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Neuroprotective effects of α-lipoic acid on long-term experimental autoimmune encephalomyelitis

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Abstract. – OBJECTIVE: Experimental autoimmune encephalomyelitis (EAE) is an animal model commonly used in research on the acute phase of multiple sclerosis (MS), but studies on the pathology and pathogenesis of EAE with a long disease course are seldom conducted. Besides its antioxidant properties, the comprehensive mechanisms through which α-lipoic acid (LA) affects EAE remain obscure. We here conducted the study to explore the possible mechanisms.

MATERIALS AND METHODS: In this study, the following methods were used for investigating the effects of LA on long-term EAE: hematoxylin-eosin staining (HE) and electron microscopic examinations of pathological changes; Western blotting of β-amyloid precursor protein (β-APP) and myelin basic protein (MBP); Enzyme-linked immunosorbent assay (ELISA) of tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), superoxide dismutase (SOD), malondialdehyde (MDA) as well as flow cytometry of CD4+CD25+FoxP3+ regulatory T cells (Tregs).

RESULTS: The results showed: (1) diverse pathological features of long-term relapsing-remitting EAE; (2) relatively increased MBP and reduced β-APP expression in LA recipients 180 days after onset; (3) down-regulated TNF-α and up-regulated TGF-β levels in LA recipients 7 days after onset; (4) lower MDA and higher SOD levels in LA recipients 180 days after onset; (5) increased Treg levels in LA recipients 7 days after onset.

CONCLUSIONS: Aside from oxidative stress, LA possessed anti-inflammatory and immunomodulatory effects on EAE. LA might be a promising candidate for MS treatment.

Key Words:

Multiple sclerosis, Experimental autoimmune encephalomyelitis, Oxidative stress, Inflammation, Immunomodulation, Neural regeneration.

Introduction

Multiple sclerosis (MS) is the most common autoimmune inflammatory demyelinating disease of the central nervous system (CNS). Affecting young adults globally, it results in motor, sensory and cognitive impairment[1]. Experimental autoimmune encephalomyelitis (EAE) in animals shares many features with MS, including the pattern of the clinical disease course, histopathological lesions of the CNS, the underlying pathophysiological mechanism and associated symptoms. EAE is thus widely used for the investigation of MS and for the assessment of novel treatments[2]. The typical pathological hallmarks of MS are the focally demyelinated lesions within the CNS white matter, along with a variable degree of inflammation, axonal loss, incomplete remyelination, and oligodendroglial involvement[3]. The impact of axonal pathology on clinical deficits has recently become a focus of major interest[4]. Inflammatory demyelinating changes of optic nerve can also be observed in multiple sclerosis[5]. Autoimmune disorders, inflammation and oxidative stress serve as the main pathogenesis of multiple sclerosis, in which encephalitogenic myelin-reactive T cells and cytokines play a crucial role[6]. α-lipoic acid (LA) has been proved to suppress oxidative stress-related diseases. In the current work, the positive anti-inflammatory and immunomodulatory roles of LA were confirmed through its effects on inflammatory demyelination, axonal degeneration and immunoregulation.

Materials and Methods

Drugs and Antibodies

Complete Freud's adjuvant (CFA) (Sigma-Aldrich Corp., St. Louis, MO, USA); α-LA (Sig-

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ma-Aldrich Corp., St. Louis, MO, USA) was administered i.p. for 7, 14 or 180 days following disease onset, 100 mg/kg/day; anti-myelin basic protein (MBP), anti-amyloid precursor protein (APP) for Western blotting, ELISA kits for tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), superoxide dismutase (SOD), malondialdehyde (MDA) (Wuhan Boster Biological Technology, Ltd., Wuhan, China); anti-mouse CD4 (FITC), anti-mouse CD25 (PE) and anti-mouse FoxP3 (PE-Cy5) (eBioscience, San Diego, CA, USA). FoxP3 Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA, USA). All drugs and antibodies were used according to the protocols recommended by the manufacturers. The breeding and use of the experimental animals all met the relevant requirements of Experimental Animal Ethics Committee of Hebei Medical University (SYXK 2016-003).

Histopathology Examination

The intact spinal cords, brains and optic nerves were removed from the sacrificed mice 7, 14 and 180 days after disease onset. The brains and spinal cords were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), dehydrated in a graded ethanol series, and sliced into 5 μm-thick axial sections for hematoxylin-eosin staining (HE). Some optic nerves and lumbosacral enlargements were fixed in a 5% glutaraldehyde solution (Sigma-Aldrich, St. Louis, MO, USA) and further prepared for transmission electron microscopic observation.

Western Blotting Analysis of MBP and APP

Anti-MBP and anti-APP antibodies were diluted to a concentration of 1:200. The spinal cords from C57BL/6 mice were individually homogenized in a RIPE buffer at 4°C, resolved in a 12% SDS-polyacrylamide gel at 200 V (20-50 μg per lane), and then transferred to nitrocellulose membranes. After blocking with 5% nonfat milk for 1 h, the membranes were sequentially incubated with MBP/APP antibodies and rabbit secondary antibodies (1:2000) conjugated to horseradish peroxidase (HRP). The reaction products were visualized with an ECL Western Blotting detection kit (Sangon Biotech, Shanghai, China), and Gel-Pro analysis software ImageJ 1.49 was used for protein quantification (National Institutes of Health, Bethesda, MD, USA). The densitometric values were normalized to those of GAPDH.

Enzyme-Linked Immunosorbent Assay (ELISA) of Cytokines

After eyeball removal, mouse blood was immediately dropped into ice-chilled EDTA-coated tubes containing 140 μg aprotinin; following a 2 h interval, the tubes were centrifuged for 15 min (3000 rpm, 4°C). The plasma was collected and stored at -80°C. TNF-α, TGF-β, SOD and MDA levels were detected with commercial ELISA kits following recommended protocols.

Flow Cytometry of CD4+CD25+FoxP3+Tregs

Freshly isolated splenocytes ($5x10^5$) were washed with PBS-azide and incubated with anti-CD4 and anti-CD25 (30 min, 4°C). After further washing of the cells, intracellular detection of FoxP3 was conducted following a FoxP3 staining protocol. Data acquisition and analysis were performed using a BD FACSCanto flow cytometry system and FACSDiva software (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

Data were presented as mean ± standard deviation and analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Statistical analyses between two groups were analyzed by the Student's *t*-test, while differences between three or more groups were analyzed by one-way analysis of variance followed by the Newman-Keuls multiple comparison test. The value of $p < 0.05$ was considered statistically significant.

Results

Clinical Manifestations

To assess the effects of LA, daily neurological scores of experimental mice were recorded along the disease course (Figure 1), and progression inhibition effects were observed (triangles). Although the onset time of EAE was not delayed, LA treatment reduced the mean maximum neurological score and relapse frequency. Compared to saline recipients, the LA recipients showed a significantly milder condition after LA treatment for 7 days (squares). We inferred that LA alleviated disease severity of EAE in the acute phase.

Histopathological Findings: Hematoxylin-Eosin Staining (HE) and Electron Microscopic Examination

Perivascular inflammatory cell infiltration, demyelination accompanied by incomplete remye-

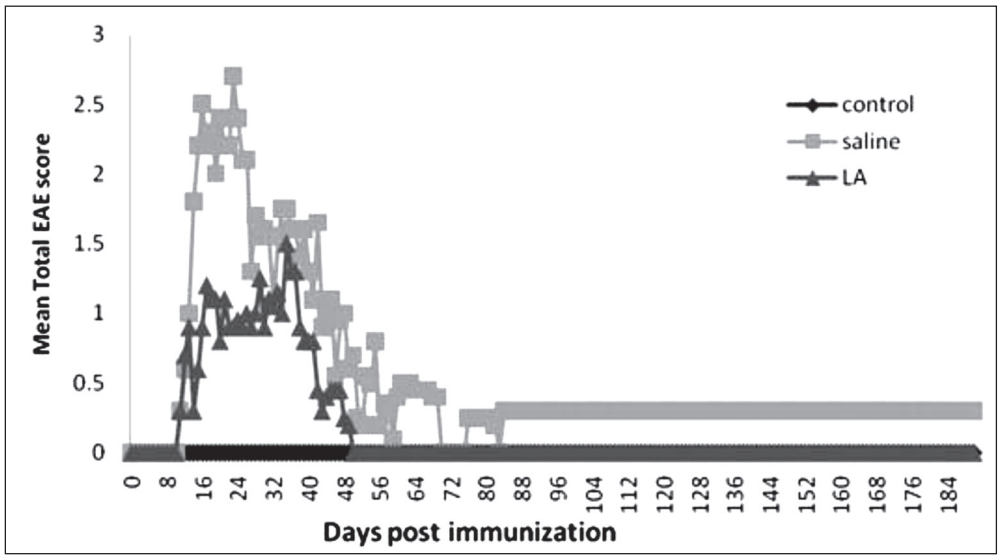

Figure 1. Manifestations of EAE mice. Inhibition effects of LA on EAE progression with markedly reduced mean maximum neurological scores and relapse frequency (triangles, $p < 0.05$, one- way analysis of variance). LA administration significantly alleviated disease severity compared to saline recipients 7 days after onset (squares, $p < 0.05$, one-way analysis of variance).

lination, and axonal injury are typical pathological features of EAE[3]. To evaluate inflammatory cell infiltration, HE staining was performed (Figures 2). There were scarcely any inflammatory cells in samples from control group mice (A, B). A large number of inflammatory cells presented with perivascular cuff formation in saline recipients (C), but evidently less in LA recipients 7 days after EAE onset (E). No differences in inflammatory cell infiltration were observed be-

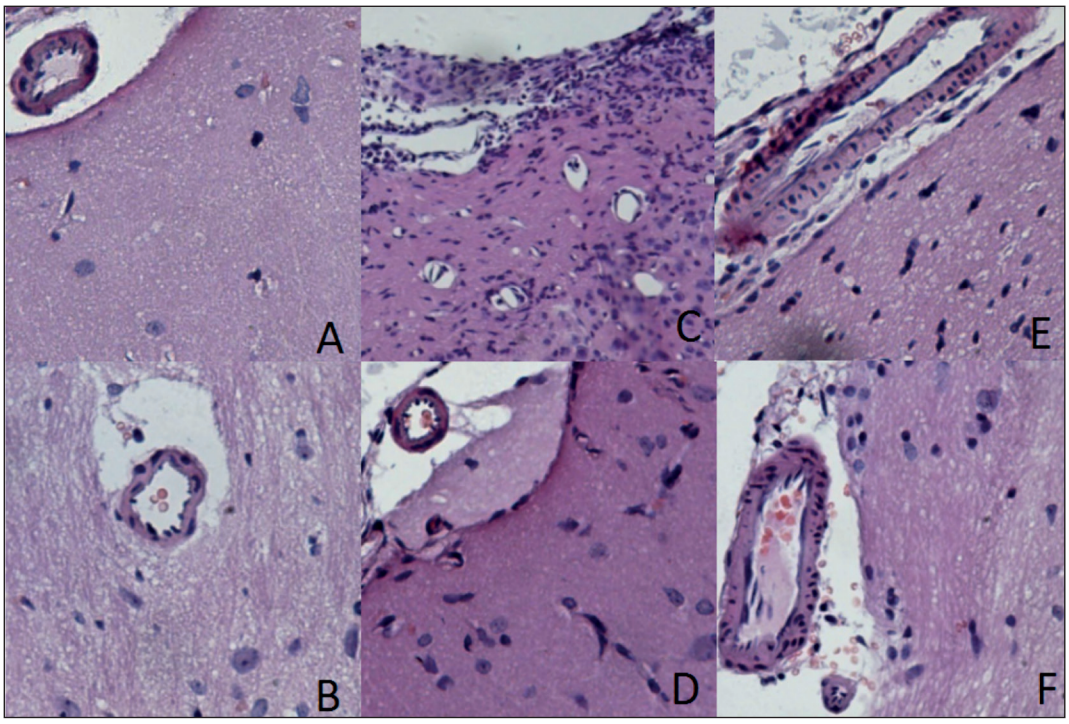

Figure 2. HE staining of brain slices. Histologic examination of brain slices from control group mice ***(A, B)***, saline recipients ***(C*** and ***D)*** or LA recipients *(E* and ***F)***, 7 days and 180 days after onset. Reduced inflammatory infiltrates in LA recipients compared to saline recipients 7 days after onset ***(C*** *vs.* ***E)*** (magnification × 400)

tween saline (D) and LA (F) recipients 180 days after onset (Magnification 400 x). In summary, significantly reduced inflammatory cell levels in LA recipients 7 days after EAE onset attested to the remarkable anti-inflammatory effects of LA. It is further speculated that LA exerted its anti-inflammatory effects in the acute phase of EAE rather than in the long-term course of the disease.

Electron micrographs of optic nerve sections showed diverse pathological features of EAE, including: loose myelin sheaths, degenerating axons, demyelinated axons, and cellular debris in axons (Figures 3). Degenerating axons with unstable myelin were apparent in the optic nerves of saline recipients (C, D). The axons were comparatively well-preserved in the optic nerves of LA recipients (E, F). The conclusion was thus drawn that LA played a protective role on axons.

Western Blotting of MBP and APP

Demyelination is the classic pathological feature of EAE. As the main component of myelin, MBP is generally considered to be a specific marker of myelin injury. In the present study, Western blotting of MBP was performed to evaluate demyelination (Figures 4). The results showed no differences between the saline, LA recipients and control group 7 days after onset. Compared to saline recipients, higher MBP expression was observed in LA recipients 180 days after onset. These findings suggested that LA may possess positive effects on suppressing myelin loss in long-term EAE.

Axonal injury has more recently been identified as one of the characteristic pathological changes of EAE. With significant aggregation, APP is thought to be the rapid response protein in axonal injury. In the present study, axonal injury was evaluated by APP Western blotting (Figures 5). Constant axonal injury was observed in long-term EAE in saline recipients. Statistical results indicated that axonal injury continued to progress even after LA administration. Nevertheless, LA somewhat alleviated axonal injury, as statistical differences between LA and saline recipients were observed both 7 and 180 days after onset.

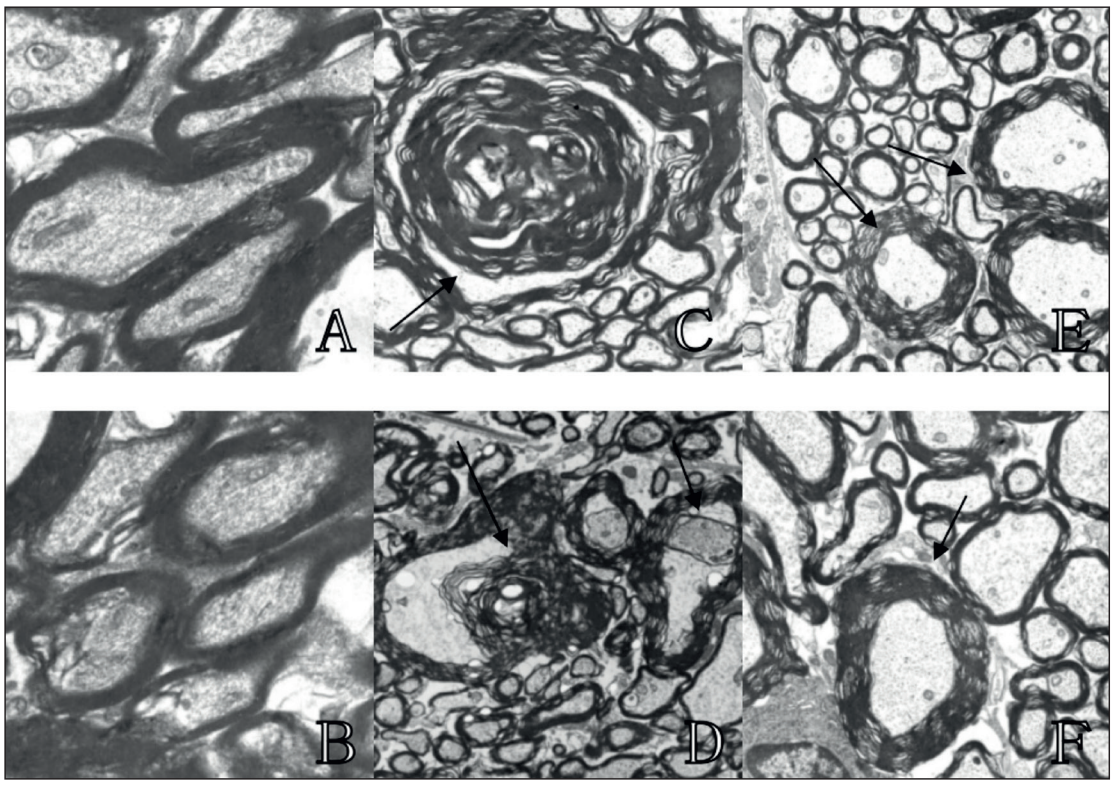

Figure 3. Electron micrographs of optic nerve slices. Diverse pathological features of EAE: loose myelin sheath, degenerating axons, demyelinated axon, and cellular debris in an axon. Optic nerves of control group mice ***(A, B)***. Degenerating axons with unstable myelin in optic nerves from saline recipients 7 and 180 days after onset ***(C, D)***. Comparatively well-preserved axons in the optic nerves from LA recipients 7 and 180 days after onset (***E, F)*** (magnification 400 x).

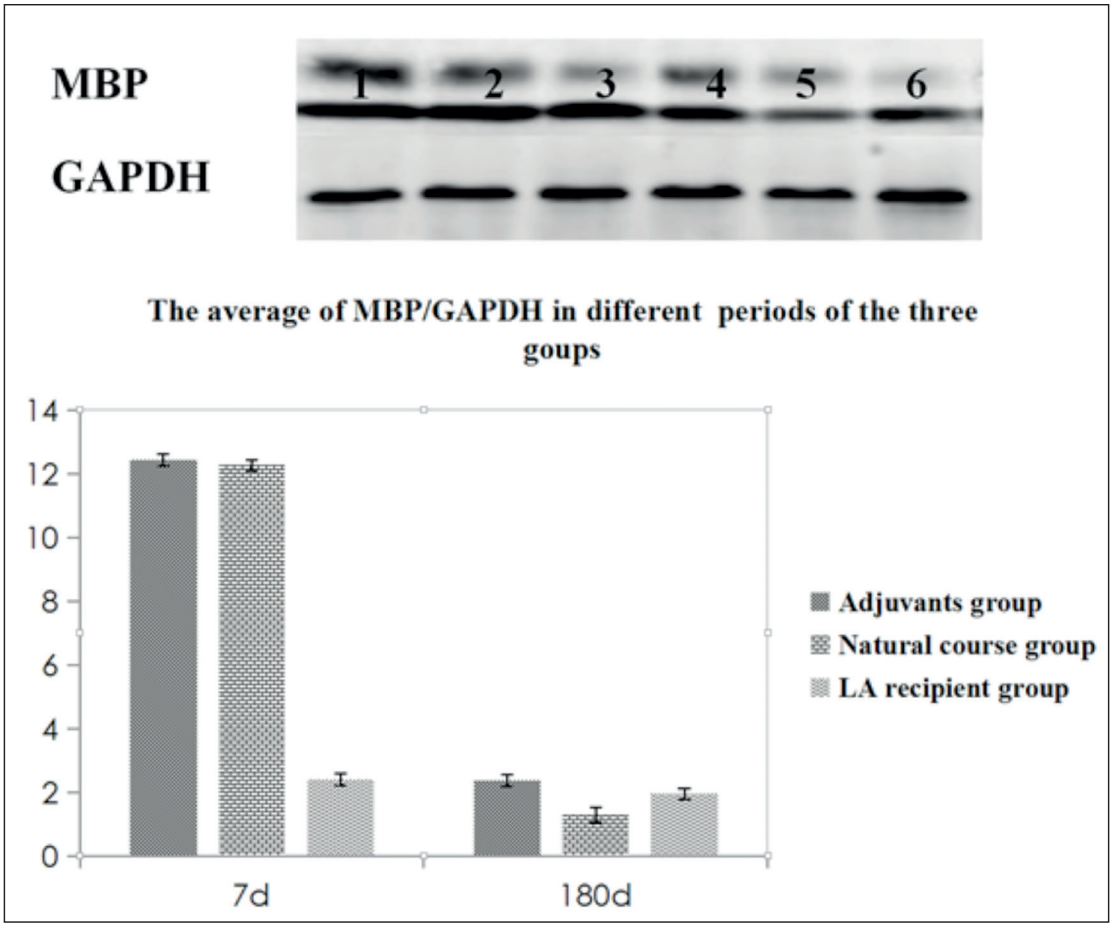

Figure 4. The protective effects of LA on myelin sheaths. Compared to saline recipients, there was higher MBP expression in LA recipients 180 days after onset ($p < 0.05$, one-way analysis of variance).

Overall, LA demonstrated distinct protective effects on axons both in the acute phase and the long-term course of the disease.

ELISA of TNF-α, TGF-β, MDA and SOD

TNF-α was closely associated with demyelination in EAE as an inflammatory effector. To explore the expression changes of TNF-α in either EAE alone or in EAE after LA administration, ELISA tests were conducted. Markedly increased levels of TNF-α were detected in saline and LA recipients both 7 and 14 days after onset, indicating that increased expression of TNF-α contributed to the progression of EAE (Figure 6). Compared to the EAE group, TNF-α levels decreased in LA recipients both 7 and 14 days after onset (Figures 6). In summary, the conclusion could be drawn that LA inhibited the inflammatory response in early EAE through down-regulating TNF-α expression.

TGF-β has been confirmed to be a protective factor in MS. It inhibits the proliferation and activation of microglia and T cells. In the present study, statistically lower levels of TGF-β were observed in both EAE and LA recipients compared to control group 7 days after onset, suggesting that a decreased TGF-β level was associated with disease activity in the acute phase of EAE (Figure 7). TGF-β expression was up-regulated in the LA recipients compared to EAE mice 7 days after onset and was simultaneously accompanied with disease remission (Figure 7). In summary, LA significantly alleviated the severity of EAE in acute phase through up-regulating TGF-β expression.

MDA is the major aldehyde product of lipid hydroperoxide injury, and is thus a preferred marker for oxidative stress and free radical damage. MDA levels were measured to evaluate oxidative damage in the present work (Figure 8). Compared to those 7 days after onset,

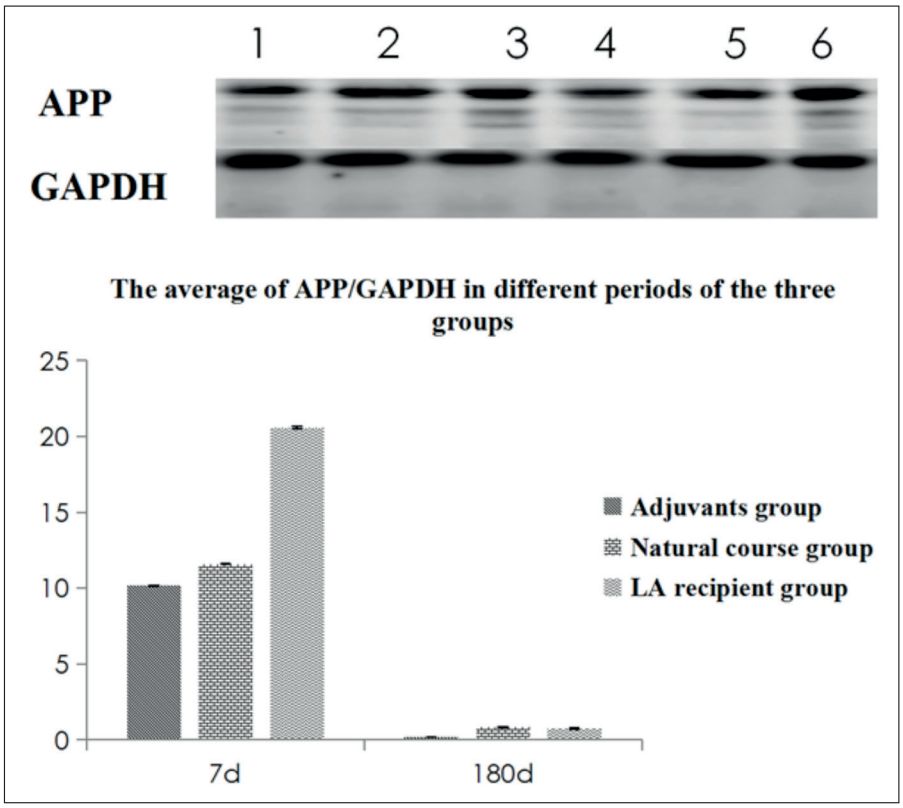

Figure 5. The protective effects of LA on axon. There were significant differences of APP expression between LA and saline recipients 7 and 180 days after onset ($p < 0.05$, respectively, one-way analysis of variance). EAE mice in both saline and LA recipient groups showed persistent axonal injury during the long-term disease course ($p < 0.01$, respectively, 7 *vs.* 180 days after onset, one-way analysis of variance).

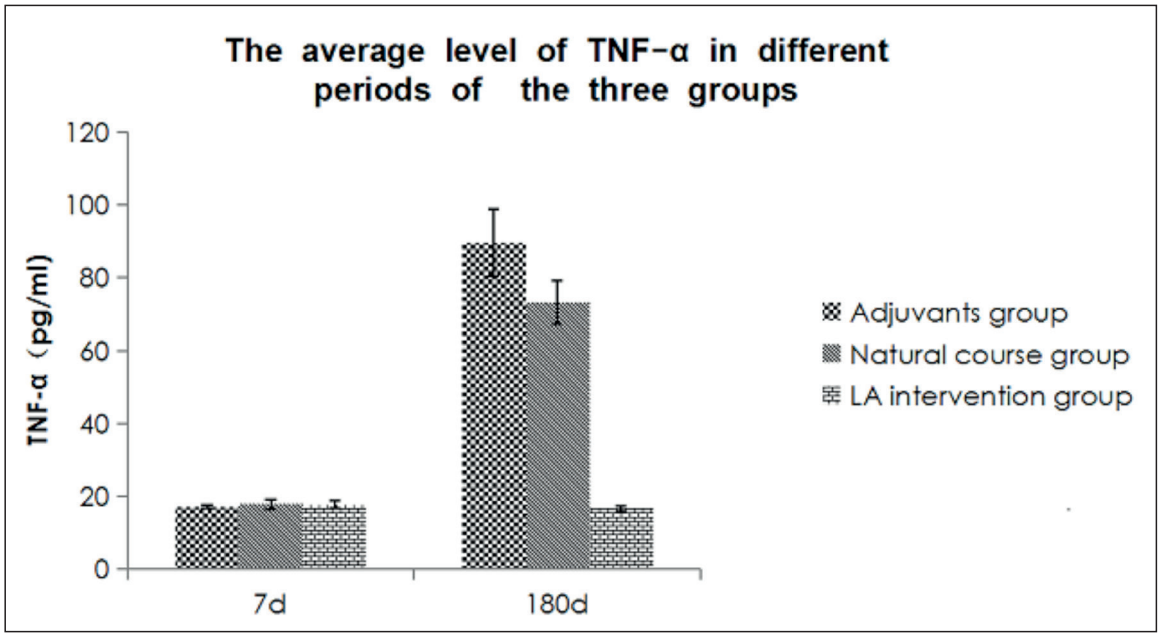

Figure 6. LA intervention reduced TNF-α expression. Associated with EAE progression, TNF-α levels increased in both saline and LA recipients 7 and 14 days after onset compared to control groups ($p < 0.01$, one-way analysis of variance). Compared to natural course groups, TNF-α levels decreased in LA intervention groups both 7 and 14 days after onset ($p < 0.01$ and $p < 0.05$, respectively, one-way analysis of variance).

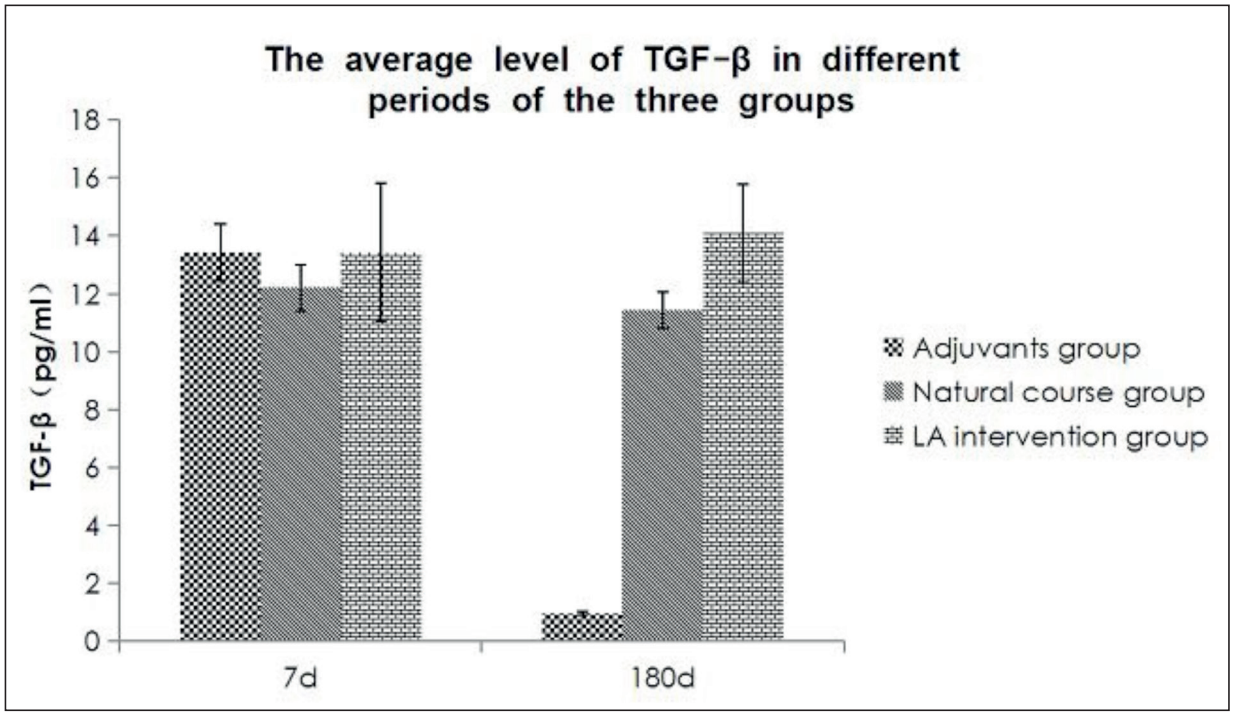

Figure 7. LA intervention increased the expression of TGF-β. Compared to natural course groups, TGF-β expression increased in LA recipient groups 7 ($p < 0.01$, one-way analysis of variance) and 14 days ($p < 0.05$, one-way analysis of variance) after onset. The expression trends of TGF-β were related to disease remission.

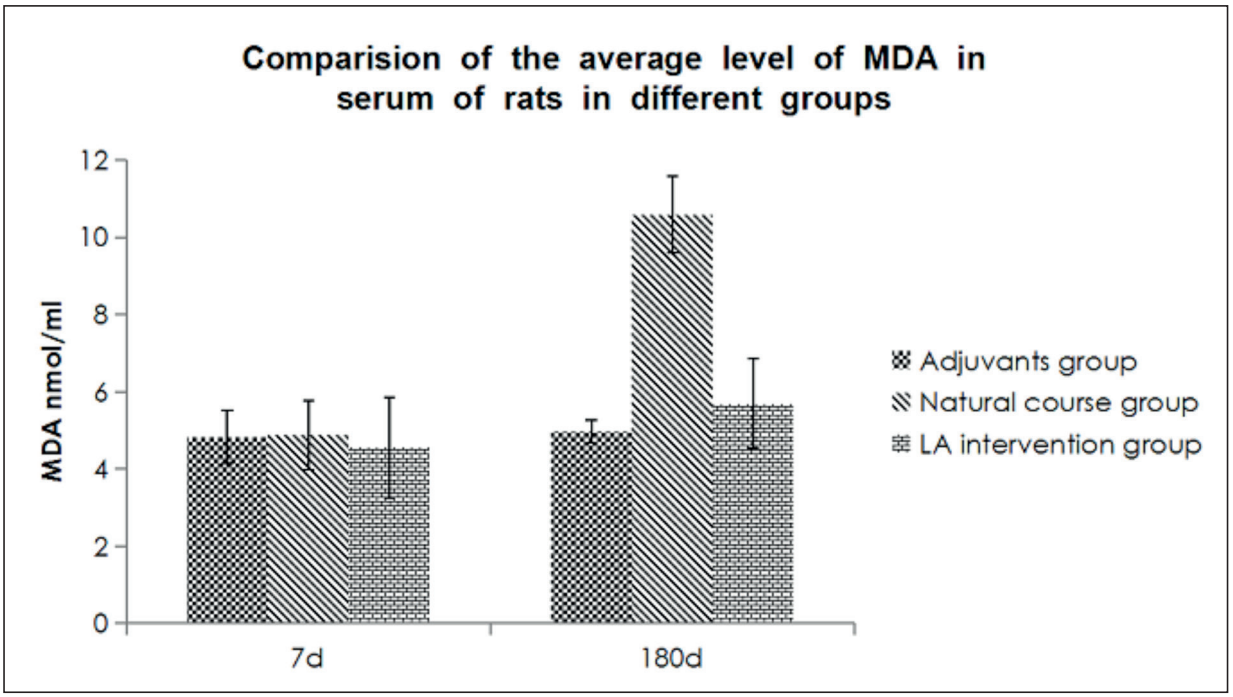

Figure 8. LA reduced lipid hydroperoxide injury. MDA expression was increased in the natural course group 7 days *vs.* 180 days after onset ($p < 0.01$, one-way analysis of variance). Compared to natural course groups, MDA expression was reduced 180 days after onset in the LA intervention group ($p < 0.05$, one-way analysis of variance).

MDA levels increased significantly 180 days after onset in the saline recipients, suggesting that oxidative stress persisted during the long-term development of EAE lesions. The levels of MDA in saline recipients were significantly higher than those in LA recipients 180 days after onset. In conclusion, it was inferred that LA might inhibit oxidative stress and alleviate lipid hydroperoxide injury in EAE in a long-term manner.

As an important antioxidant enzyme, SOD is involved in neuroinflammatory disorders. Existing researches have confirmed that SOD gene transcription and expression are increased both in EAE and MS. In our research, ELISA tests were conducted to assess SOD level changes (Figure 9). There were significantly higher levels of SOD in saline recipients compared to LA recipients 7 days after disease onset, which might partly be interpreted as the result of severe oxidative stress. As there were decreasing levels of SOD in the LA recipients, we further suggested that with LA treatment, the intervention of an exogenous antioxidant inhibited the oxidative stress of EAE and was accompanied by reduced endogenous antioxidant production. Compared to 7 days after onset, the levels of SOD were significantly elevated 180 days after onset in LA recipients, indicating that LA enhanced SOD production in a long-term manner and played an antioxidant role during the long-term course of EAE.

Flow Cytometry of Tregs

With the role of immunoregulation, CD4+CD25+FoxP3+ regulatory T cells (Tregs) affect the progression and prognosis of autoimmune diseases. Tregs were detected by flow cytometry in the present study to investigate the effects of LA on immunoregulation (Figures 10). There was a reduction in the quantity of Tregs in the peripheral blood during the acute phase of EAE but an increase during remission in both LA recipients and EAE mice. Compared to that of EAE mice, Treg levels of LA recipients increased obviously 7 days after onset. Accordingly, we suggested that LA played a role in immunomodulation through up-regulating Treg expression, thereby promoting the remission of EAE.

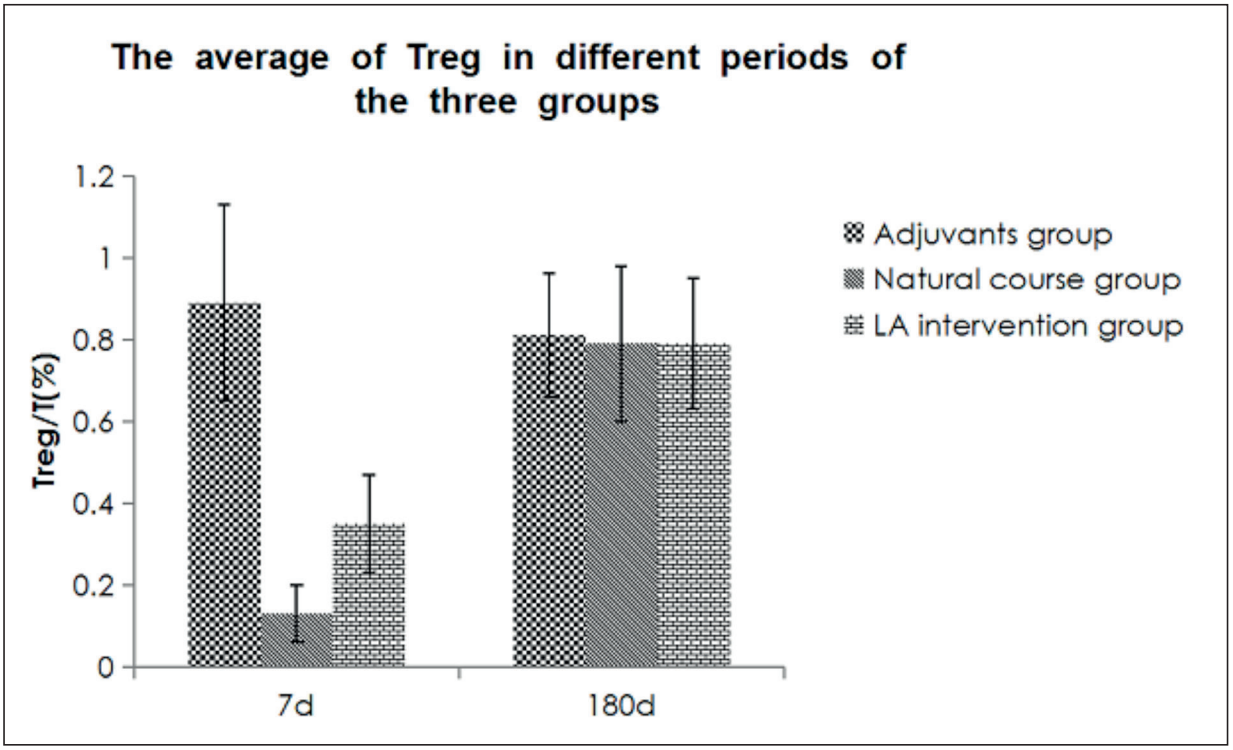

Figure 9. LA increased the expression of SOD. Compared to 7 days after onset, the levels of SOD were significantly elevated 180 days after onset in LA recipients ($p < 0.01$, one-way analysis of variance), indicating that LA enhanced SOD production in a long-term manner and play an antioxidant role during the long-term course of EAE.

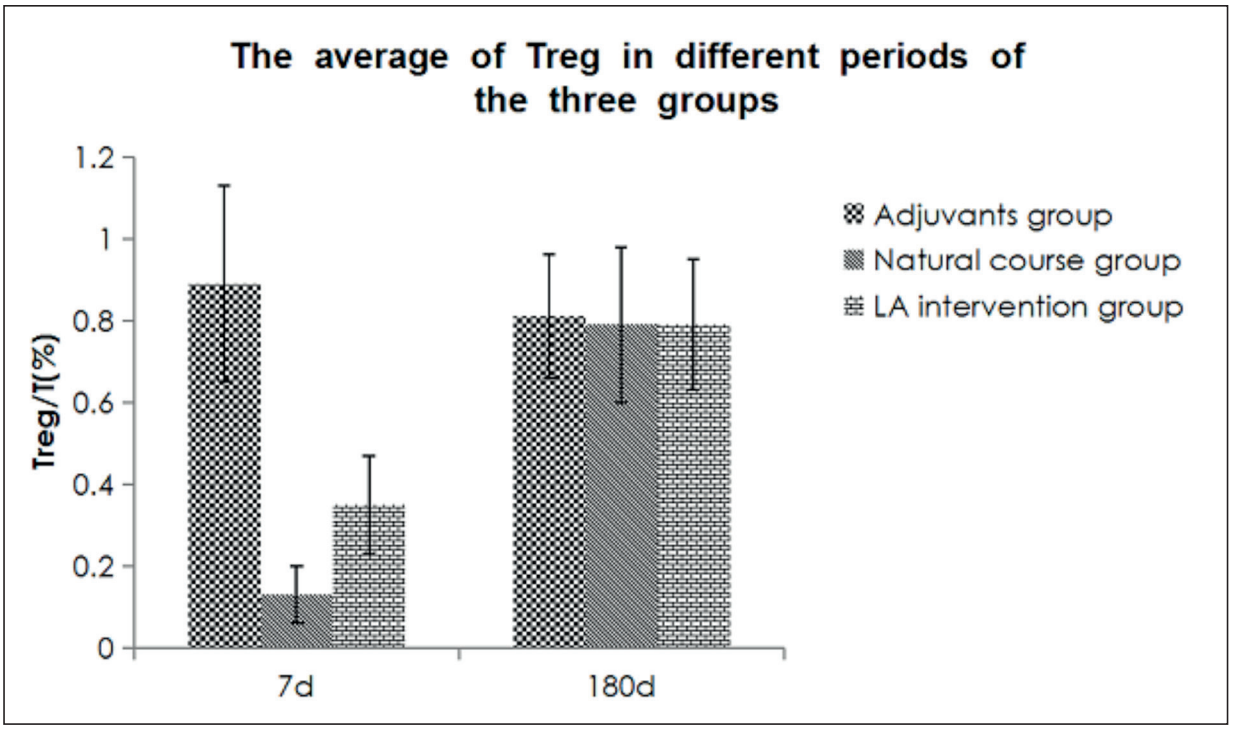

Figure 10. LA intervention increased the expression of Tregs. Compared to natural course groups, Treg expression increased in LA recipient groups 7 ($p < 0.01$, one-way analysis of variance) and 14 days ($p < 0.05$, one-way analysis of variance) after onset. The expression trends of Tregs were consistent with that of TGF-β, and they both were related to disease remission.

Discussion

MS is an inflammatory demyelinating disease of the CNS leading to multiple chronic neurological disabilities in early to middle adulthood, especially in females[7]. As a widely used animal model of MS, EAE can be induced in various species through different immunization protocols[8], and is particularly important for mechanistic investigation and treatment assessment[2]. Traditionally, focal inflammatory demyelinated plaque with axonal preservation and reactive astrocytic scar formation in the white matter had been regarded as typical pathological features of MS[9]. researches[10] have discovered that there were earlier pathological changes involved the axons and neurons even preceding myelin destruction. The losses of axons and whole neurons have been detected both in normal appearing white matter (NAWM) and cortical gray matter, even outside the sites of demyelination[11]. Recent studies have supported that APP might primarily be a marker of early axonal damage[12], as well as a biomarker of disease progression in both MS and EAE[13]. The reduction of APP expression observed in EAE might reflect a protective role possessed by certain neuroactive agents. Constant axonal injury was found during the long-term disease course of EAE, even after LA treatment. When compared to saline recipients, LA administration somewhat alleviated axonal injury, manifesting a distinct axonal protective effect both in the acute and the long-term course of disease. As an essential myelin component, MBP is believed to be one of the main targets for autoreactive lymphocytes involved in MS. Destruction and abnormal distribution of MBP contributed to neuronal damage that occurred in the progressive phase of MS[14]. Increased expression of MBP genes involved in myelin repair and neuroregeneration indicated the therapeutic and neuroprotective implications of certain drugs[15]. In the present study, higher MBP levels in LA recipients compared to saline recipients 180 days after onset demonstrated that LA might alleviate myelin loss during the long-term disease course. Immunological inflammation is regarded as the most important pathogenesis of multiple sclerosis. With different cytokine profiles, the roles of various T cell subsets in the pathophysiology of MS have been well-established[16]. The immune

response in MS is generally considered to be shifted towards Th1 cytokine production, including TNF-α[17]. TNF-α presents with elevated levels in both the serum and active lesions of MS patients[18], and is an important participant in the attack stage of EAE[19]. The present study congruously demonstrated that TNF-α was associated with disease deterioration in the acute phase of EAE. Furthermore, LA might exert an anti-inflammatory effect on EAE through down-regulating TNF-α expression. TGF-β is a pleiotropic cytokine that has been shown to influence the differentiation and functioning of T cells, and thus playing an important role in immune-mediated diseases[20]. Immune tolerance in EAE might be achieved by increasing the production of TGF-β from Tregs[21,22]. As an immunoregulatory factor, TGF-β inhibits microglia activation and the production of related cytokines, promotes cytotoxic T lymphocyte-associated antigen-4 (CTLA-4)-mediated immunosuppression, and enhances the production of Tregs [23]. Consistent with previous investigations, we observed that decreasing TGF-β levels were associated with disease activity in the acute phase of EAE. Moreover, LA up-regulated TGF-β production accompanied with EAE remission. In summary, LA alleviated acute phase disease severity through up-regulating TGF-β expression. With immunomodulatory effects, Tregs affect the onsets and prognoses of many autoimmune diseases. FoxP3 transcription factor is pivotal for Tregs to carry out their immunomodulatory functions. Immune incompetence as well as the immunosuppression characteristics of Tregs were implemented through cell-to-cell contacts or through the cytokine-medated pathway[24,25]. Quantity and/or function abnormalities of Tregs in MS patients were associated with the fluctuation of patients' conditions[26,27]. Clinical remission of MS was correlated with the restoration of the inhibitory function of memory T cell-derived Tregs. In the present study, a similar relationship between Tregs variability and EAE condition was observed. With increased Treg levels, LA exerted immunomodulatory effects on EAE and promoted disease remission. Oxidative stress (OS) has recently attracted increasing attention in the pathogenesis study of MS[28]. With high oxygen consumption, low antioxidant capacity and large amounts of polyunsaturated fatty acids, the CNS is vulnerable to oxidative stress[29]. Oxidative stress leads to demyelination and axonal injury through multiple mechanisms [30,31]. Malondialdehyde (MDA), the major aldehyde product of lipid hydroperoxide injury, is considered as a preferred marker of oxidative stress[32]. In the present study, MDA levels were measured to assess oxidative damage. The findings showed that oxidative stress persisted in long-term EAE lesions, and that LA inhibited oxidative stress and reduced lipid hydroperoxide injury with decreasing levels of MDA. Superoxide dismutase (SOD) is an important antioxidant enzyme in the anti-free radical system of the body. Former investigations demonstrated that SOD was involved in neurodegenerative and neuroinflammatory disorders. In active lesions of both MS and EAE, cell matrix SOD (SOD1) and mitochondrial SOD (SOD2) gene transcription and expression were significantly elevated, facilitating the elimination of reactive oxygen species[33,34]. Based on the results of this study, the authors suggested that, accompanied by reduced endogenous antioxidant production, LA alleviated oxidative stress in the acute phase of EAE and played a long-term role in antioxidant stress during the whole of the disease course observed. LA has attracted great research interest in the areas of oxidative stress and immunomodulation as a natural antioxidant[35]. With neuroprotective properties, LA has been applied to various neurological disorders, including MS[36]. LA plays anti-inflammatory and immunomodulatory roles by inhibiting the expression of intercellular adhesion molecules and the migration of T cells into the spinal cord[37]. This study showed that, compared to saline recipients, LA treatment reduced inflammatory cell infiltration within active lesions and decreased myelin damage and axonal injury so as to achieve long-term neuroprotective effects. LA down-regulated the expression of TNF-α, increased the levels of TGF-β and Tregs, exerted anti-inflammatory and immunomodulatory effects on EAE.

Conclusions

We demonstrated that LA ameliorated the severity of EAE, and played anti-inflammatory, immunomodulatory roles in the acute phase of EAE in addition to its antioxidant stress effect. Demyelination and axonal damage were markedly reduced by LA in the long-term course of EAE. Therefore, with multiple beneficial effects on EAE, LA might also be applicable to the treatment of MS.

Acknowledgements
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Conflict of Interest
The Authors declare that they have no conflict of interests.

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