

Protective effect of picroliv against lipopolysaccharide-induced cognitive dysfunction and neuroinflammation by attenuating TLR4/NF- κ B pathway

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Folia Neuropathol 2018; 56 (4): 337-345

DOI: <https://doi.org/10.5114/fn.2018.80867>

Abstract

Introduction: Present investigation determines the beneficial effect of picroliv against lipopolysaccharide (LPS)-induced neuronal inflammation and injury.

Material and methods: Neuronal injury was induced by LPS 250 μ g/kg, i.p. for the period of one week, and picroliv 12.5 and 25 mg/kg was given i.p. 30 min prior to the administration of LPS for the duration of 12 days. The effect of picroliv was determined on the cognitive function by Morris water maze (MWM). Mediators of inflammation were estimated by using enzyme-linked immunosorbent assay (ELISA) and western blot, reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical analysis was done to determine the expressions of several proteins.

Results: Data of the study reveal that picroliv ameliorates the reduced memory impairment and cognitive dysfunction in LPS-induced mice. Moreover, expressions of inflammatory protein and β -amyloid protein and level of inflammatory mediators were found to be reduced in the picroliv-treated group as compared to the negative control group. Data of RT-PCR reveal that the gene of Toll-like receptor 4 (TLR-4), α -synuclein, neurotrophic factor (BDNF) and interleukin-1 β (IL-1 β) protein were also decreased in the picroliv-treated group as compared to the negative control group. In addition picroliv attenuates the altered level of nuclear factor- κ B (p-NF- κ B), amyloid- β (A β), α -synuclein and glial fibrillary acidic protein (GFAP) positive cells in the brain of LPS-induced mice.

Conclusions: The report concludes that picroliv protects the neuroinflammation and injury in LPS-induced mice by regulating the inflammatory pathway.

Key words: neuroinflammation, picroliv, β -amyloid, apoptosis, TLR4, NF- κ B.

Introduction

Neurodegenerative diseases like Huntington's, Parkinson's and Alzheimer's diseases are commonly associated with neuroinflammation [7]. Endotoxin isolated from the bacterial membrane is known as

lipopolysaccharide (LPS) [11]. It activates the immune system and also stimulates the process of inflammation in the brain. The literature reveals that the process of inflammation was induced by up-regulating the inflammatory mediators such as nuclear factor- κ B (NF- κ B), interleukin (IL)-1, IL-6, tumor necrosis factor- α

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(TNF- α), cyclooxygenase-2 (COX-2) and prostaglandin-E2 (PGE2) [13]. Moreover, initiation of response of the immune system and sensitivity to body pathogens was done with the help of Toll-like receptors (TLRs) [9]. Reports reveal that lipopolysaccharide (LPS) induces neurodegeneration and neuroinflammation by activating the NF- κ B/TLR4 pathway [2]. It is well documented that LPS induces neuroinflammation for the long-term period by a single dose of administration in mice [5]. Moreover, LPS administration induces the activity of β -secretase which further enhances the production of β -amyloid [21]. The increased production of β -amyloid has a role in the degeneration of neurons and it is one element of the pathogenesis of Alzheimer's disease [10]. Conventional treatment available for the management of neurodegeneration has several limitations and thus a therapy for it needs to be developed.

In the last few decades, alternative medicines have shown promising effects in the management of neurodegeneration. *Picrorhiza kurroa* is traditionally used as a hepatoprotective, cardioprotective, antioxidant, anti-diabetic and anti-cancer agent in Ayurveda [15]. Picroliv is chemically an iridoid glucoside isolated from *Picrorhiza kurroa* (*Scrophulariaceae*) [17]. Moreover picroliv is reported for its potential antioxidant, hepatotoxicity, immunomodulatory, anti-allergic, anti-ulcer and anti-inflammatory activity [4,8,12,22]. In addition, picroliv inhibits the activation of NF- κ B and thereby shows anti-inflammatory activity.

Material and methods

Animals

Albino mice (age: 3 weeks; weight: 17-23 g) were used in the study. All the animals were procured from the Experimental Animal Centre of the Academy of Military Medical Sciences, China. Standard guidelines (humidity: $60 \pm 5\%$; temperature: $24 \pm 3^\circ\text{C}$) were used to store the animals for 12 hr light and dark cycle. Protocols of the investigation were approved by the Institutional Animal Care and Use Committee of People's Hospital of Hangzhou Medical College, China (IACUC/PH-HMC/ZPPH/2017/15).

Chemicals

Picroliv was kindly gifted by the Shanghai Institute for Biological Sciences, China. ELISA kits for IL-6, IL-1 β , COX-2 and TNF- α were purchased from Ebioscience, USA. NF- κ B, iNOS and TLR-4 antibodies were purchased from Thermo Fisher Scientific, USA,

and CD14, APP, A β and β -actin antibodies were procured from Abcam, California, USA.

Experiments

All the animals were divided into four different groups including the control group, negative control group which receives LPS 250 $\mu\text{g}/\text{kg}$, *i.p.* for the period of one week, and picroliv 12.5 and 25 mg/kg was given *i.p.* 30 min prior to the administration of LPS for the duration of 12 days. Later, animals were sacrificed with anesthesia and the brain was isolated carefully.

Determination of behavioral changes

Morris water maze apparatus was used to determine the behavioral changes. Water maze having height and diameter of 40 and 100 cm, respectively, and the platform was of the depth of 15.5 cm. Four quadrants were created in the apparatus with a thread and in one of the quadrant platforms was placed in such a way that it was not able to be visualized. Observation of swimming behavior was done for the duration of six days continuously and escape latency was determined. Later, by escaping the platform, spatial memory of each mouse was determined and the effect of picroliv was also estimated in the LPS-induced cognitive dysfunction mice.

Assessment of proinflammatory mediators

Hippocampus was dissected from the isolated brain samples and RIPA buffer was used to prepare the samples of hippocampus. The tissue sample was centrifuged for the duration of 5 min at 12000 rpm. Further, ELISA kits were used to determine the concentration of IL-6, IL-1 β , COX-2 and TNF- α in the tissue homogenate as per the direction given by the manufacturer of kits.

Western blot assay

All the animals were sacrificed and hippocampi and cortices were dissected from the isolated brain samples. Later pro-prep extraction solution was used to homogenize the brain tissue to collect the protein samples. Novex 4-12% Bis-Tris Plus gels were used to separate the collected protein and transfer the same into polyvinylidene difluoride (PVDF) membrane. Membrane was further incubated with primary antibodies such as

NF- κ B (dilution of 1 : 500), iNOS (dilution of 1 : 1000), TLR-4 (dilution of 1 : 500), CD14 (dilution of 1 : 1000), APP (dilution of 1 : 500), A β (dilution of 1 : 1000) and β -actin (dilution of 1 : 1000). Thereafter, secondary antibodies were incubated with the membrane. Ez West Lumi western blotting detection reagent was used to visualize the immunocomplex and Sigma Gel software was used to estimate the optical density blots.

mTLR-4 Forward: T AG AACTGTGCCATGCCT
Reverse: CGCTGGTGCCTTCGCTATGGT
mIL-1 β Forward: CTTCGTCTATTGCCTGTCCT
Reverse: GTCTTCTCAGGATATCACC
mBDNF Forward: GCTGCTTGGCCGAAGAG
Reverse: CCTGGTAGTCTGCGTCTCTT
mGAPDH Forward: GGCATATGTGAAGCAGACGC
Reverse: GGAACACTGCTGGTAGGAGAG

RT-PCR

Separated hippocampus from isolated brain was used to isolate the RNA by Trizol Reagent. RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) was used to reversely transcribe the RNA. Primers mentioned below were mixed with RT 2 SYBR Green Master to determine the gene expression by Quantitative SYBR Green PCR assays.

Gene	Sequence
α -synuclein	Forward: ATAGGCTGGTTTGACATCC Reverse: AGAGTACTCTGGGAGGTTGG

Immunohistochemical analysis

Brain was isolated from all the mice and tissues were fixed with paraformaldehyde (4%) and later tissues were sectioned coronally into thickness of 14 μ m by using microtome. The sections of brain tissues were stained with primary antibodies such as p-NF- κ B, A β , α -synuclein and GFAP overnight. Secondary antibodies were further incubated with the brain tissues and fluorescent mounting medium was used to mount the tissue. Confocal laser scanning microscope was used to take the images and observe the changes in the brain tissues.

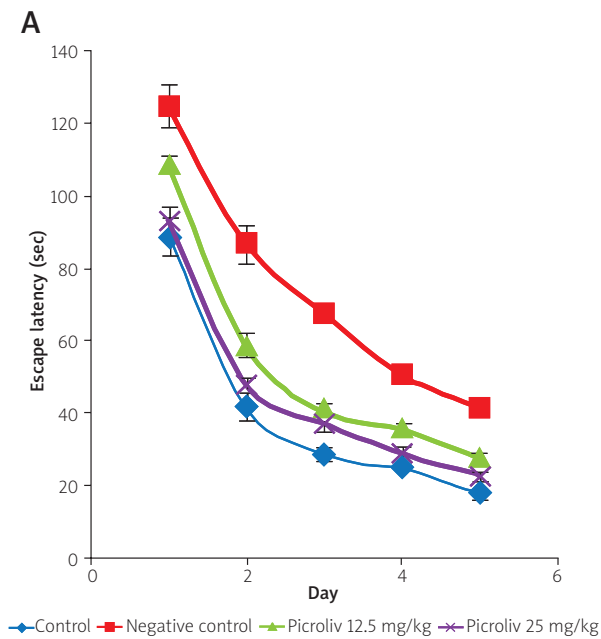
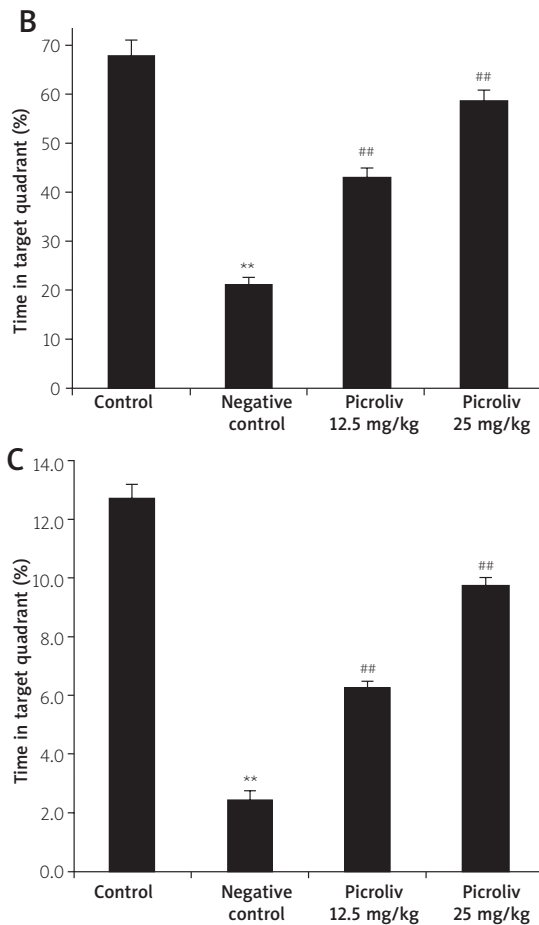


Fig. 1. Picroliv attenuates the cognitive function such as spatial memory in lipopolysaccharide-induced neuronal injured mice model by using Morris water maze. Mean \pm SEM ($n = 10$); ** $p < 0.01$ as compared to the control group; ## $p < 0.01$ as compared to the negative control group.



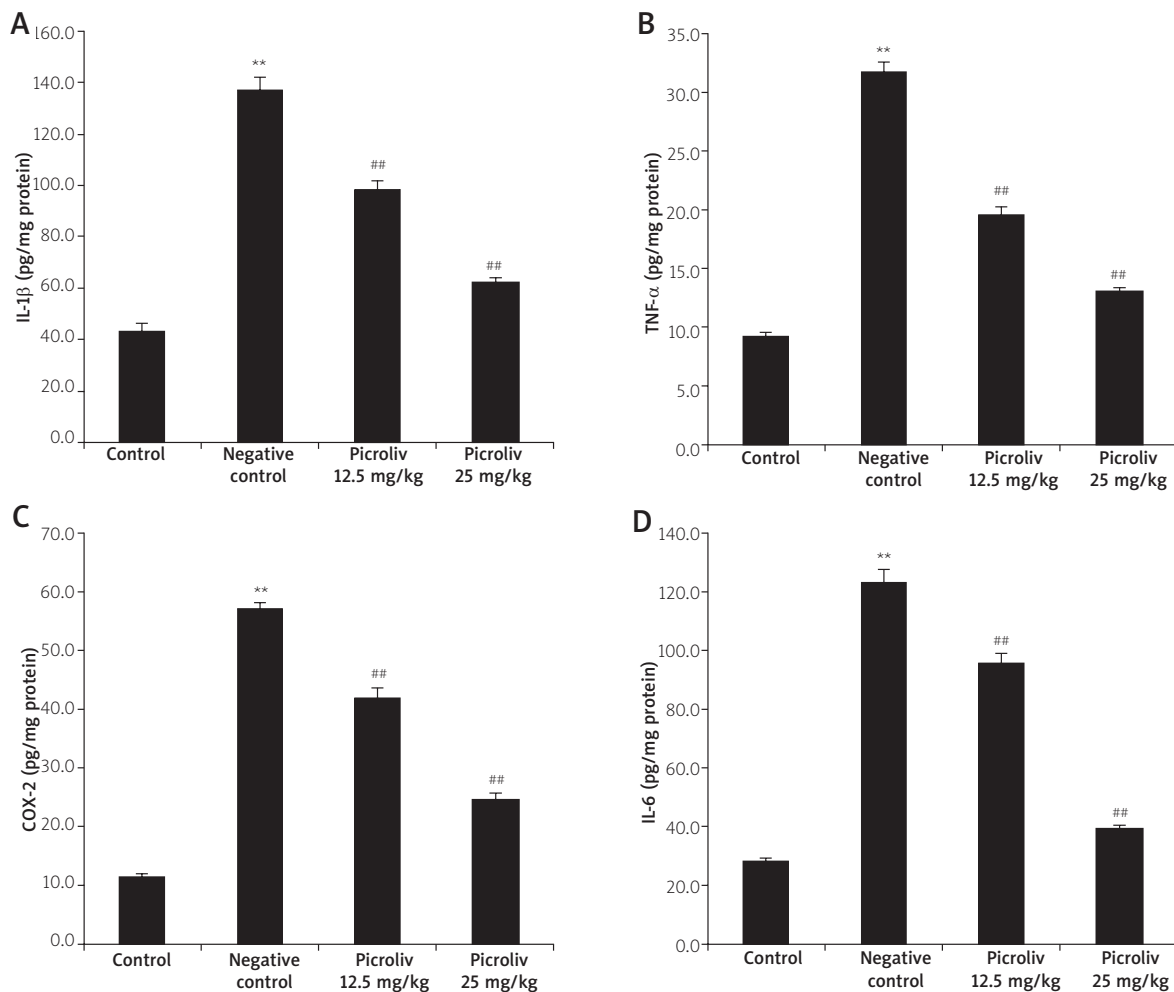


Fig. 2. Picroliv attenuates the level of proinflammatory mediators such as IL-6, IL-1 β , COX-2 and TNF- α in the lipopolysaccharide-induced neuronal injured mice model. Mean \pm SEM ($n = 10$); ** $p < 0.01$ as compared to the control group; ## $p < 0.01$ as compared to the negative control group.

Statistical analysis

All data were expressed as mean \pm SEM ($n = 10$). The statistical analysis was performed using one way ANOVA. Post-hoc comparison of means was carried out by Dunnett’s post hoc test (Gradpad Prism 6.1., CA, USA) multiple comparisons. The level of statistical significance was set at $p < 0.05$.

Results

Picroliv attenuates the cognitive function

Assessment of memory impairment in picroliv- and LPS-treated group is shown in Figure 1. The percentage of crossing and time spent in the target quadrant

was found to be less and escape latency was higher in the negative control group than in the control group. Of note, picroliv was reported to improve the memory as the percentage of crossing and time spent in the target quadrant was enhanced and escape latency was reduced in the picroliv group as compared to the negative control group.

Picroliv attenuates the level of proinflammatory mediators

Picroliv inhibits the parameters responsible for inflammation in LPS-induced mice as shown in Figure 2. There was an increase in the level of pro-inflammatory mediators such as IL-6 (123.3 pg/mg), IL-1 β (137.2 pg/mg), COX-2 (57.2 pg/mg) and TNF- α

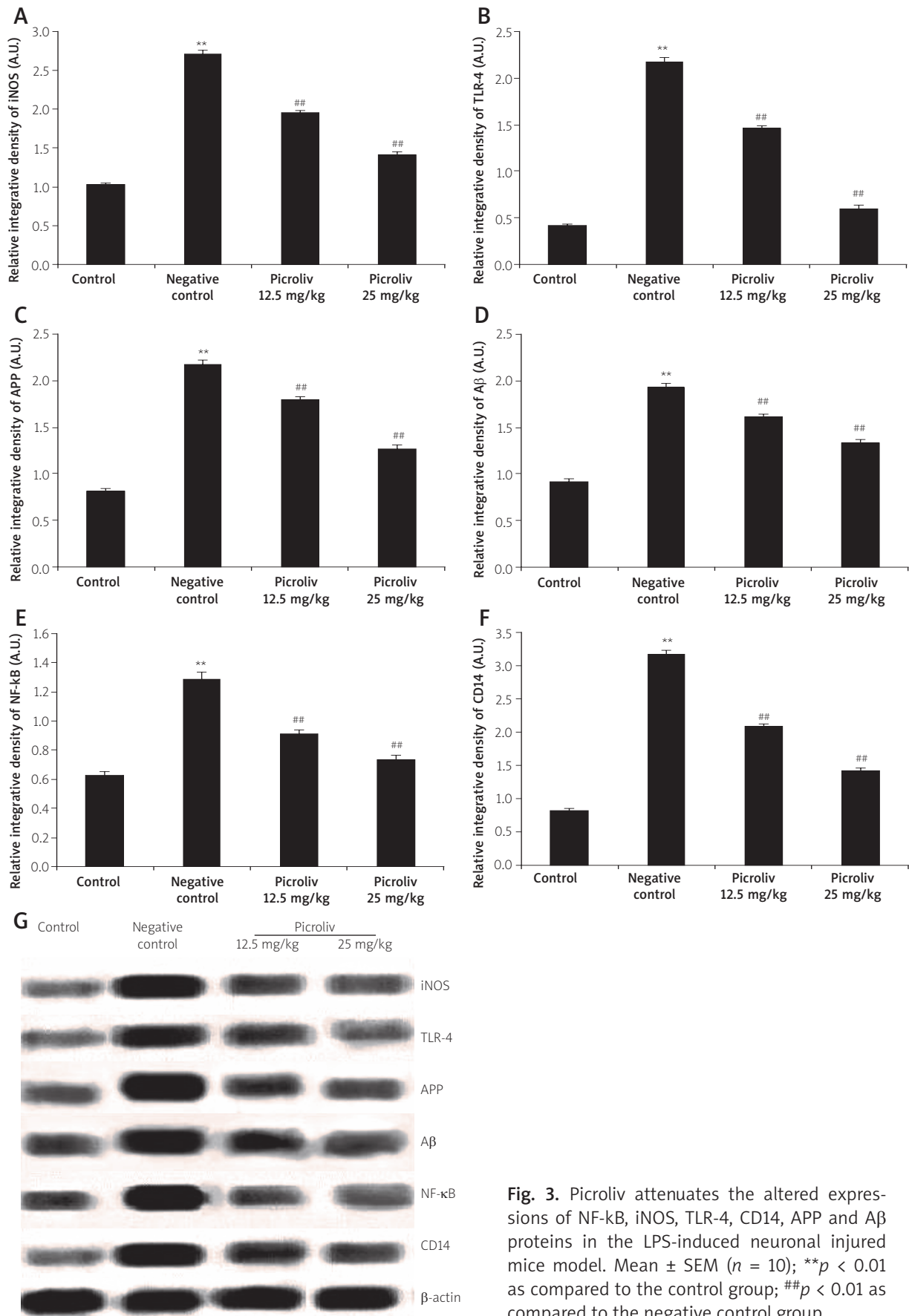


Fig. 3. Picroliv attenuates the altered expressions of NF- κ B, iNOS, TLR-4, CD14, APP and A β proteins in the LPS-induced neuronal injured mice model. Mean \pm SEM ($n = 10$); ^{**} $p < 0.01$ as compared to the control group; ^{##} $p < 0.01$ as compared to the negative control group.

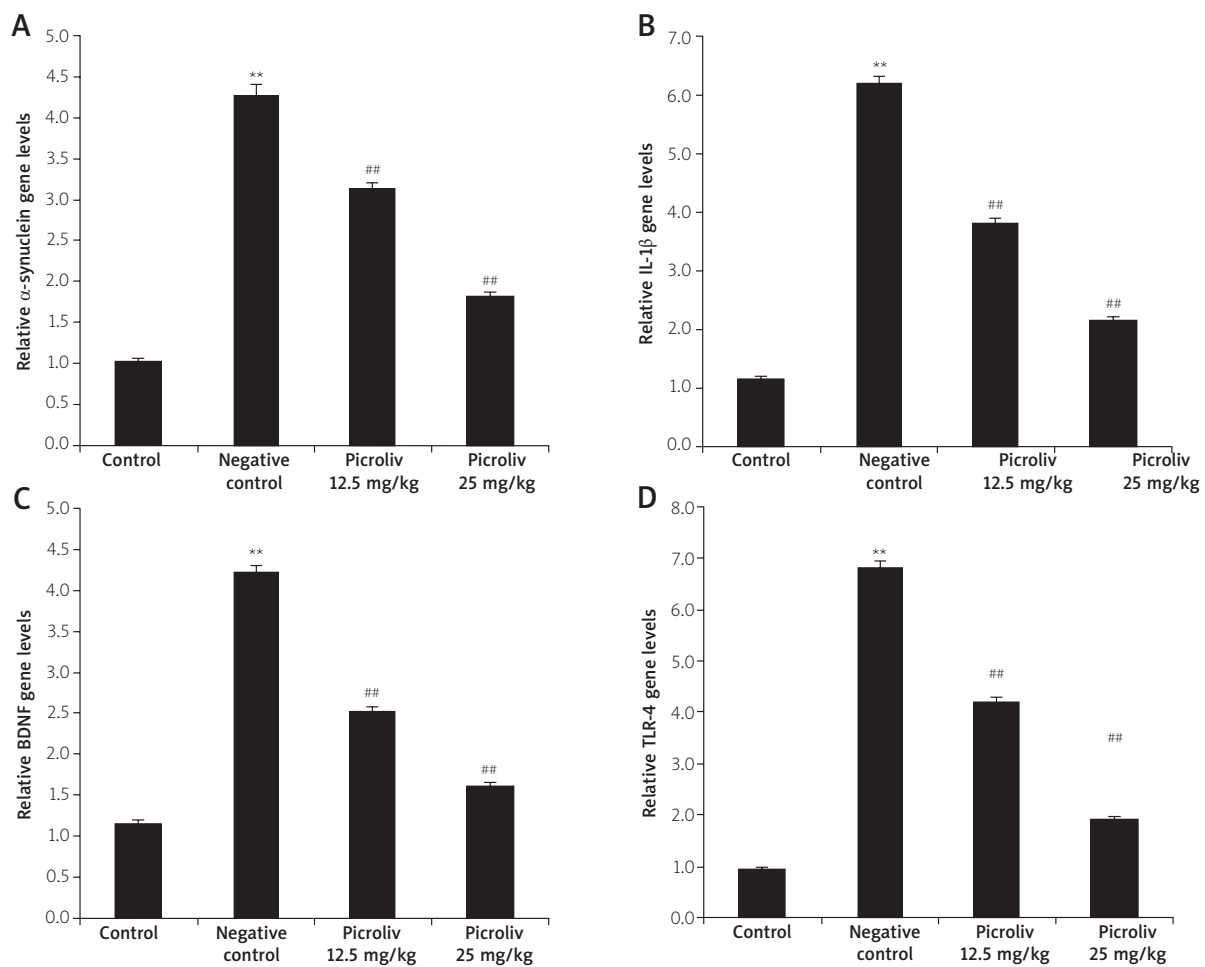


Fig. 4. Picroliv attenuates the altered m-RNA expressions of TLR-4, α -synuclein, BDNF and IL-1 β proteins in the LPS-induced neuronal injured mice model. Mean \pm SEM ($n = 10$); ** $p < 0.01$ as compared to the control group; ## $p < 0.01$ as compared to the negative control group.

(31.8 pg/mg) in the negative control group as compared to the control group. Picroliv treatment results in a significant decrease in the level of mediators of inflammation as compared to the negative control group in a dose-dependent manner.

Picroliv attenuates the expressions of NF-kB, iNOS, TLR-4, CD14, APP and A β

The expression of NF-kB, iNOS, TLR-4, CD14, APP and A β protein was determined in the tissue homogenate of LPS-induced mice as shown in Figure 3. There was a significant increase in the expression of NF-kB, iNOS, TLR-4, CD14, APP and A β protein in the brain tissue of the negative control group as compared to the control group. However, treatment with picroliv reduces the expression of NF-kB, iNOS,

TLR-4, CD14, APP and A β protein in the tissue homogenate as compared to the negative control group.

Picroliv attenuates the m-RNA expressions of TLR-4, α -synuclein, BDNF and IL-1 β

The effect of picroliv on the gene level of TLR-4, α -synuclein, BDNF and IL-1 β is shown in Figure 4. m-RNA expressions of TLR-4, α -synuclein, BDNF and IL-1 β were enhanced significantly ($p < 0.01$) in the negative control group as compared to the control group. It was assessed that the gene level of TLR-4, α -synuclein, BDNF and IL-1 β protein is reduced in the picroliv-treated group as compared to the negative control group.

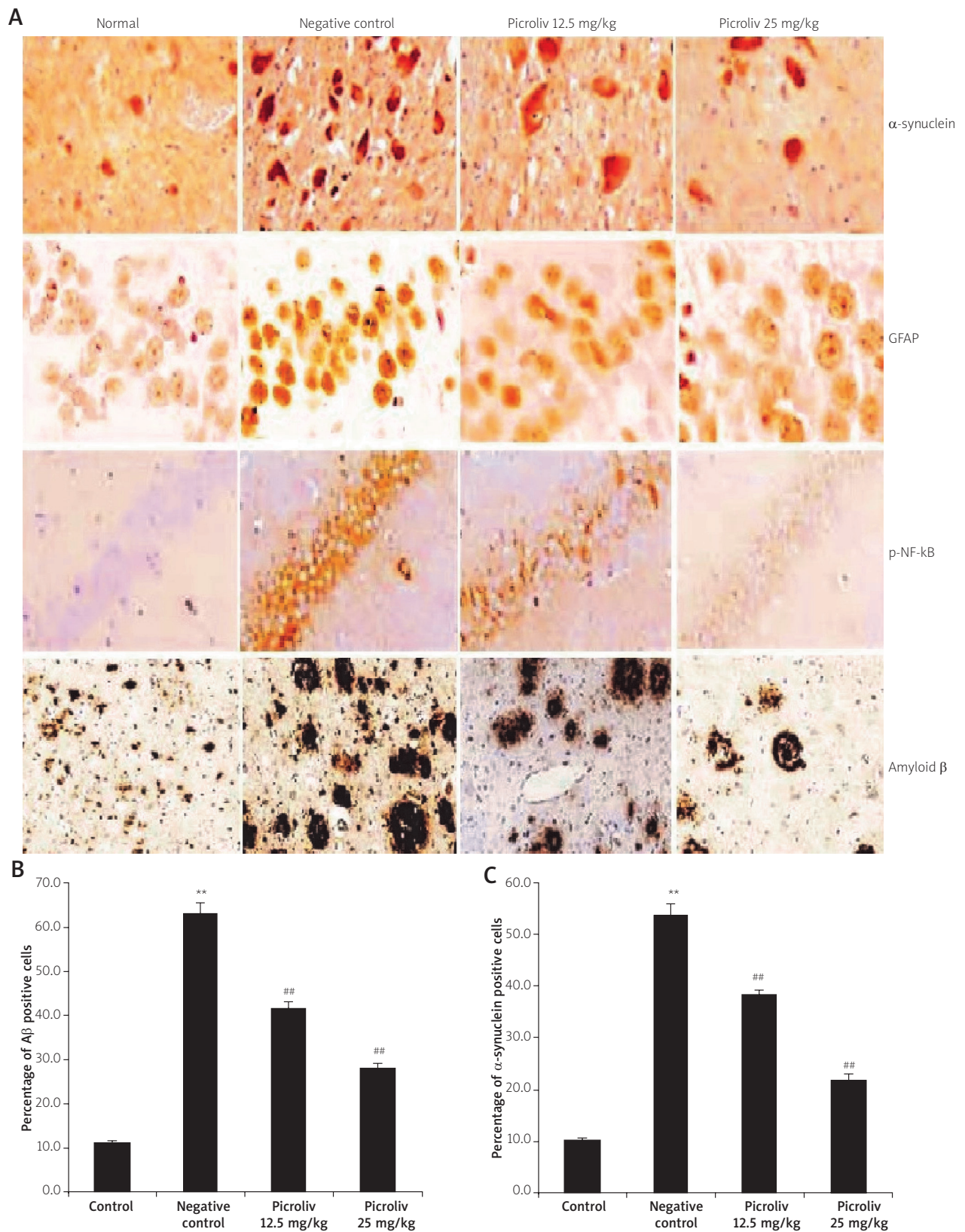


Fig. 5. Picroliv attenuates the number of A β (A), α -synuclein (B), GFAP (C) and p-NF-kB (D) positive cells in the LPS-induced neuronal injured mice model. Mean \pm SEM ($n = 10$); ** $p < 0.01$ as compared to the control group; ## $p < 0.01$ as compared to the negative control group.

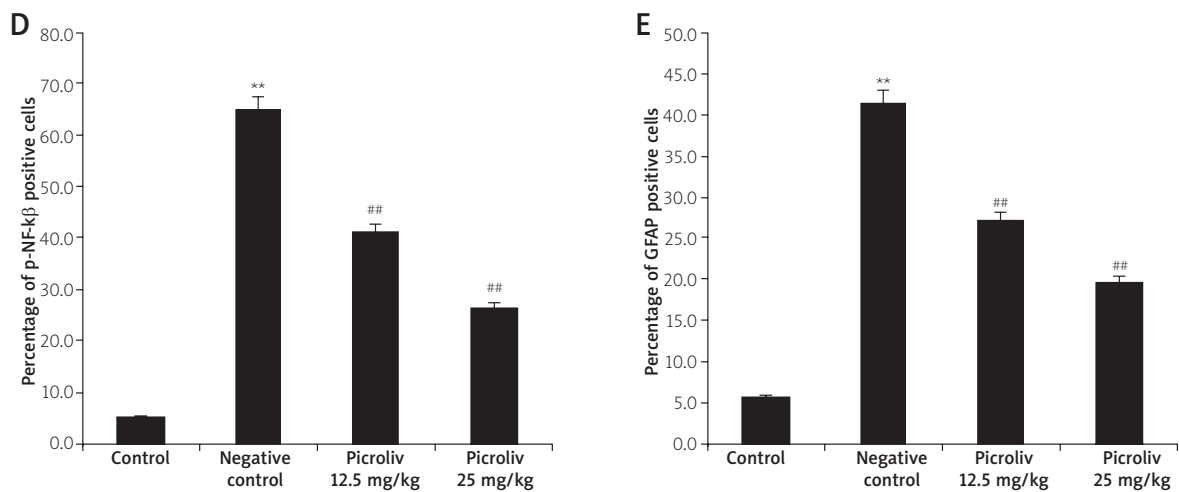


Fig. 5. Cont.

Picroliv attenuates the number of A β , α -synuclein, GFAP and p-NF-kB positive cells

Immunohistochemical analysis was done to determine the effect of picroliv on the number of A β , α -synuclein, GFAP and p-NF-kB positive cells (Fig. 5). In the negative control group, the number of A β , α -synuclein, GFAP and p-NF-kB positive cells was found to be higher than in the control group. However, treatment with picroliv significantly decreases ($p < 0.01$) the number of A β , α -synuclein, GFAP and p-NF-kB positive cells as compared to the negative control group.

Discussion

Neuroinflammation involved in the development of neuronal injury induced by LPS and picroliv has been reported for its strong anti-inflammatory properties [20]. Present investigation evaluates the protective effect of picroliv on neuronal injury and also postulates the possible mechanism of action. Neuronal injury was induced by LPS and the effect of picroliv was determined on the cognitive function by MWM. Mediators of inflammation were estimated by using ELISA and western blot, RT-PCR and immunohistochemical analysis was done to determine the expressions of several proteins.

Neurodegenerative diseases are commonly characterized with cognitive dysfunction and impairment of memory [3]. Data of presented investigation report that LPS induction results in cognitive dysfunction and treatment with picroliv ameliorates

the cognitive function in LPS-induced mice. The literature reported that the process of inflammation induced due to activation of receptors like CD14 and TLR4 [20]. LPS was reported to trigger the immune response and inflammation by activating the CD14 and TLR4 receptors [18]. Moreover inflammatory mediators such as iNOS, IL-6, IL-1 β and COX-2 induce the release by activating NF-kB signaling pathway due to stimulation of CD14 and TLR4 receptors [6]. The result of the study suggested that picroliv ameliorates the enhanced level of proinflammatory mediators in LPS-induced mice. Moreover the expression of CD14, TLR-4 and NF-kB proteins is reduced in the brain tissues of the picroliv-treated group as compared to the negative control group.

In addition, progression of neuroinflammation occurs due to activation of microglia and astrocytes [16]. Reported data reveal that synthesis of neurotrophic factor (BDNF) is enhanced in astrocytes and BDNF is responsible for the growth of neurons. Data of investigations suggested that picroliv reduces the expression of BDNF in LPS-induced mice.

α -Synuclein, GFAP and β -amyloid are the neuro peptides involved in the normal functioning of brain cells and are also found to have a role in the development of neurodegenerative disorders [19]. α -Synuclein was reported to induce the expressions of inflammatory protein like TLR-4. Deposition of β -amyloid is enhanced by TLR-4 and thereby causes activation of microglia and neuroinflammation [14]. Data of the investigation suggested that picroliv significantly reduces the expression of TLR-4 and β -am-

ylويد in the neuronal tissues as compared to the negative control group. Moreover, results of immunohistochemical analysis and RT-PCR suggest that the number of β -amyloid, α -synuclein and TLR-4 is reduced in the picroliv-treated group as compared to the negative control group.

Conclusions

In conclusion, data of this study suggest that picroliv ameliorates the neuronal injury and protects the cognitive function in LPS-induced neuronal injured mice. Moreover, picroliv reduces the neuroinflammation by regulating NF- κ B/TLR-4 inflammatory pathway and inhibits neuronal degeneration by reducing the deposition of β -amyloid.

Acknowledgments

Authors of the manuscript are thankful to Zhejiang TCM youth talent fund (2018ZQ006) for providing the support to carry out the presented work.

Disclosure

The authors declare no conflict of interest.

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