Piperine Improves Experimental Autoimmune Encephalomyelitis (EAE) in Lewis Rats through its Neuroprotective, Anti-inflammatory and Anti-Oxidant Effects

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Research Article

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Abstract

Inflammation, demyelination, glial activation, and oxidative damage are the most pathological hallmarks of multiple sclerosis (MS). Piperine, a main bioactive alkaloid of black pepper, possesses antioxidant, anti-inflammatory and neuroprotective properties whose therapeutic potential has been less studied in the experimental autoimmune encephalomyelitis (EAE) models. In this study, the efficiency of peprine on progression of EAE model and myelin repair mechanisms was investigated. EAE was induced in female Lewis rats and piperine and its vehicle were daily administrated intraperitoneally from day 8 to 29 post immunization. We found that peperine alleviated neurological deficits and EAE disease progression. Luxol fast blue and H&E staining and immuno-staining of lumbar spinal cord cross sections confirmed that piperine significantly reduced the extent of demyelination, inflammation and immune cell infiltration and inhibited microglia and astrocyte activation. Gene expression analysis in lumbar spinal cord showed that piperine treatment decreased the level of pro-inflammatory cytokines (TNF-α, IL-1β) and iNOS and enhanced IL-10, Nrf-2, HO-1and MBP expressions. Piperine supplementation also enhanced the total antioxidant capacity (FRAP) and reduced the level of oxidative stress marker (MDA) in the CNS of EAE rats. Finally, we found that piperine has anti-apoptotic and neuroprotective effect in EAE through reducing caspase-3 (apoptosis marker) and enhancing BDNF and NeuN expressing cells. This study strongly indicates that piperine has a beneficial effect on the EAE progression and could be considered as a potential therapeutic target for MS treatment. Upcoming clinical trials will provide the deep understanding of piperine's role for the treatment of the MS.

Introduction

Multiple sclerosis (MS) is the typical inflammatory demyelinating disease of the central nervous system (CNS) and a major cause of neurological disability in young adults worldwide [1]. Experimental autoimmune encephalomyelitis (EAE) is a Th-1 mediated inflammatory demyelinating disease of the CNS that best represents the pathology of MS [2, 3]. Inflammation, demyelination, gliosis (astrocytes and microglial activation), oligodendrocyte death and axonal loss are the most pathological hallmarks of MS and EAE [4, 5]. Glial activation is implicated in EAE and MS disease development and progression [6, 7]. The reactivated microglia and astrocytes release inflammatory factors such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), IL-17, IL-6, and reactive oxygen species (ROS), which lead to the destruction of myelin, oligodendrocytes and axons in MS and EAE [8]. It has been indicated that an inflammatory reaction against myelin can lead to acute neuronal cell loss by apoptosis [9]. Oxidative stress and NO derived from inducible nitric oxide synthase (iNOS) is thought to play an important role in the pathobiology of EAE and MS diseases [10, 11].

Despite extensive research to develop effective drugs for MS treatment to reduce progression of neurological disability, there are no effective therapies to halt progression of disease. The current therapeutic strategy is based on immunomodulatory drugs like interferon beta (IFN-β), glatiramer acetate, natalizumab, fingolimod, etc. [12, 10]. The therapeutic efficacy of these compounds is limited by significant side effects and the high variability of individual drug response probably because of disease

heterogeneity [13–15]. These limitations have prompted the search for new and safer treatment strategies. Previous studies have widely used the anti-inflammatory or antioxidant agents as promising medications in different neurodegenerative diseases management [16–18]. Moreover, natural products with antioxidant and anti-inflammatory properties have been considered as the potential therapeutic target in MS treatment [19, 20, 13, 21, 22].

Piperine is the main bioactive alkaloid ingredient of black pepper which has a wide range of pharmacological potencies including: antioxidant [23], anti-inflammatory [24, 25], anti-apoptotic [17], antidepressant [26] and anticonvulsant [27] effect. Researchers have demonstrated that piperine has free radical-quenching effects and reduces oxidative damage [28]. It has also been proved that piperine reduces inflammation by suppressing iNOS activity and activating the nuclear factor erythroid 2-related factor 2 (Nrf-2) / heme oxygenase-1 (HO-1) [29, 30]. Recent study revealed that piperine decreased T-cell proliferation via dihydrooratate dehydrogenase inhibition, which provides a therapeutic strategy for MS disease [31]. Furthermore, piperine was effective in various animal models of neurological diseases such as Alzheimer [16, 32], Parkinson [33, 34], epilepsy [35] and local model of demyelination [30].

The present study was designed to investigate the therapeutic efficacy of piperine on EAE symptoms and myelin repair. We first evaluated the effect of piperine on development and progression of EAE model using daily clinical scores monitoring. Next, we examined the demyelination and remyelination processes by LFB and MBP staining. H&E staining and immunostaining against CD45 were performed to assess inflammatory scores and immune cell infiltration. Furthermore, we evaluated the effect of piperine on expression level of inflammatory (TNF-α, IL-1β), anti-inflammatory (IL-10) and oxidative stress (iNOS, Nrf2, HO-1) mediators by qPCR. The total antioxidant capacity and oxidative stress status of the CNS was analyzed by FRAP and TBARS assays. Moreover, the microglia and astrocyte activation was confirmed using immune-labeling against Iba1 (as a microglia marker) and GFAP (as an astrocyte marker). The neuroprotective role of piperine was also evaluated by gene expression analysis of MBP and BDNF. Finally, we assessed the anti-apoptotic and neuroprotection role of piperine using immunostaining against Caspase-3 (marker of apoptosis) and NeuN (mature neuron marker).

Material And Methods

Animals

Adult female Lewis rats weighing 180-200g were obtained from Daru-Pakhsh pharmaceutical Company (Tehran, Iran). All rats were housed under standard conditions with a 12h light and 12h dark cycle and allowed to free access to pellet and water. The room was maintained with appropriate temperature (22 ± 2°C) and humidity (60%). Guinea pig was purchased from Razi institute (Tehran, Iran) to prepare the Guinea pig spinal cord homogenate (GPSCH) for EAE induction. All experimental procedures of this study were conducted according to the international guidelines for care and use of laboratory animals and approved by the ethical committee of Babol University of Medical Sciences (Ethic cod number: IR.MUBABOL.HRI.REC.1398.315).

Chemicals

Piperine (94.62.2, > 98% purity), Complete Freund's adjuvant (F5881) and Pertussis toxin (P7208) were purchased from Sigma-Aldrich (St.Louis, Mo, USA). Antibodies including: anti-myelin basic protein (MBP) (MAB386, Millipore), anti-glial fibrillary acidic protein (GFAP) (Z0334-Dako), anti-Iba1 (019-19741-Wako), anti-CD45 (14-0451-82 -Invitrogen), anti-Caspase-3 (9661-Cell signaling) and anti-NeuN (MAB-377-Millipore) were used for immunohistochemistry assays. All information related to primary and secondary antibodies are available in Table S1. Piperine dose was selected based on our previous study [30]. Piperine was freshly prepared by dissolving in Tween 80 and diluting in 0.9% saline (final concentration of solvent 0.2%).

Experimental groups

Thirty two Lewis rats were randomly divided into four equal groups (n = 8) as follows: (1) control group/untreated rats, (2) EAE group/immunized rats (3): Vehicle group/immunized rats that daily received i.p. injection of 200µl of tween 80 0.2% as piperine solvent, 8 days post immunization until the day 29 and (4) Piperine treated group/immunized rats that daily received i.p. injection of 200µl of piperine (5mg/kg/day), 8 days post immunization until the day 29.

EAE Induction and interventions

For EAE induction, guinea pig spinal cord homogenate (GPSCH) was prepared as previously described [21, 36]. Each gram of spinal cord was homogenated in 1 ml of distilled water. GPSCH was mixed with complete Freund's adjuvant (CFA) (1:1, v/v), to obtain the immunization emulsion. CFA contained 500 mg/ml heat inactivated Mycobacterium tuberculosis (M.t., strain H37 RA, Difco, Germany). Rats were immunized with subcutaneous injections of 0.4 ml immunization emulsion at the base of their tail (0.2 ml each side) on day 0. Rats received intraperitoneal injections (i.p.) of 250 ng pertussis toxin in 0.2 ml of distilled water, on the day of immunization and 48 h later. Animals were daily monitored for clinical signs of EAE and scored as previously described [21] as follows: score 0, no symptoms; score 1, complete tail paralysis; score 2, mild paresis of hind limbs; score 3, complete paralysis of one hind limb; score 4, bilateral hind limb paralysis; score 5, complete paralysis (tetraplegia), moribund state, or death. We gave the average score if the rats were at borderlines of two different scores. Piperine treatment was started at day 8 post immunization with dose 5mg/kg/day during the experimental procedure (29 days post immunization). Animal in vehicle group received the same amount of piperine solvent in the same way.

Quantitative Real-Time PCR (q-PCR)

Lumbar spinal cord of rats was dissected on ice, snap-frozen in liquid nitrogen and stored at -80^{o} C until processed for RNA extraction. Total RNA was isolated using RNA extraction kit (FABRK001, Yektatajhiz Azma, and Tehran-Iran) following the manufacturers instruction. RNA concentration was evaluated by nanodrop spectrophotometer (Thermo scientific, Wilmington, DE USA), and two micrograms of RNA were run on agarose gel to verify the integrity of isolated RNA. We synthesized cDNA from 1µg of total RNA with a reverse transcription reagent kit as described by the manufacturer's protocol (YT4500, Yektatajhiz

Azma kit, Tehran, Iran). The cDNA pool was subjected to quantitative real-time PCR (q-PCR) by using a Cyber®Green Master Mix (Qiagen, Germany, Cat No. IZ.30 – 5). Rotor-Gene® Q real Time PCR System device (Qiagen GmbH, Hilden, Germany) was used to perform q-PCR. The selective forward and reverse primers for the GAPDH gene as reference gene and tumor necrosis factorα (TNF-α), interleukin1β (IL-1β), interleukin 10 (IL-10), induced nitric oxide synthase (iNOS), Heme oxygenase1(HO-1), Nuclear factor erythroid 2 related factor 2(Nrf2), brain derived neurotrophic factor (BDNF) and myelin basic protein (MBP) as target genes were designed (Fazapajooh, Tehran, Iran) and their sequence is shown in Table 1. Quantitative real-time PCR reactions were performed in duplicate in three independent experiments. The q-PCR reaction mixture consists of two microliter of cDNA, 10µl SYBR™ green master mix and 1 µl of each primer (5µM) in a total volume of 20µl mixtures of each reaction. Forty cycles (94^{o}C for 30s, 60 ^{o} C for 45s and 72 ^{o} C for 45s) were exerted in PCR reaction. An optimal annealing temperature for each primer was mentioned in Table 2. The cycle threshold (CT) of target genes was normalized to GAPDH as the reference gene to minimize the effect of sample variations and calculate the relative expression level of the target genes by the 2-delta-delta CT method [37].

Table 1
The primers sequences used in the qPCR.

Sequence (5’-3’) Forward Reverse	Accession ID	Gene Symbol
ACGGCAAGTTCAACGGCACAG	NM _017008.4F	**GAPDH**
GACATACTCAGCACCAGCATCACC	NM _017008.4R	
GGACCCAAGCACCTTCTTTT	NM_03512.2F	**IL-1β**
AGACAGCACGAGGCATTTT	NM_03512.2R	
ACCACGCTCTTCTGTCTACTG	NM_012675.3F	**TNF-α**
CTTGGTGGTTTGCTACGAC	NM_012675.3R	
CACTGCTATGTTGCCTGCTC	NM_012854.2F	**IL-10**
TGTCCAGCTGGTCCTTCTTT	NM_012854.2R	
TGGTGAGGGGACTGGACTTT	NM_012611.3F	**iNOS**
ATCCTGTGTTGTTGGGCTGG	NM_012611.3R	
GGGAAGGCTTTAAGCTGGTGA	NM_012580.2F	**Hmox-1**
GTGGGGCATAGACTGGGTTC	NM_012580.2R	
GTCCCAGCAGGACATGGATT	NM_031789.2F	**Nrf2**
GTTTGGGAATGTGGGCAACC	NM_031789.2R	
GTCGCACGGTCCCCATTG	NM_001270630.1F	**BDNF**
ACCTGGTGGAACTCAGGGT	NM_001270630.1R	
CTATAAATCGGCTCACAAGG	NM_001020462.1F	**MBP**
AGGCGGTTATATTAAGAAGC	NM_001020462.1R	

Table 2
Optimal annealing temperatures for each primer.

Primer Name	Annealing Temp
GAPDH	**60**
IL-1β	**63**
TNF-α	**60**
IL-10	**62**
iNOS	**62**
Hmox-1	**63**
Nrf2	**63**
BDNF	**60**
MBP	**60**

Biochemical assays

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay is a simple, quick and inexpensive method for measuring the total antioxidant activity of a sample [38, 39]. The FRAP assay was done as previously described [38]. The reduction of the ferric ion (Fe3+) to the ferrous ion (Fe2+) as the signal or reaction was measured as a FRAP value, which is tied to a color change by donor electrons in the sample [40]. In brief, we mixed 50 µl of sample from each group with 1500 µl of FRAP reagent (10 volumes of 300mM acetate buffer, pH 3.6 + 1 volume of 10mM TPTZ in 40mM HCl + 1 volume of 20mM iron (III) chloride) and incubated at 37^{o} C for5-min and the absorbance was read at 593 nm after the incubation time. We calculated the FRAP value as follows and results are expressed in µmol/l.

$$\frac{\text{Absorbance at 593nm of test sample reaction mixture}}{\text{Absorbance at 593nm of } Fe^{2+} \text{ standard reaction mixture}} \times Fe^{2+} \text{ standard concentration (µmol/L)}$$

Thiobarbituric acid reactive substances assay (TBARS)

Malondialdehyde (MDA) is one of the end products of Lipid peroxidation that is considered as a marker of oxidative damage. The TBARS assay was performed as described before [41]. TBARS assay measures reaction of MDA with thiobarbituric acid (TBA) to produce a pink colored dimeric compound. Briefly, we mixed 100µl of sample with 20ml of TBAR solution (15 %(v/v) TCA, 0.375 %(w/v) TBA and 0.25M HCL)

and incubated the mixture in a boiling water bath for 15 min. After cooling the solution, we centrifuged the mixture at 1000g for 10 min and the absorbance was read at 535nm. MDA value expressed in µmol/l and calculated as previously explained [41].

Histological analysis

Luxol Fast Blue (LFB) staining

Luxol fast blue (LFB) staining was done to evaluate demyelination process in the spinal cord of the experimental groups as previously explained [42]. Rats deeply anesthetized and perfused with PBS (0.1 M) followed by paraformaldehyde 4% (pH 7.4). Then, brains were removed, post-fixed overnight in PFA 4%, and dehydrated in a graded series of alcohol. After processing, tissues were incubated with xylene and embedded and blocked in paraffin. Serial coronal sections (6 µm) from the lumbar part of spinal cord were obtained by using a microtome (Leica RM2135, Germany). After deparaffinization with xylene and rehydration in a graded alcohol, sections were stained by 0.1% LFB (Atom Scientific, UK) at 60°C for 3 h. Next, sections were counterstained with 0.1% cresyl violet (Sigma, Germany) for 10 min at room temperature followed by washing with distilled water. Again, the slides were washed with distilled water, dehydrated in alcohol series and cleaned with xylene and mounted by entellan (Merck Chemicals, Germany). Slides were observed under an Olympus Cx-23 microscope. We captured Images from spinal cord with C-P6 OPTIKA camera and the demyelination extension was calculated as the ratio of demyelinated area/total area with Image J software (National Institutes of Health, USA) [42, 43]. The images were analyzed from 12 different points of the spinal cord (100 µm interval) from coronal serial sections (6 µm).We averaged 10 sections from a single animal in each group (n = 3); therefore, 30 sections were analyzed for each experimental group. We also measured the optical density (OD) of the spinal cord in MBP-stained sections with the ImageJ software and corrected against background signal levels [44]. Optical density was expressed in arbitrary units. We exclusively selected neuropil staining to set threshold values and the percentage of the total area was quantified. We calculated the mean OD of three sections for each rat (n = 3), which was used as the observed value for group comparisons.

Hematoxylin and Eosin (H&E) staining

H&E staining was performed to analyze the inflammatory scores and cell infiltration. After deparaffinization with xylene, the sections were rehydrated in alcohol series and stained with Hematoxilin for 5 min at room temperature. Then, sections were cleared in xylene and after several washes counterstained with Eosine for 10 min at room temperature, and then washed and cover slipped. Slides were observed under an Olympus Cx-23 microscope. We captured Images from spinal cord with C-P6 OPTIKA camera for later analysis. The inflammation scores were assessed by at least five sections of the lumber spinal cord per rat as previously described [45]. We determined inflammation scores as follows: **0**: no inflammation; **1**: cell infiltration located only in the perivascular areas and meninges; **2**: mild cellular infiltration (inflammatory cells are infiltrated into less than one third part of the white matter); **3**: moderate cellular infiltration (inflammatory cells are infiltrated into more than one third part of the white matter); and **4**: inflammatory cells are infiltrated into the whole white matter [46].

Immunohistochemistry

The immunostaining procedure was performed based on our previous study [37, 47]. The lumbar spinal cord of rats was dissected after perfusion and post-fixed 2 h in PFA (4%) and then incubated in 20% sucrose solution overnight. Next, tissue was embedded and frozen in an embedding medium (Thermo Scientific). A Cryostat (MICROM HM 525, Thermo Scientific) was used to obtain coronal sections (12 μm) of spinal cord collected on superfrost plus slides (Thermo Scientific). Before the IHC experiment, sections were air-dried for 1 h and rehydrated in PBS for 15 min. Antigen retrieval protocol for immune-detection of MBP was performed with ethanol 70% for 10 min prior to antibody incubation. After several washes with PBS, blocking buffer (4% bovine serum albumin (BSA) in PBS and 0.3% Triton X-100) was applied on sections for 1 h at room temperature. Next, primary antibodies were incubated overnight at 4°C including GFAP (1:500, Dako, Z0334), Iba1 (1:250, 019-19741-Wako), anti-CD45 (1/100, MCD4500-Invitrogen), anti-Caspase-3 (1/50, 9661-Cell signaling), anti-NeuN (1/50, MAB-377-Millipore) and MBP (1:100, AB980, Millipore). After washing several times, we applied appropriate secondary antibodies on the sections and incubated for another 2h at room temperature and then washed them with PBS. In the final step, to visualize the cell nuclei sections were incubated with Hoechst (33342) 5μg/ml for 30 min. Then, after several washes with PBS slides were mounted by Fluor-mounting medium (SouthernBiotech, USA). Images were acquired with a BX-51 fluorescent microscope (Olympus, Japan). For quantification of IHC data we used Image J software as described earlier [42, 47]. The number of CD45+, GFAP+, Iba1+, Caspase-3 + and NeuN + cells was counted and averaged from three sections in each slide and three slides from each animal (150 μm apart) (n = 3 animals/group). Data are expressed as the mean number of cells ± SEM per square millimeter.

Statistical Analysis

The result is expressed as means ± SEM of at least three independent experiments. Statistical differences were analyzed using t-test Student or one-way ANOVA followed by Tukey test as indicated in the figure legend for each experiment. Statistical analysis was done by the Graph-Pad prism 6 software (Graph-Pad software, CA, USA) and $P < 0.05$ was considered as the minimum level of significance.

Results

Piperine treatment attenuated EAE Clinical Courses

Experimental autoimmune encephalomyelitis (EAE) is a valuable animal model for human MS [48]. To study the effect of piperine on EAE progression, rats were immunized with suspension consisting of GPSCH and CFA and received pertussis toxin to induce EAE [21]. In treatment group animals daily received piperine injection (5mg/kg/day/IP) [30] eight days post immunization until day 29. Then we monitored the neurological symptoms of EAE in rats as mean clinical score, peak clinical score and

cumulative score. Animals in EAE and EAE + veh groups showed the peak neurological score up to level 5 between 10 and 12 days post immunization. The acute phase was followed by a remitting phase between days 15 and 19 post immunization, and second phase of neurological impairment started between 20 and 23 days post immunization (Fig. 1A). Piperine treatment significantly decreased daily mean clinical score of EAE compared to untreated groups over the course of the study ($p < .0001$). Relapse of disease signs was also significantly attenuated after treatment with piperine, when compared to EAE and EAE + veh groups (Fig. 1A, $p < 0.0001$, Student's t-test, $n = 8$). Piperine treated rats showed lower peak and cumulative scores when compared to EAE and EAE + Veh groups (Fig. 1B-C, 0.0001, Student's t-test, $n = 8$).

Piperine treatment improved myelin repair process in EAE model

To study the effects of piperine on EAE-induced demyelination, lumbar spinal cord of rats was dissected at day 22 post immunization (12 days after piperine treatment). After tissue processing, sections were stained with luxal fast blue (LFB) and immune-labeled with myelin basic protein (MBP), which has an important role in myelin compaction (Fig. 2A). Histological analysis of LFB stained sections showed that demyelination extension in piperine treated group was considerably less than that in EAE and EAE + veh groups (Fig. 2B, $p < 0.001$, One way ANOVA, Tukey Post-hoc, $n = 3$). Moreover, results were further confirmed by analyzing optical density (OD) in MBP stained sections (Fig. 2C). Severe demyelination was observed in EAE and EAE + Veh groups, and the OD significantly decreased in both compared to the control group (Fig. 2A-C, $p < 0.01$, One Way ANOVA, Tukey Post-hoc, $n = 3$). Piperine treatment improved myelin repair process as the level of OD increased compared to EAE and EAE + veh groups (Fig. 2A-C, $p < 0.05$, One Way ANOVA, Tukey Post-hoc, $n = 3$). We also evaluated the gene expression level of MBP as an essential protein for the formation and stabilization of the myelin membrane in lumbar part of spinal cord in different experimental groups. Our results showed that in EAE and EAE + Veh groups the expression level of MBP was significantly lower than that in the control (Fig. 3A, $p < 0.01$, One Way ANOVA, Tukey Post-hoc, $n = 4$). Piperine treatment enhanced MBP expression level compared to EAE and EAE + Veh groups (Fig. 3A, $p < 0.0001$, One Way ANOVA, Tukey Post-hoc, $n = 4$). BDNF is a member of the neurotrophic family and has an important role in remyelination process [49]. Tukey post hoc analysis showed that the gene expression level of BDNF in EAE and EAE + Veh groups was significantly lower than that in the control group (Fig. 3B, $P < 0.01$). However, the BDNF expression level increased more significantly in the piperine treated group than that in the EAE and EAE + Veh groups ($p < 0.0001$) and even the control group ($P < 0.001$) (Fig. 3B, One Way ANOVA, Tukey Post-hoc, $n = 4$). These data suggested that piperine treatment facilitates myelin repair in EAE model through enhancing MBP expression at gene (Fig. 3A) and protein (Fig. 2A) levels and BDNF (Fig. 3B) as an important neurotrophic factor in myelin repair.

Piperine reduced infiltrated cells in the spinal cords and modulated the expression level of pro-inflammatory and anti-inflammatory cytokines in EAE model

To evaluate the effect of piperine on inflammatory processes in EAE model, histopathological and molecular analyses were done in different experimental groups. To examine the degrees of infiltration of immune cells into the CNS, lumbar spinal cord sections from different experimental groups were stained with Hematoxilin and Eosin (H&E). We determined inflammation scores as described in the methods [45]. H&E staining showed that the infiltration of inflammatory cells into the spinal cord at widespread area considerably increased in EAE and EAE + Veh groups and inflammatory scores in these groups were significantly higher than that in control group (Fig. 4A-B, $p < 0.0001$, One Way ANOVA, Tukey Post-hoc, $n = 3$). Treatment with piperine significantly decreased infiltrated cells and inflammatory scores compared to EAE and EAE + Veh groups ($p < 0.001$), while inflammatory score was higher than that in control ($p < 0.05$) (Fig. 4A-B, One Way ANOVA, Tukey Post-hoc, $n = 3$). To further confirm our data, we immune-stained lumbar spinal cord sections with CD45 antibody. CD45 is considered as a leukocytes common antigen with tyrosine phosphatase activity [50]. Quantification of CD45 positive cells in the lumbar spinal cord sections indicated that the number of CD45 positive cells in EAE and EAE + Veh groups was significantly more than that in control group ($P < 0.001$). However, in piperine treated group the number of CD45 positive cells was significantly lower than those in EAE and EAE + Veh groups (Fig. 4A-C, $p < 0.01$, One Way ANOVA, Tukey Post-hoc, $n = 3$). The infiltration of immune cells was observed at the same site of demyelinated area. Thus, the severe paralysis seen in EAE and EAE + Veh groups is correlated with significant increases of inflammation and demyelination in the spinal cord. Piperine treatment through reduction of inflammation and demyelination in the treated animals negatively regulates the progression of EAE.

Next, we measured the expression level of TNF-α and IL-1β as inflammatory cytokines and IL-10 as an anti-inflammatory cytokine by qPCR. Our results revealed that in EAE and EAE + Veh groups, the expression level of TNF-α and IL-1β significantly increased compared to control group (Fig. 5A-B, $p < 0.0001$, One Way ANOVA, Tukey Post-hoc, $n = 4$). Daily treatment with piperine at 5mg/kg effectively downregulated the expression levels of TNF-α and IL-1β genes compared to EAE and EAE + Veh groups (Fig. 5A-B, $p < 0.0001$, One Way ANOVA, Tukey Post-hoc, $n = 4$). However, the gene expression level of TNF-α and IL-1β in piperine treated group was significantly higher than control ($P < 0.01$, $P < 0.001$ respectively). In piperine treated group the expression level of IL-10 was higher than those in EAE, EAE + Veh and control groups (Fig. 5C, $p < 0.0001$, One Way ANOVA, Tukey Post-hoc, $n = 4$). Although the IL-10 gene expression in EAE and vehicle groups was lower than that in control, the change was not statistically significant. Therefore, piperine has immunomodulatory effect in EAE model through reduction of inflammatory cytokines and augmentation of anti-inflammatory cytokine.

Piperine reduced the astrocytes and microglial activation in EAE model

The activated microglia and astrocytes in EAE animals produce pro-inflammatory factors that are toxic and promote inflammatory infiltration into the CNS [51, 52]. We therefore explored the effect of piperine on activated microglia and astrocytes. We performed immunostaining against ionized-calcium binding adaptor protein (Iba-1) as microglia marker and glial fibrillary acidic protein (GFAP) as astrocyte marker in

lumbar spinal cord sections (Fig. 6A-B). Our results showed that the number of Iba-1 positive cells significantly elevated in EAE and EAE + Veh groups compared to the control ($P < 0.01$). Following piperine treatment the number of Iba-1 positive cells considerably decreased compared to EAE and EAE + Veh groups (Fig. 6A-C, $p < 0.05$, One Way ANOVA, Tukey Post-hoc, $n = 3$). The number GFAP positive cells in EAE and EAE + Veh groups was significantly higher than that in control (Fig. 6D, $P < 0.0001$). In piperine treated group the number GFAP positive cells was significantly reduced compared to EAE and EAE + Veh groups (Fig. 6B-D, $p < 0.001$, One Way ANOVA, Tukey Post-hoc, $n = 3$). These results together demonstrated that inhibition of microglia/astrocytes activation by piperine treatment could suppress EAE progression and enhance repair processes.

Piperine decreased oxidative stress factors and enhanced antioxidant capacity in the EAE model

The effect of piperine on the gene expression level of iNOS, Nrf2 and HO-1 in the lumbar part of spinal cord was evaluated by q-PCR. iNOS is one of the main oxidative related enzymes that plays a major role in cytotoxicity, inflammation and oxidative stress damage [53]. The iNOS expression level in EAE and EAE + Veh groups was significantly more than control group ($P < 0.0001$). Piperine treatment significantly reduced the expression level of iNOS compared to EAE and EAE + Veh groups (Fig. 7A, $p < 0.001$, One Way ANOVA, Tukey Post-hoc, $n = 4$). Nrf2 is a transcription factor which is responsible for cellular antioxidant responses [54]. We found that the expression level of Nrf2 in piperine treated group was significantly more than that in EAE, EAE + Veh and control groups. However, there was no significant difference in Nrf2 expression level between EAE and control groups (Fig. 7B, $p < 0.0001$, One Way ANOVA, Tukey Post-hoc, $n = 4$). HO-1 catalyzes the heme degradation and has anti-inflammatory and anti-oxidant properties [55]. The assessment of HO-1 gene expression in spinal cord of rats indicated that in piperine treated group, the level of HO-1 was significantly higher than that in EAE, EAE + Veh and control groups (Fig. 7C, $p < 0.001$,$P < 0.05$, One Way ANOVA, Tukey Post-hoc, $n = 4$). The expression level of HO-1 in EAE group was also more than in the control group (Fig. 7C, $P < 0.05$).

To further confirm the protective effect of piperine against oxidative damage we measured malondialdehyde (MDA) (TBARS assay) and total antioxidant capacity (FRAP assay) in the lumbar part of spinal cord tissue. MDA is the main product of lipid peroxidation and can be considered as diagnostic indices of oxidative injury [56]. We found that, MDA content in EAE and EAE + Veh groups was more than that in control ($P < 0.05$). Piperine treatment significantly decreased MDA compared to EAE and EAE + Veh groups (Fig. 7D, $P < 0.01$, One Way ANOVA, Tukey Post-hoc, $n = 4$). The ferric reducing ability of plasma (FRAP) can be considered as a measure of antioxidant power [39]. Our data indicated that piperine treated group has strong reducing power capacity compared to EAE, EAE + Veh ($p < 0.05$) and control groups ($p < 0.001$) (Fig. 7E, One Way ANOVA, Tukey Post-hoc, $n = 4$). Thus, piperine decreases oxidative stress and increases total antioxidant capacity of the CNS in EAE model.

Piperine reduced caspase-3 expression and neuronal loss in the EAE model

In order to prove the neuroprotective effect of piperine, immunostaining against neuronal nuclear protein (NeuN) as a mature neuronal marker and caspase-3 as a marker of apoptosis was done in lumbar spinal cord sections (Fig. 8A-B). We quantified the number of NeuN positive cells in the lumbar spinal cord sections. The number of NeuN expressing cells in EAE and EAE + Veh groups was considerably lower than that in control group ($P < 0.001$). In piperine treated rats the number of NeuN positive cells significantly increased compared to EAE and EAE + Veh groups (Fig. 8A-C, $P < 0.05$, One Way ANOVA, Tukey Post-hoc, $n = 3$). Caspase-3 is a member of cysteinyl-aspartate-specific proteases which is considered as a main mediator of apoptosis in neuronal cells [57]. We showed that the number of caspase-3 positive cells significantly increased in EAE and EAE + Veh groups compared to control group ($P < 0.0001$). Piperine treatment reduced the caspase-3 expressing cells compared to EAE and EAE + Veh groups (Fig. 8B-D, $P < 0.001$, One Way ANOVA, Tukey Post-hoc, $n = 3$). Consequently, piperine protected EAE induced neuronal apoptosis via blocking the caspase-3 and enhancing neuronal cell survival.

Discussion

We investigated whether piperine had a beneficial effect on the experimental autoimmune encephalomyelitis (EAE) as an animal model of multiple sclerosis (MS). We found that treatment with piperine attenuated clinical symptoms of EAE and spinal cord demyelination, which were accompanied by the reduction of infiltration of immune cells, activation of microglia and astrocytes and expression of inflammatory mediators (TNF-α, IL-1β and iNOS) in the spinal cord of EAE rats. Moreover, treatment with piperine decreased the level of MDA as an oxidative stress marker and increased the total antioxidant capacity and the expression level of Nrf2 and HO-1 as antioxidant mediators. Also, the level of IL-10 as an anti-inflammatory cytokine in piperine treated group was higher than that in EAE group. Furthermore, treatment with piperine enhanced the expression level of BDNF and MBP in EAE model. Finally, we showed that piperine reduced the level of caspase-3 and enhanced NeuN positive cells in spinal cord of EAE rats. The findings suggest that piperine has a neuroprotective and therapeutic effect on progression of EAE by suppressing inflammatory responses and oxidative stress.

We previously revealed that piperine enhanced memory function and myelin repair in hippocampal local model of demyelination [30]. Here, we used EAE model to further confirm the efficacy of piperine on the animal model that best represents the pathology of MS. We checked several categories of mechanisms including cell infiltration and glia activation, the profile of pro-inflammatory and anti-inflammatory cytokines, the state of oxidative stress parameters, apoptosis, neuronal survival and myelin repair to understand the possible mechanisms of beneficial effects of piperine in EAE model. EAE models are of interest in investigation and understanding of the pathological changes during the MS disease course and drug screening for development of MS therapeutics [2].

EAE is a Th-1 mediated inflammatory demyelinating disease of the central nervous system (CNS), which resembles MS in many respects [3]. EAE is characterized by inflammatory infiltrates of T lymphocytes, B

lymphocytes, macrophages and focal demyelinating plaques in the CNS [58]. An activated immune system and infiltration of mononuclear cells into the CNS in the early phase leads to disease onset and progression in MS and EAE model [59]. Therefore, suppression of the early activated immune system is very important. Here, we used rat EAE model that was actively induced in Lewis rats using guinea pig SCH, CFA, and *M.tuberculosis* [21]. Animals showed the typical pattern of acute phase–remitting phase–first relapse which is considered as a chronic EAE. We found that infiltrated cells (mostly CD45 + cells) into spinal cord of EAE rats extensively increased. CD45 is a lymphocyte common antigen which is essential for T cells activation [50]. Piperine significantly decreased the number of infiltrated T cells. Piperine treatment significantly suppressed clinical symptoms, inflammatory scores and infiltrated cells (CD45 + cells) compared to those of EAE rats. The anti-inflammatory effects of piperine were well-documented in different animal models of inflammatory diseases [24, 60–62].

The activation of antigen-specific T cells, in the early stage of MS disease causes the secretion of TNF-α, IL-1β and IFN-γ, which are critical for inducing relapse in MS and EAE [63, 59, 64]. However, the rise of anti-inflammatory cytokines (IL-4, IL-10 and TGF-β) seems to mediate disease remission [65]. Our results indicated that piperine reduced the expression level of pro-inflammatory cytokines such as TNF-α and IL-1β and enhanced the level of IL-10 in the EAE rats. In this regard, various reports have revealed that piperine treatment suppresses the production of TNF-α, IL-1β and iNOS in the animal models of Parkinson [17, 33], local model of demyeination [30] and epilepsy [66, 35]. Moreover, piperine is an effective inhibitor of NF-κB [67], which is an important transcription factor to initiate production of several pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-12 [68]. On the other hand, we showed that the expression level of IL-10 significantly increased in piperine-treated group compared to EAE. IL-10, which is a multifunctional cytokine, can inhibit Th-1 cells and inflammatory macrophages and also block NF-κB activity [69, 70]. It has been confirmed that the expression level of IL-10 reduced during the relapse phase [71], and enhanced in the remitting phase of MS patients [72]. It was also indicated that IL-10 is important for recovery of EAE [73] and the absence of IL-10 results in a more severe EAE [74]. Therefore, piperine modulated pro-inflammatory and anti-inflammatory cytokines and could play an anti-inflammatory role in EAE model. These data supported our former data about piperine effect on lysolecethin-induced local demyelination model [30].

Microglia and astrocytes play a major role in the pathogenesis of MS and EAE [75]. Their activation and maturation are critical to disease development and progression [76] and ablation of microglia suppresses EAE development [77]. In the demyelinated lesions of MS and EAE, activated microglia and macrophages produce inflammatory mediators and enhanced cell infiltration. Therefore, the regulation of microglial and astrocytes activation may be an attractive therapeutic strategy for MS and EAE [78]. In line with this, we found that microglia and astrocytes were activated in EAE model as the expression of Iba1 and GFAP extensively increased in spinal cord of EAE group compared to the control. The cytokines and other factors released by the infiltrating cells and activated glial cells (astrocytes and microglia) induce inflammation, demyelination, axonal loss and gliosis [79]. Treatment with piperine also inhibited microglial and astrocyte activation in the spinal cords of EAE rats corresponding to attenuation of EAE scores and spinal demyelination. In this regard, several studies reported that minocycline (a broad

spectrum tetracycline antibiotic), galectin-1 (an endogenous glycanbinding protein), and MW01-5-188WH (an orally bioavailable and brain-penetrating small molecule compound) prevent clinical scores, inflammation, and demyelination via inhibiting microglial activation in relapsing-remitting EAE model [80, 81, 18]. In addition, piperine was found to protect neuronal degeneration by inhibiting microglia activation and production of pro-inflammatory cytokines in mouse models of Parkinson disease [82]. Curcumin, which has been reported as antioxidant and anti-inflammatory compound to inhibit microglia and astrocytes activation, has multiple therapeutic effects against various neurodegenerative disorders including MS [20]. Our results are consistent with previous studies and suggest that piperine has a beneficial effect in EAE model through suppressing astrocyte and microglial activation.

Reactive oxygen species (ROS), generated as a result of the inflammatory process and reactivation of microglia and astrocytes are supposed to play a role in the pathobiology of EAE and MS [83]. Oxidative stress is involved in the initiation and progression of MS [84]. Malondialdehyde (MDA), a well-known end product of lipid peroxidation, is a marker of oxidative stress [85]. Nitric oxide (NO) produced from inducible nitric oxide synthase (iNOS), the main oxidative related enzymes, is involved in inflammation, cytotoxicity, neuronal death, and oxidative injury in several neurological diseases [86]. Several lines of evidence indicated that the iNOS expression and MDA content was increased in demyelinating diseases [21, 30, 87, 88]. Consistent with this, we found that the levels of MDA and iNOS were significantly increased in EAE group while the level of FRAP, Nrf2 and HO-1 was not affected. Piperine treatment remarkably reduced the level of MDA and iNOS expression and enhanced total antioxidant capacity (FRAP) and the expression level of Nrf2 and HO-1, as antioxidant defense elements that contributed to the reduction of oxidation. Furthermore, the level of FRAP, Nrf2 and HO-1 in piperine treated rats was higher than that in the control (Fig. 7). Nrf2 is the main transcription factor which regulates a wide range of antioxidant genes including HO-1 (downstream target of the Nrf2-ARE (antioxidant responsive element) pathway) during the oxidative stress conditions [54, 89]. Previous studies have shown that Nrf2 could also inhibit the activation of NF-kB [90]. HO-1 is an inducible enzyme that catalyzes the heme degradation to produces bilirubin, reduces the oxidative injury and regulates apoptosis and inflammation [55] [55]. It was reported that dimethyl fumarate, which enhances Nrf2, considerably reduced disability and relapse phases in MS during the phase 3 clinical trials [91]. Moreover, another study proved that HO-1 has protective effect and its overexpression reduced the clinical severity of EAE in rats [92]. Furthermore, induction of EAE in HO-1 knockout mice developed a more severe form of EAE than did wild-type [93]. It was also shown that piperine activates the Nrf2/HO-1 pathway and inhibits the iNOS activity [29]. Together, we suggest that Nrf2-HO-1 pathway is one of the mechanistic targets of piperine for its protective effect in EAE model.

Neuron loss and axonal injury are the main causes of long-term disabilities in MS [94] and EAE [9]. Several studies have confirmed that apoptosis is an important feature in the pathogenesis of MS and EAE [95, 9]. Caspase-3 is considered as the main effector caspase involved in neuronal apoptosis during development or after exposure to injury [96]. Previous study has described that activation of caspase-3 causes neuronal apoptosis during EAE [97]. To understand how piperine exerts its neuroprotective effect in EAE model, we evaluated the number of caspase-3 and NeuN positive cells in the lumbar section of rat

spinal cord in different experimental groups. The neuronal nuclear protein (NeuN) is commonly considered as a marker of mature neuron [98] and quantification of NeuN is an indicator of neuron health [99]. Our results are consistent with previous studies showing that the number of caspase-3 expressing cells increased in EAE compared to control group. We also found that the number of NeuN + cells significantly diminished in the spinal cord of EAE rats. In the present study, treatment with piperine significantly decreased the number of caspase-3 positive cells and enhanced the NeuN expressing cells in the spinal cord of EAE rats. Likewise, several studies had proved that treatment with piperine has anti-apoptotic and neuroprotective effect in the different animal models of neurological diseases [35, 34, 17]. In addition, natural products with antioxidant, anti-inflammatory and neuroprotective properties such as curcumin [21], quercetin [22], and ginseng [13] protect neuron and alleviate neurological symptoms of EAE model.

To better understand the mechanism of piperine on myelin repair process, we evaluated the expression level of MBP as the main structural proteins of myelin and BDNF as a neurotrophic factor. BDNF has an essential role in the development, maintenance of the CNS and myelin repair process [49]. BDNF has an anti-apoptotic effect on neurons through enhancing the Bcl-2 which inhibits the caspase-3 [100]. Several studies reported that BDNF is a critical factor to promote myelin repair in different animal models of central demyelination [49, 101]. BDNF can also regulate the expression level of MBP [101] and enhance oligodendrocyte progenitor's proliferation, migration and differentiation in the demyelination context [101, 102]. It has been reported that the level of BDNF in MS patients was significantly less than that in the healthy controls [103]. On the other hand, treatment with BDNF diminished the clinical score and pathological severity of EAE [100]. The clinical administration of BDNF has been limited because of its poor penetration to the blood brain barrier (BBB). Therefore, drugs with potential to enhance BDNF expression can be beneficial for MS treatment [104]. Quite similarly, we found that the expression of BDNF and MBP was significantly reduced in EAE group compared to control animals. Our results indicated that piperine treatment significantly enhanced the level of BDNF and MBP which were accompanied by the reduction of demyelination in the spinal cord of EAE rats. Consistent with our data, several lines of evidence showed that piperine treatment up-regulated the BDNF expression in the animal model of depression [105] and local model of demyelination [30]. The BBB penetration of piperine was detected in an in vitro and in vivo tissue distribution analysis [106] and this feature of piperine may lay a foundation for its superior pharmacological activity in the CNS. Our findings suggest that piperine facilitates myelin repair process through enhancing BDNF and MBP level in the CNS which is also associated with its anti-inflammatory, anti-oxidant and neuroprotective effect in the EAE model.

Conclusion

In summary, we demonstrate that intraperitoneal administration of piperine (5mg/kg) eight days post immunization alleviated neurological deficits and EAE disease progression through its neuroprotective, anti-inflammatory, anti-apoptotic and antioxidant properties. Piperine inhibits microglia and astrocyte activation and infiltration of immune cells into spinal cord of EAE rats. Importantly, piperine treatment decreased the level of pro-inflammatory cytokines (TNF-α, IL-1β), iNOS and oxidative stress marker

(MDA). Piperine supplementation also enhanced the level of IL-10, Nrf2, HO-1 and MBP in the CNS of EAE rats. Finally, we found that piperine has anti-apoptotic and neuroprotective effect in EAE through reduction of caspase-3 (apoptosis marker) and enhancement of BDNF and NeuN expressing cells. This study strongly indicates that piperine has a beneficial effect on the EAE progression and could be considered as a potential therapeutic target for MS treatment. Further research is needed to prove the exact mechanistic pathways of piperine in order to develop strategies to better follow and treat these neurological patients and open up new avenues for immuno-modulatory treatment. Upcoming clinical trials will provide the deep understanding of piperine's role for the treatment of the MS.

Declarations

Author Declarations section

Ethics approval

All experimental procedures of this study were conducted according to the international guidelines for care and use of laboratory animals and approved (Ethic cod number: IR.MUBABOL.HRI.REC.1398.315) by the ethical committee of Babol University of Medical Sciences.

Consent to participate and or Consent for publication

It is not applicable in our study because this article does not contain any studies involving human participants performed by any of the authors.

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of interest/Competing interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Authors' contributions

All authors contributed to the study conception and design. Material preparation and data collection were performed by Reza Nasrnezhad, Sohrab Halalkhor and Fereshteh Pourabdolhossein. Data analysis and interpretation was done by Reza Nasrnezhad, Farzin Sadeghi and Fereshteh Pourabdolhossein. The first draft of the manuscript was written by Reza Nasrnezhad and its major revision was performed by Fereshteh Pourabdolhossein. All authors read and approved the final manuscript.

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Compliance with Ethical Standards section

Disclosure of potential conflicts of interest

The authors declare no conflict of interest related to this study.

Research involving Human Participants and/or Animals

In this study all experimental procedures were conducted according to the international guidelines for care and use of laboratory animals and approved by the ethical committee of Babol University of Medical Sciences (Ethic cod number: IR.MUBABOL.HRI.REC.1398.315).

Informed consent

It is not applicable in this study.

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Figures

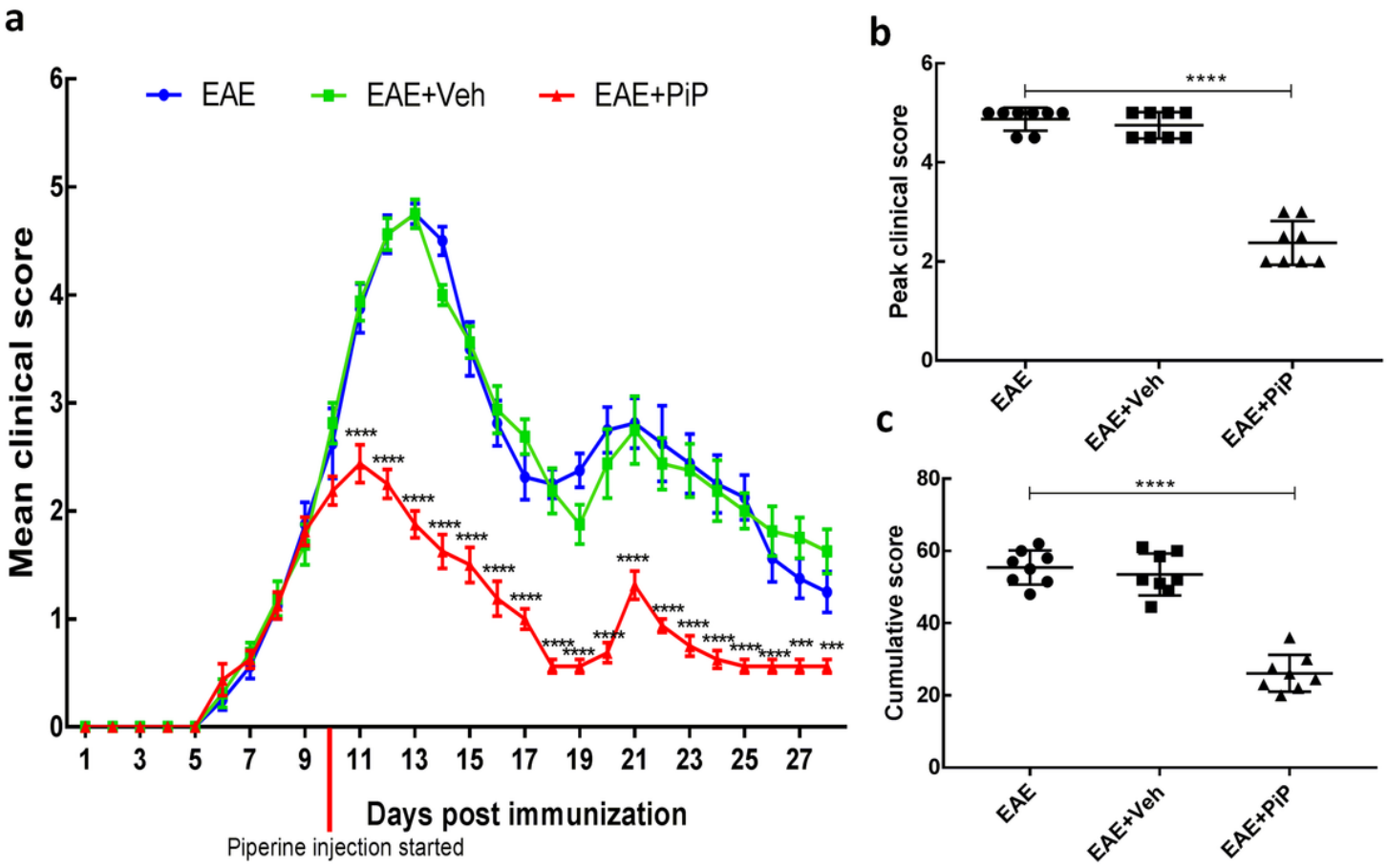

Figure 1

Piperine attenuates clinical score and severity of EAE. a Daily mean EAE clinical score of Lewis rats (8 rats per group) on EAE, EAE+veh and EAE+PiP (5mg/kg) groups. Piperine and its vehicle were administered through daily intraperitoneally injection at day 8 after EAE induction until day 29. Data are presented as mean ± SEM and Student's t-test is done, ***P < 0.001, ****P < 0.0001. b Comparison of the average of peak score shows that piperine significantly reduced the peak clinical score compared to EAE or EAE+veh (****P < 0.0001). c Comparison of the sum of EAE scores in different groups indicates that

piperine significantly decreased the cumulative scores compared to other groups (****P < 0.0001). Data is shown as mean ± SEM and Student's t-test is performed, **P < 0.01, ***P < 0.001.

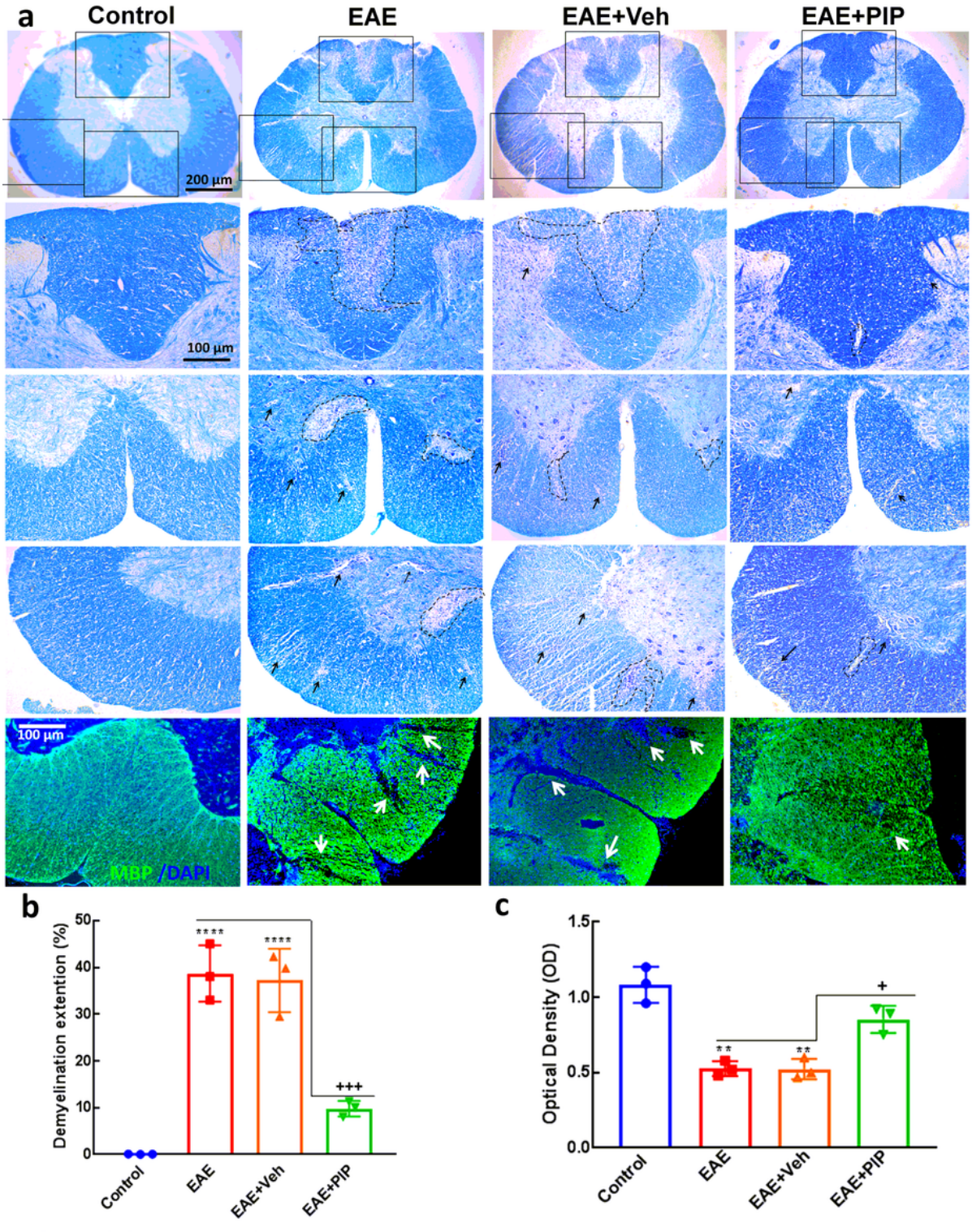

Figure 2

The extent of demyelination is reduced in piperine treated animals. a Representative micrographs were prepared from the lumbar spinal cord sections of control, EAE, EAE+Veh and EAE+PiP groups stained with Luxol fast blue and MBP. Boxes show magnified image of demyelinated areas, arrows and dashed line

show demyelinated region in the magnified images. Scale bar is 100μm for magnified images and 200μm for the first row images. b Quantitative analysis of the extent of demyelinated area in different experimental groups in LFB stained images. Piperine significantly reduced the extent of demyelination compared to EAE and EAE+Veh groups+++P < 0.001, One way ANOVA, post hoc Tukey is done. c Optical density (OD) was calculated to measure the severity of demyelination with internal control in MBP-stained images. Quantitative analysis of OD shows piperine enhances remyelination compared to EAE and EAE+Veh groups+P < 0.05, One way ANOVA, post hoc Tukey was done. Data is expressed as mean ± SEM. n=3 for histological analysis.

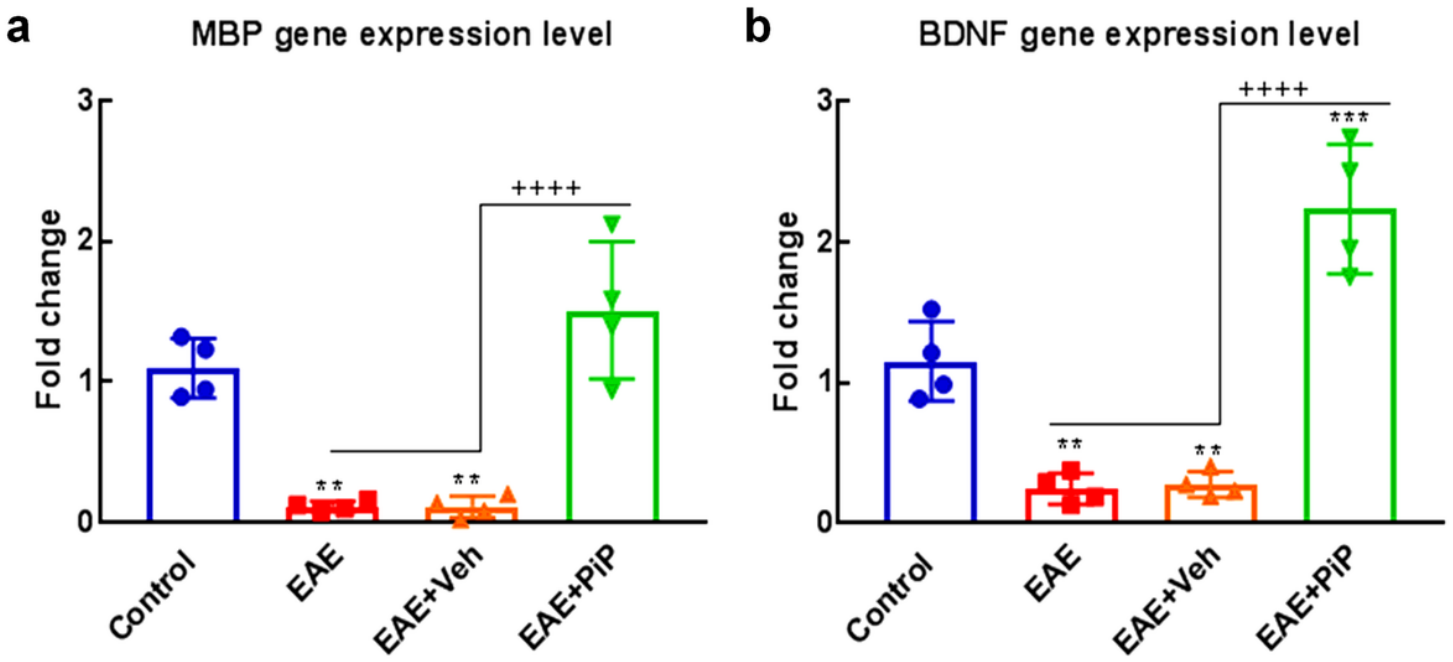

Figure 3

Piperine treatment enhances MBP and BDNF gene expression in EAE animals. Lumbar spinal cord of rats was harvested and evaluated for gene expression analysis. The data confirmed piperine significantly increased a MBP (++++P < 0.0001) and b BDNF (++++P < 0.0001) expression levels compared to EAE and EAE+Veh groups. One way ANOVA, post hoc Tukey was used. Data is stated as mean ± SEM.

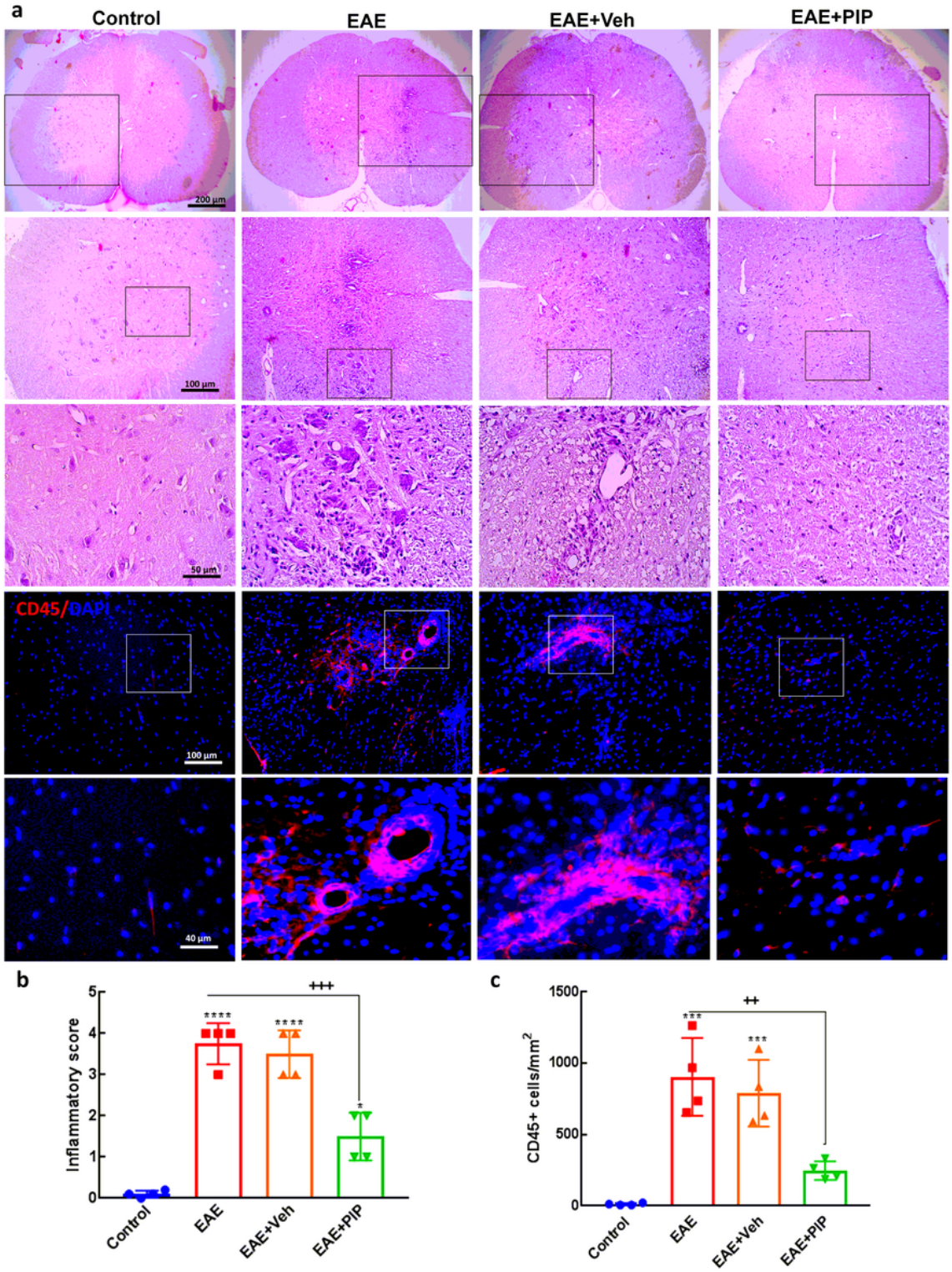

Figure 4

Piperine treatment reduces immune cells infiltration. a Representative micrographs were prepared from the lumbar spinal cord sections of control, EAE, EAE+Veh and EAE+PiP groups stained with H&E and CD45 (red). Boxes show magnified image of selected areas. Scale bars for images of H&E in the first row are 200µm, second row, 100µm and third row 50 µm. Scale bars for images of CD45 in the fourth row are 100 µm and fifth row 40 µm. b Quantitative analysis of the inflammatory score. Higher inflammatory

scores were observed in EAE group compared to piperine treated animals (+++P < 0.001). In each group 20 sections were scored by a pathologist who was blinded to the experimental groups. c Quantitative analysis of the number of CD45+ (red) cells in the inflammatory regions of the lumbar spinal cord sections. CD45 is a lymphocyte common antigen which is essential for T cells activation. Piperine treatment significantly reduced the number of CD45+ cells in the lumbar spinal cord of EAE rat (++P < 0.01). ***P < 0.001, ****P < 0.0001 for comparison between control, EAE and EAE+veh groups. n=4 for histological analysis, One way ANOVA, post hoc Tukey was used. Data is shown as mean ± SEM.

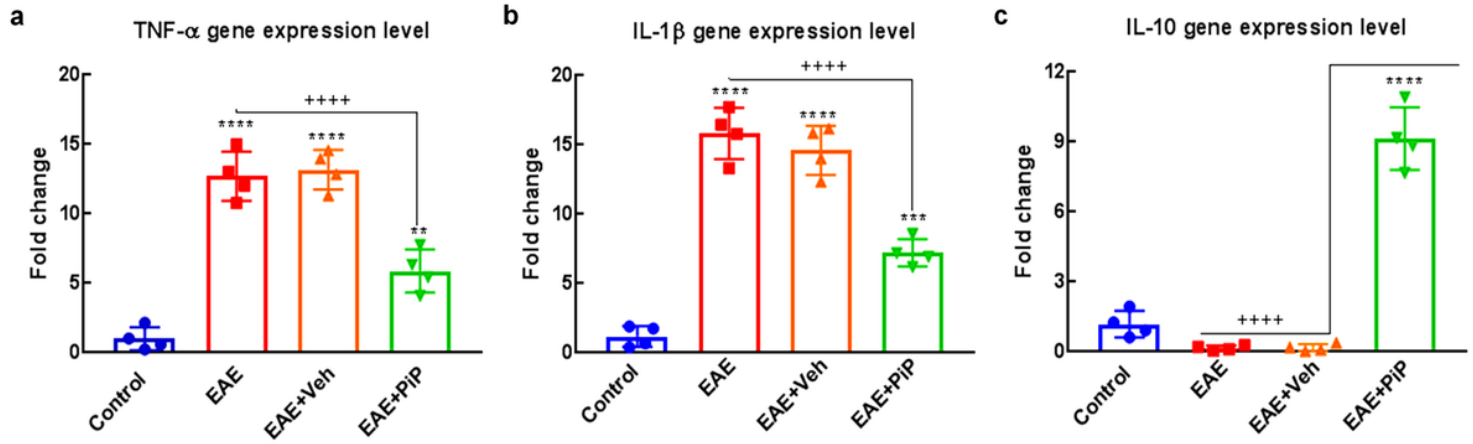

Figure 5

Piperine treatment suppressed the gene expression level of the inflammatory cytokines and enhanced IL-10 in EAE rats. Lumbar spinal cord of rats was harvested and evaluated for the expression of a TNF-α, b IL-1β and c IL-10 by real time-PCR. a TNF-α and b IL-1β expression level considerably increased in EAE and EAE+veh groups compared to control (****P < 0.0001). Piperine treatment could effectively diminish the level of TNF-α and IL-1β gene expression compared to EAE and EAE+veh groups (++++P < 0.0001). However, the level of TNF-α (**P < 0.01) and IL-1β (***P < 0.001) gene expression in piperine treated animals was higher than that in control. c The expression level of IL-10 as an anti-inflammatory cytokine in piperine treated group was significantly higher than those in EAE, EAE+veh (++++p < 0.0001) and control groups(****P < 0.0001). n=4 for gene expression analysis, One way ANOVA, post hoc Tukey was used. Data is shown as mean ± SEM.

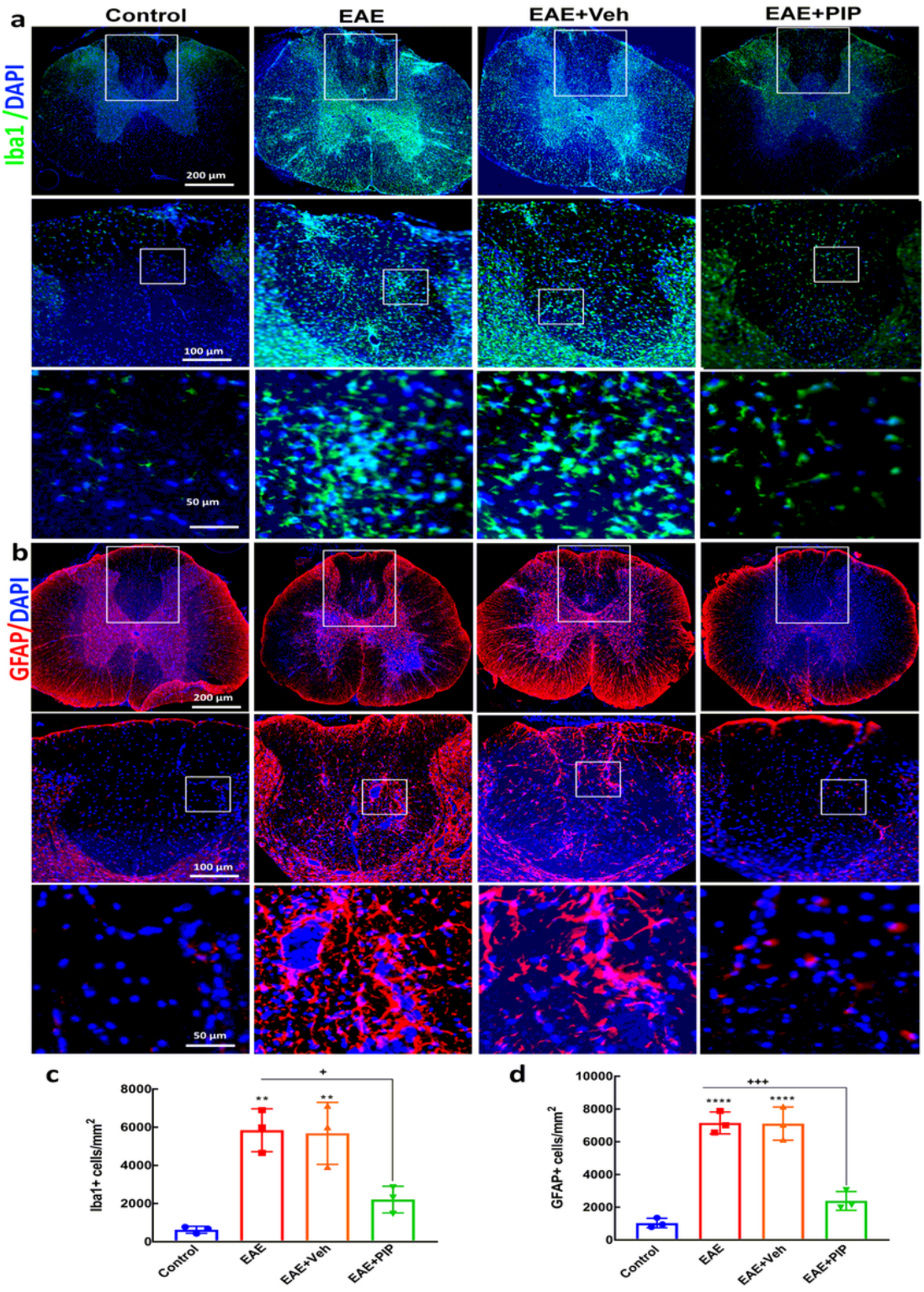

Figure 6

Piperine treatment efficiently inhibited microglia and astrocyte activation. a Immunostaining against Iba1 (green) as a microglia and DAPI (blue) as a nucleus marker was performed on a lumbar spinal cord sections from different experimental groups including: control, EAE, EAE+Veh and EAE+PiP groups. Boxes show magnified image of selected areas. Scale bar for images from the first row are 200µm, second row, 100µm and third row 50 µm. b Immunostaining against GFAP (red) as an astrocyte marker and DAPI

(blue) as a nucleus marker was done in all experimental groups. Boxes show magnified image of selected areas. Scale bar for images from the first row are 200μm, second row, 100μm and third row 50 μm. c Quantitative analysis of the number of Iba1+ cells (microglia) in the lumbar spinal cord sections. The number of Iba1+ cells was considerably increased in EAE and EAE+veh compared to control group (**P < 0.01). Piperine treatment significantly reduced the number of Iba1+ cells compared to EAE and EAE+veh (+P < 0.05). d Quantification of GFAP+ cell number in the lumbar spinal cord showed that the number of GFAP+ cells in EAE and EAE+veh groups was significantly more than that in control (****P < 0.0001). Piperine treatment significantly decreased the number of GFAP+ cells compared to EAE and EAE+veh groups (+++p < 0.001). Data is displayed as mean ± SEM. One way ANOVA, post hoc Tukey was used, n=3.

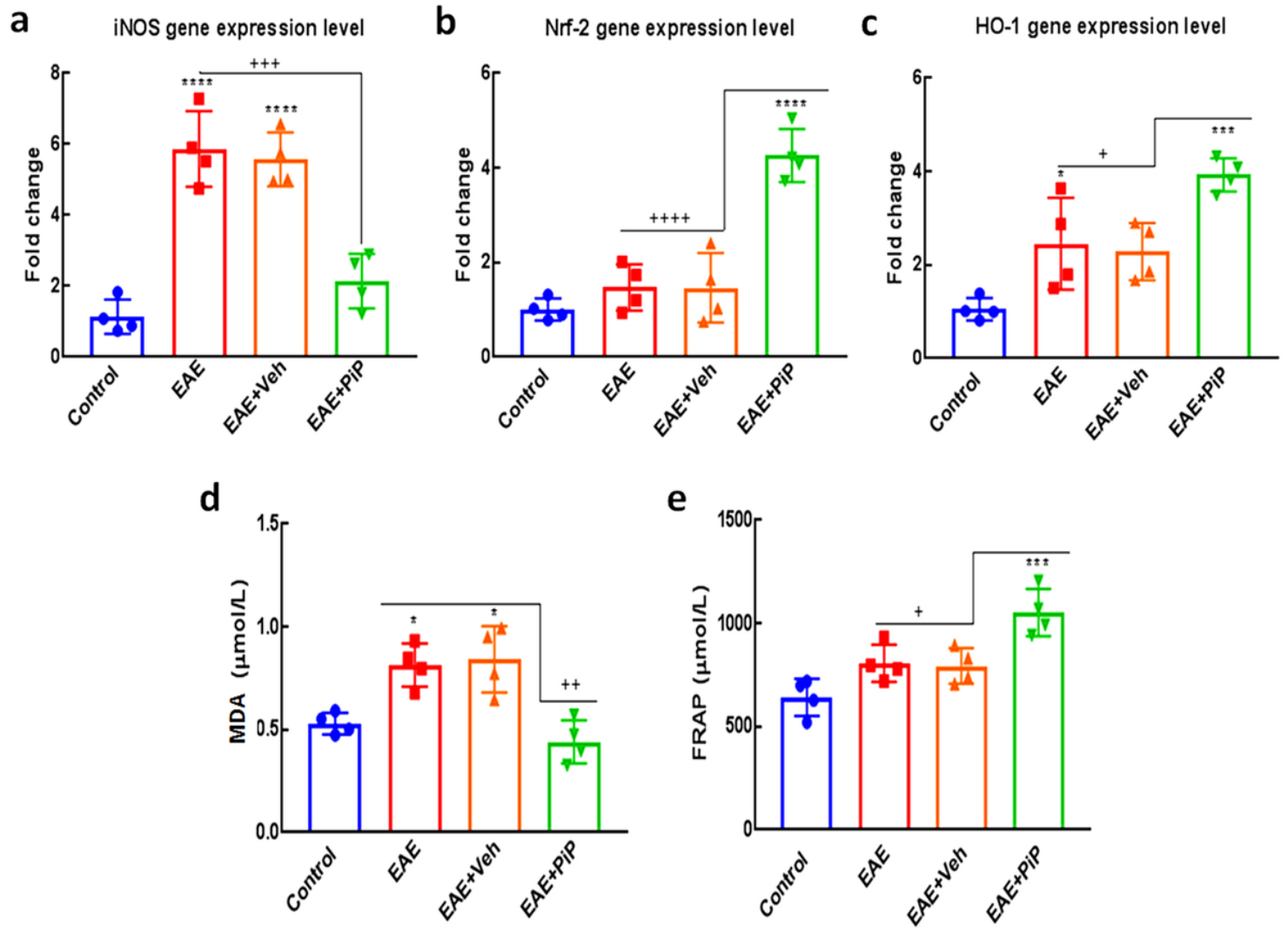

Figure 7

Piperine treatment modulated the level of oxidant and antioxidant mediators EAE rats. Lumbar part of spinal cord was harvested and evaluated for the gene expression analysis of iNOS a Nrf-2 b and HO-1 c. a The results indicated that the expression level of iNOS in EAE and EAE+veh groups was significantly more than control group (****P < 0.0001). Piperine treatment significantly decreased the level of iNOS

compared to EAE and EAE+veh groups (+++p < 0.001). b The level of Nrf-2 in piperine treated group was significantly higher than those in EAE and EAE+veh (++++p < 0.0001) and control (****P < 0.0001) groups. c The level of HO-1 was significantly increased in EAE(*P < 0.05) and piperine-treated groups (***P < 0.001) compared to control. Moreover, the level of HO-1in Piperine-treated animals was significantly higher than that in EAE and EAE+veh groups (+p < 0.05). d The level of MDA (as an oxidative stress marker) was measured by TBARS assay. The level of MDA in EAE and EAE+veh groups was considerably higher than that in control (*P < 0.05). However, piperine treatment significantly decreased the MDA level compared to EAE and EAE+veh groups (++p < 0.01). e we measured the total antioxidant capacity of the lumbar spinal cord with FRAP assay. The level of FRAP in piperine-treated group was significantly higher than those in EAE and EAE+veh (+p < 0.05) and control (***P < 0.001) groups. Data is presented as mean ± SEM. One way ANOVA, post hoc Tukey was used, n=4.

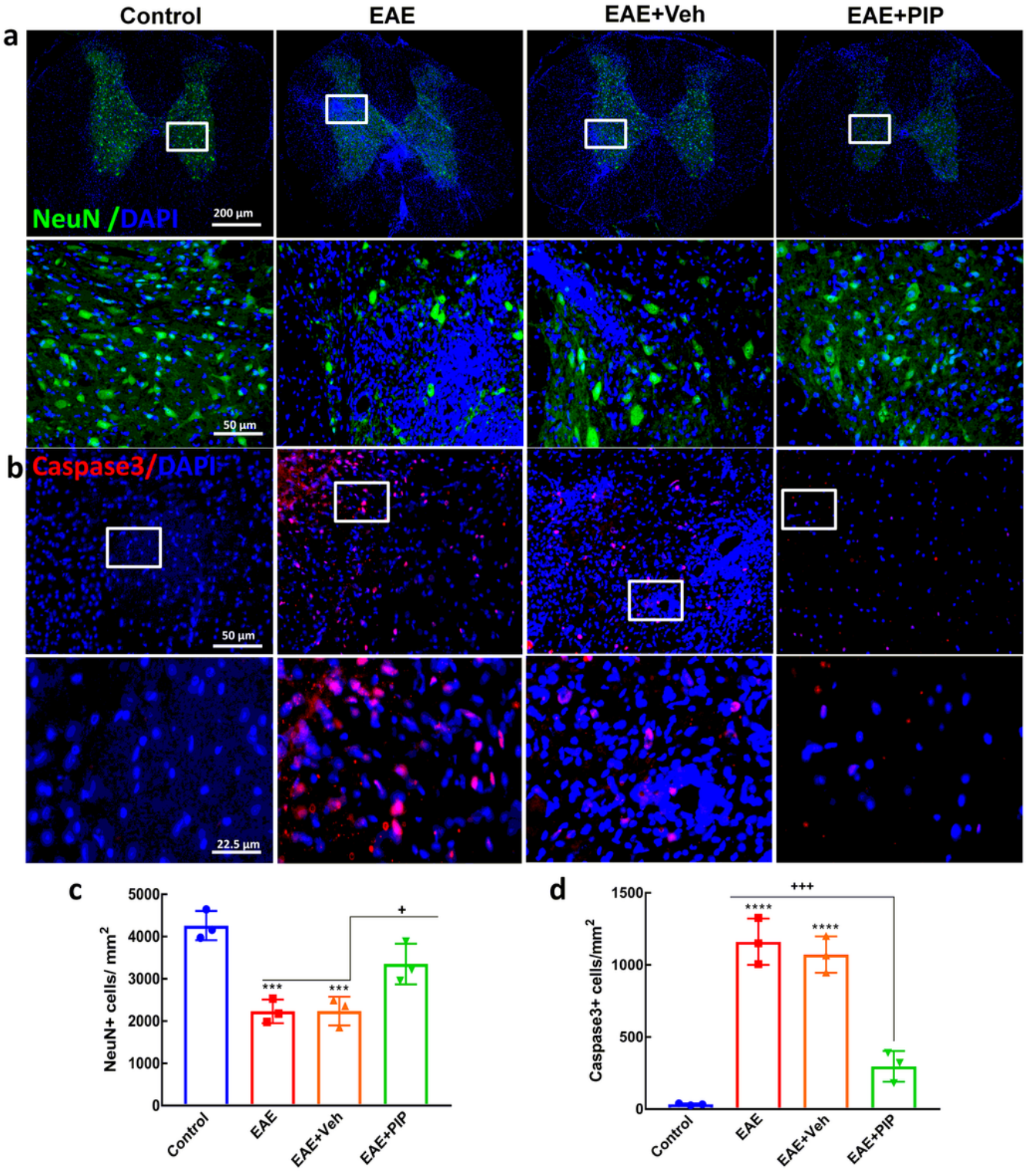

Figure 8

Piperine inhibited apoptosis in EAE model. Representative micrographs were prepared from the lumbar spinal cord sections of all experimental groups stained with NeuN as mature neuron marker (green) a and caspase-3 as marker of apoptosis (red) b and DAPI (blue) as a nucleus marker. Boxes show magnified image of selected areas. a Scale bar for NeuN images from the first row are 200µm and second 50 µm. b Scale bar for caspase-3 images from third row are 50 µm and the fourth row 22.5 µm. c Quantitative

analysis of the number of NeuN+ cells (neuron) in the lumbar spinal cord sections. The number of NeuN+ cells was considerably decreased in EAE and EAE+veh compared to control group (***P < 0.001). However, the number of NeuN+ cells in piperine-treated group was significantly higher than that in EAE and EAE+veh groups (+P < 0.05). d Quantification of the number of caspase-3+ cells in the lumbar spinal cord sections indicated that it significantly increased in EAE and EAE+veh compared to control group (****P < 0.0001). Piperine treatment effectively inhibited caspase-3 expression compared to EAE and EAE+veh groups (+++P < 0.001). Data is shown as mean ± SEM. One way ANOVA, post hoc Tukey was used, n=3.

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