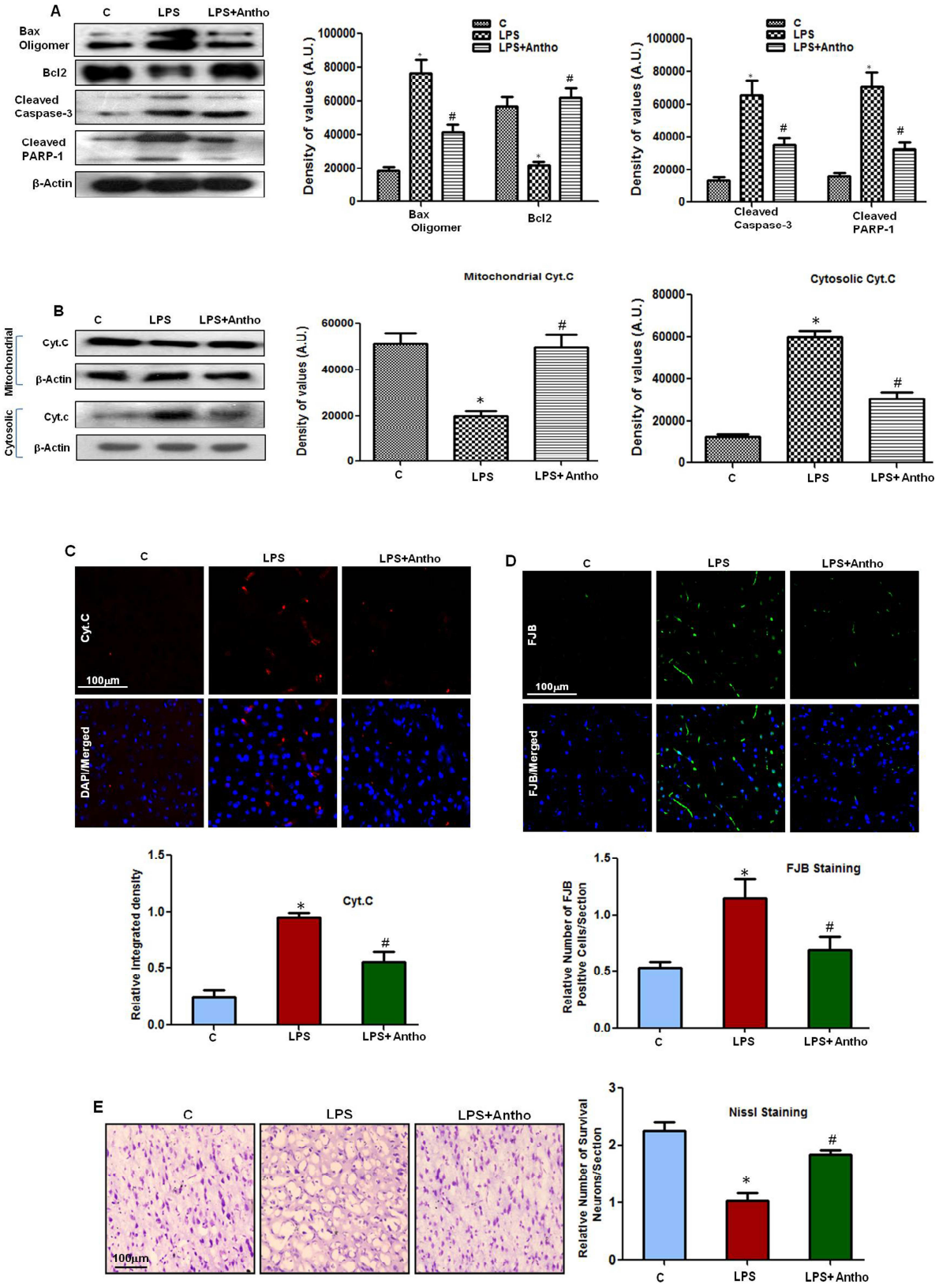
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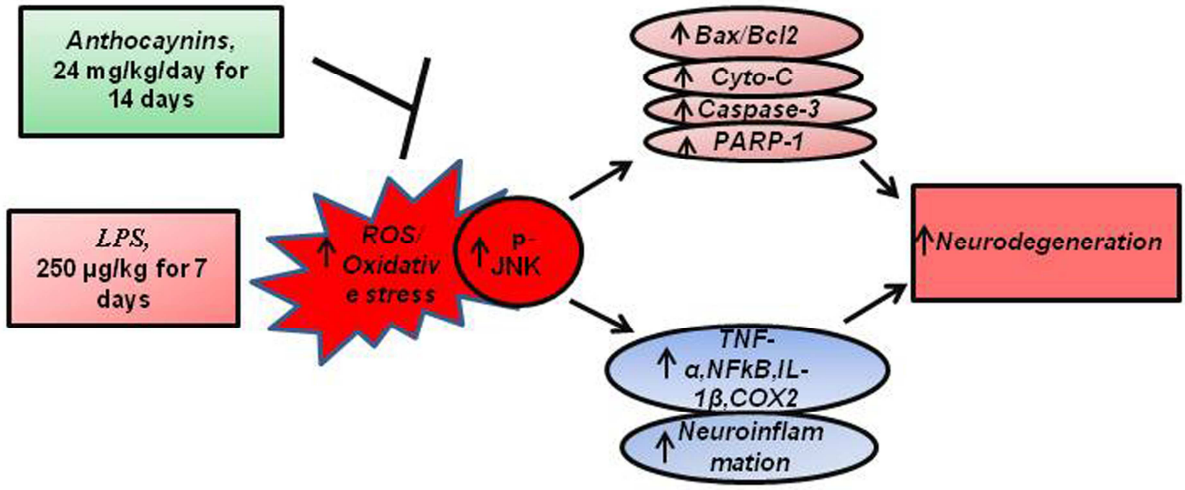

Highlights

- Anthocaynains (24 mg/kg, i.p. 14days) reduce oxidative stress in the LPS-injected mice
- Anthocyanins ameliorates activated microglia and astrocytes in the LPS-injected mice
- Anthocyanins attenuates various inflammatory mediators in the LPS-injected mice
- Anthocyanins prevents apoptosis and neurodegeneration in the LPS-injected mice