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Article

Cornuside ameliorated experimental autoimmune encephalomyelitis by limiting the recruitment of CD4+ T lymphocytes in the spinal cord

Rongbo Zhang[1], Shuijing Zhang[2], Bin Xu[1], You Wu[1], Shunli Liang[1], Bonan Hou[1], Mimi Wang[3], Jin Liu[4], Qiang Yuan[4,*]

[1]Department of Neurology, The Second Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310005, China, [2]Department of Neurological Rehabilitation, Zhejiang Rehabilitation Medical Center, Hangzhou, Zhejiang 310051, China, [3]Department of Pharmacy, The Second Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310005, China, [4]College of Pharmacy, Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310053, China

We conducted this study to determine whether cornuside could improve the neurological deficit symptoms of experimental autoimmune encephalomyelitis (EAE) rats, as well as determine the potential involvement of $CD4^+$ T lymphocytes, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and tumor necrosis factor-α (TNF-α). Altogether, 32 Lewis rats were randomly divided into control, EAE, EAE/prednisolone, and EAE/cornuside, wherein their neurological function was assessed every day. $CD4^+$ T lymphocyte recruitment into the spinal cord (SC) was evaluated using immunohistochemistry. The VCAM-1, ICAM-1 and TNF-α mRNA expressions in the SC were determined by real-time quantitative PCR, and the VCAM-1 and ICAM-1 proteins were determined by western blotting. Compared to the control group, the EAE group rats with neurological deficits had enhanced $CD4^+$ T lymphocyte infiltration and higher expression levels of VCAM-1, ICAM-1, and TNF-α in the SC. Meanwhile, compared with the EAE group, the EAE/cornuside and EAE/prednisolone groups had lower neurological scores, less $CD4^+$ T lymphocyte infiltrations, and lower expression levels of VCAM-1, ICAM-1, and TNF-α in the SC. Thus, cornuside ameliorated EAE, which could be owed to the inhibition of $CD4^+$ T lymphocyte recruitment and VCAM-1, ICAM-1, and TNF-α expressions in the SC.

Keywords: Cornuside. Multiple sclerosis. Experimental autoimmune encephalomyelitis. T lymphocyte. Vascular cell adhesion molecule-1. Intercellular cell adhesion molecule-1.

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease that primarily damages the central nervous system (CNS) and mainly affects young adults, often leading to limb paralysis, sensory disturbance, and visual impairment, among others. (Dobson, Giovannoni, 2019; Oh, Vidal-Jordana, Montalban, 2018). At present, drugs for MS often have many side effects (Li *et al.*, 2020; McCall, 2019; McCool *et al.*, 2019). Therefore, it is necessary to explore more therapeutic drugs for MS treatment.

It is known that the MS pathogenesis involves virtually all innate and adaptive immune-mediated mechanisms (Lazibat, Rubinic Majdak, Zupanic, 2018). Bidirectional interactions among peripheral T and B cells, myeloid cells, CNS-resident microglia, and astrocytes contribute to MS inflammation, demyelination, and neurodegeneration (Filippi *et al.*, 2018). Nevertheless, activation of potentially autoreactive $CD4^+$ T lymphocytes is considered the key triggering event in MS pathogenesis (Constantinescu *et al.*, 2011; *Lassmann*, Bradl, 2017; Lazibat, Rubinic Majdak, Zupanic, 2018). These activated

***Correspondence:** Q. Yuan. College of Pharmacy. Zhejiang Chinese Medical University. Hangzhou, Zhejiang 310053, China. Phone: +86-0571-86613653. E-mail: yuanqiang0825@163.com. Rongbo Zhang ORCID: https://orcid.org/0000-0002-0342-0815

cells differentiate, express adhesion molecules, and gain blood-brain barrier (BBB) permeability. Upon CNS reactivation, $CD4^+$ T cells secrete inflammatory products and cytokines which directly damage myelin and axons, or indirectly facilitate inflammatory lesion formation by activating CNS-resident cells and attracting further peripheral inflammatory cells (Constantinescu *et al.*, 2011; Filippi *et al.*, 2018; Lazibat, Rubinic Majdak, Zupanic, 2018). In relation to this, experimental autoimmune encephalomyelitis (EAE) is one of the most widely used animal models of MS (Naghashpour *et al.*, 2016; Pitarokoili, Ambrosius, Gold, 2017; Xue *et al.*, 2016). Furthermore, it has been confirmed that vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) are the main factors mediating T cell BBB migration and infiltration (Abadier *et al.*, 2015; Anderson, Buffone, Hammer, 2019; Engelhardt, Ransohoff, 2012).

Cornuside, one of the cornel iridoid glycosides extracted from Corni Fructus, has been widely used in traditional oriental medicine for treating inflammatory diseases (Qu *et al.*, 2019; Yin *et al.*, 2014). For example, it has been reported that cornuside can significantly reduce tumor necrosis factor-α (TNF-α)-induced VCAM-1 and ICAM-1 expressions (Kang *et al.*, 2007). However, it remains unclear whether cornuside can inhibit CNS VCAM-1 and ICAM-1 expressions, and $CD4^+$ T lymphocyte recruitment, ultimately improving the neurological symptoms of EAE rats.

Consequently, the aim of the present study is to determine whether cornuside could improve the neurological deficit symptoms of EAE rats, as well as determine the potential involvement of $CD4^+$ T lymphocytes and VCAM-1 and ICAM-1 in these processes.

MATERIAL AND METHODS

Material

Cornuside (CAS number: 131189-57-6, purity: 99.647%) was provided by the Chengdu Herbpurify Co., Ltd, China. The molecular formula is $C_{24}H_{30}O_{14}$, and the molecular weight is 542.49.

Animals

A total of 32 male Lewis rats (7-8 weeks of age) were provided by the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Following the institutional guidelines of the Zhejiang Chinese Medical University (Hangzhou, China), these rats were placed in a specific pathogen-free animal barrier environment within the research center. They had free access to food and water during the experiment and were preserved for one week of acclimation. This study was approved by the Laboratory Animal Management and Ethics Committee of Zhejiang Chinese Medical University.

Induction and treatment of EAE

On Day 0, the rats designated for EAE induction were immunized with 0.4 mL of immunization emulsion via four subcutaneous injections (two hind footpads and two sides of the base of tail, 0.1 mL per injection) (Liu *et al.*, 2019). The immunization emulsion was made up of guinea pig SC homogenate (1 g SC: 1 mL normal saline) and complete Freund's adjuvant (1 : 1, v/v, Sigma, USA), which contained incomplete Freund's adjuvant and 1 mg/mL of *Mycobacterium tuberculosis*.

The rats were then randomly divided into four groups (8 rats per group) as follows: (1) Control, naïve/normal saline treated rats; (2) EAE immunized/normal saline treated rats; (3) Prednisolone, immunized/prednisolone (10 mg/kg) treated rats (Liu *et al.*, 2014); and (4) Cornuside, immunized/cornuside (120 mg/kg) treated rats. Daily treatment begun on Day 0 via intragastric administration of 10 mL/kg, lasting until Day 14 after immunization.

Neurological evaluation

Neurological deficits were scored daily for 14 days using the following criteria: 0, no symptoms; 0.5, mild tail weakness; 1, severe tail weakness; 2, mild hind limb weakness with uncoordinated walking; 2.5, one hind limb paralyzed; 3, two hind limbs paralyzed; 3.5, rat drags two hind limbs, and has difficulty with forelimb use; 4, complete paralysis (tetraplegia); and 5, dying

or death secondary to EAE (Pitarokoili, Ambrosius, Gold, 2017).

Tissue harvesting and sectioning

On the 14th day after immunization, the rats were anesthetized with 10% chloral hydrate and were transcardially perfused with 4% paraformaldehyde. Afterwards, the rats were sacrificed, and their SCs were removed, wherein SC parts were rapidly frozen in liquid nitrogen and stored at -80 □. Meanwhile, the other parts were immersed in 4% paraformaldehyde for 48 hours and were embedded in paraffin. After which, SC sections (4 μm) were cut for immunohistochemistry (IHC) analysis.

Immunohistochemistry

Paraffin sections were dewaxed and hydrated, and the antigen was repaired under high temperatures and pressure (0.01M ethylenediamine tetra acetic acid, pH 9.0). After rinsing, the sections were immersed in a 0.3% H2O2 solution for 20 minutes, and then incubated with primary antibody (rabbit anti-CD4, 1:400; American Abcam) for 60 minutes at 37 °C. After three rinses, the sections were incubated with a secondary antibody (polymer horseradish peroxidase conjugated goat anti-rabbit IgG antibody, 1:1000; Santa Cruz, USA) for 40 minutes at 37 °C, and were visualized with diaminobenzidine after rinsing again. The nuclei were then stained with Harris hematoxylin solution for 1 minute. After which, all the sections were dehydrated using ethanol, hyalinized dimethylbenzene, and sealed using natural gum.

For quantitative analysis of CD4 positive T lymphocytes in the SC, three sections per rat were selected and photographed using an Olympus microscope (200× magnification), selecting 3-5 fields of view per section. The number of positive cells in more than 200 cells was counted and converted into CD4 positive ratio. The percentage of $CD4^+$ T lymphocytes was calculated using this formula: the number of CD4 positive cells in the fields of view /the total number of cells in the fields of view ×100%.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from the SC using a TRIzol reagent (Bio Basic Inc, Canada) and was reversely transcribed to cDNA using the PrimeScript™ RT reagent Kit (Takara Bio Inc, Japan). The PCR was set at 95 °C (1 minute), with 40 cycles of 95 °C (15 second) and 63 °C (25 second). The selected mRNA primers are shown in Table I. The expression level of the gene encoding rat 18s rRNA was then used to normalize VCAM-1 and ICAM-1 mRNA expressions, wherein the target gene relative expression levels were calculated using the $2^{-\square\square Ct}$ method.

TABLE I - Forward and reverse primers of mRNA used in RT-PCR assay

Gene	Forward	Reverse
Vcam1	GATGTCATTGCGAGGTCGTTTAGA	GCATGAAGTTACAACAGTCAGTCCAAG
Icam1	CAGGTATCCATCCATCCCACAGA	CGTCTTCGCAAGAGGAAGAGCAGT
18s rRNA	GAATTCCCAGTAAGTGCGGGTCATA	CGAGGGCCTCACTAAACCATC
Tnfα	TCTACTGAACTTCGGGGTGATCGG	GTGGTTTGTGAGTGTGAGGGTCTG

Western blotting

The total SC protein was extracted using the tissue protein extraction reagent (Thermo Pierce, USA) and underwent quantitative analysis using the bicinchoninic acid method. The same amount of total protein (60 μg) was added to each pore, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was

performed for about 2 hours. Afterwards, the protein was transferred to polyvinylidene difluoride membranes (Millipore, USA) blocked with Tris-buffered saline and Tween 20 (TBS-T), and was then incubated overnight with primary antibodies (anti-VCAM-1, polyclonal rabbit anti-rat, 1:500, Santa Cruz, USA; anti-ICAM-1, monoclonal mouse anti-rat, 1:1000, Abcam, USA; β-actin, monoclonal mouse anti-rat, 1:1500, Santa Cruz, USA) in TBS-T at 4 °C. The horseradish peroxidase conjugated second antibody (goat anti-rabbit IgG, 1:2000, Santa Cruz, USA; goat anti-mouse IgG, 1:2000, Santa Cruz, USA) was then dissolved in TBS-T, incubating for 1 hour. Finally, the immunoreactive bands were visualized in enhanced chemiluminescence solution (GE Healthcare, USA) and exposed onto X-ray films. Following this, the relative expression levels of the target proteins were normalized to β-actin for analysis.

Statistical analysis

All data were presented as mean ± SEM. One-factor Analysis of Variance followed by Fisher's Least Significant Difference or Tamhane's T2 test was used for multiple comparisons. Statistical significance was considered at P 0.05 for all analyses.

RESULTS

Cornuside ameliorated clinical signs of experimental autoimmune encephalomyelitis

Compared to the control rats, substantial neurological deficit observed in the EAE group demonstrated that the EAE model was successfully established, showing that both prednisolone and cornuside group rats had significantly reduced mean clinical scores compared to the EAE group rats (Figure 1A). On Day 14 after immunization, the scores of the EAE, cornuside, and prednisolone groups were 3.83 ± 0.23, 1.63 ± 0.46, and 1.00 ± 0.42, respectively, with significant differences in the mean clinical score between the cornuside and EAE groups (P = 0.008). On the 11th day after immunization, EAE prevalence in the EAE group reached 100%, while it was 50% in the cornuside group (Figure 1B). Meanwhile, compared to the control group, the EAE group rats had significantly greater weight loss, while the cornuside group rats had less weight loss as compared to the EAE group from Day 11 after immunization (Figure 1C). Collectively, it is suggested that cornuside could improve the clinical symptoms of EAE.

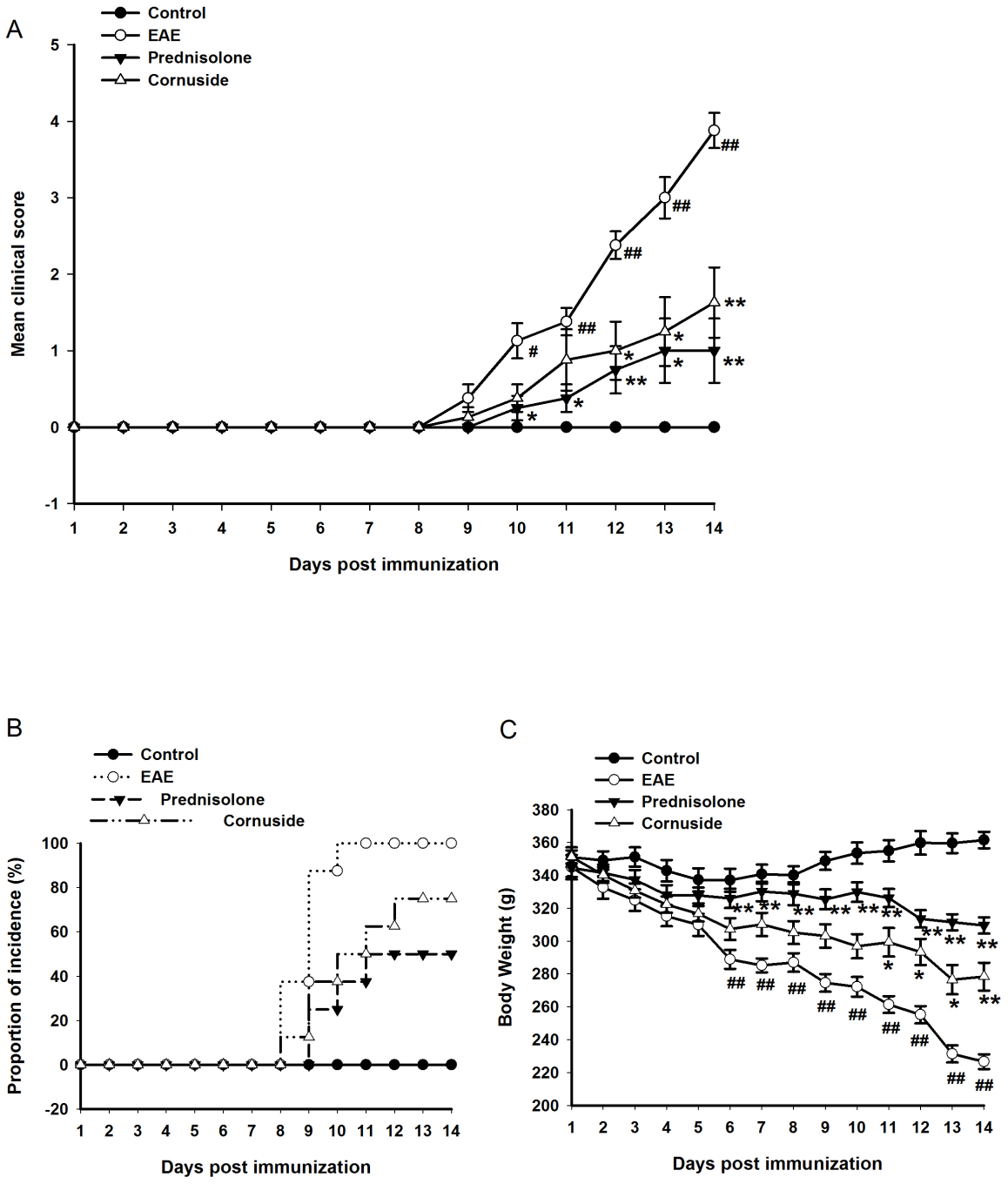

FIGURE 1 - Cornuside ameliorated clinical signs of experimental autoimmune encephalomyelitis (EAE). (A) Clinical scores of neurological deficits. (B) The proportional EAE incidence in rats of the four groups. (C) The body weight of the rats among the groups. **P*<0.05, ***P*<0.01 for the cornuside or prednisolone group versus the EAE group, $^{\#}P$<0.05, $^{\#\#}P$<0.01 for the EAE group versus the control group.

Cornuside inhibited CD4+ T lymphocyte infiltration into the spinal cord

Compared to the control group, the number of T lymphocytes measured via IHC CD4 staining in SC was significantly elevated in the EAE group rats. On the other hand, rats from both the cornuside and prednisolone groups had markedly reduced CD4 staining compared to the EAE group (Figure 2). This indicates that cornuside could inhibit CD4+ T lymphocyte infiltration into the SC.

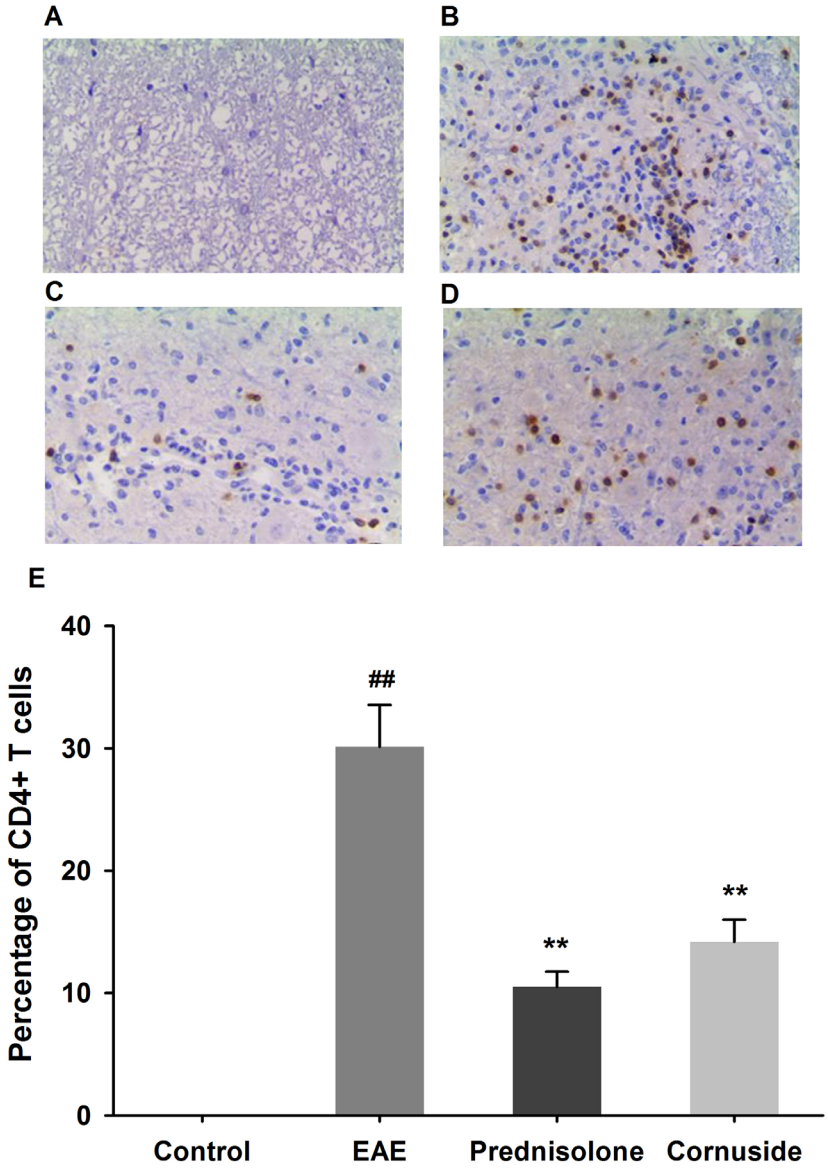

FIGURE 2 - Immunohistochemical (IHC) staining for CD4 positive T lymphocytes in the spinal cord. The representative CD4 positive T lymphocytes of the (A) Control, (B) EAE, (C) Prednisolone, and (D) Cornuside groups. (E) Percentage of CD4+ T lymphocytes. $^{**}P<0.01$ for the cornuside or prednisolone group versus the EAE group, $^{\#\#}P<0.01$ for the EAE group versus the control group. Original magnification ×200.

Cornuside inhibited VCAM-1 and ICAM-1 expressions in the spinal cord

Compared to the control group, the EAE group rats exhibited significantly increased SC VCAM-1 and ICAM-1 mRNA expressions, while rats from both the cornuside and prednisolone groups had decreased expression of both mRNAs compared to EAE group (Figure 3A). Similarly, the protein expression of VCAM-1 and ICAM-1 were elevated in the EAE group rats compared to the control group rats, while the expression of both proteins was significantly reduced in rats from the cornuside and prednisolone groups compared to the EAE group (Figure 3B).

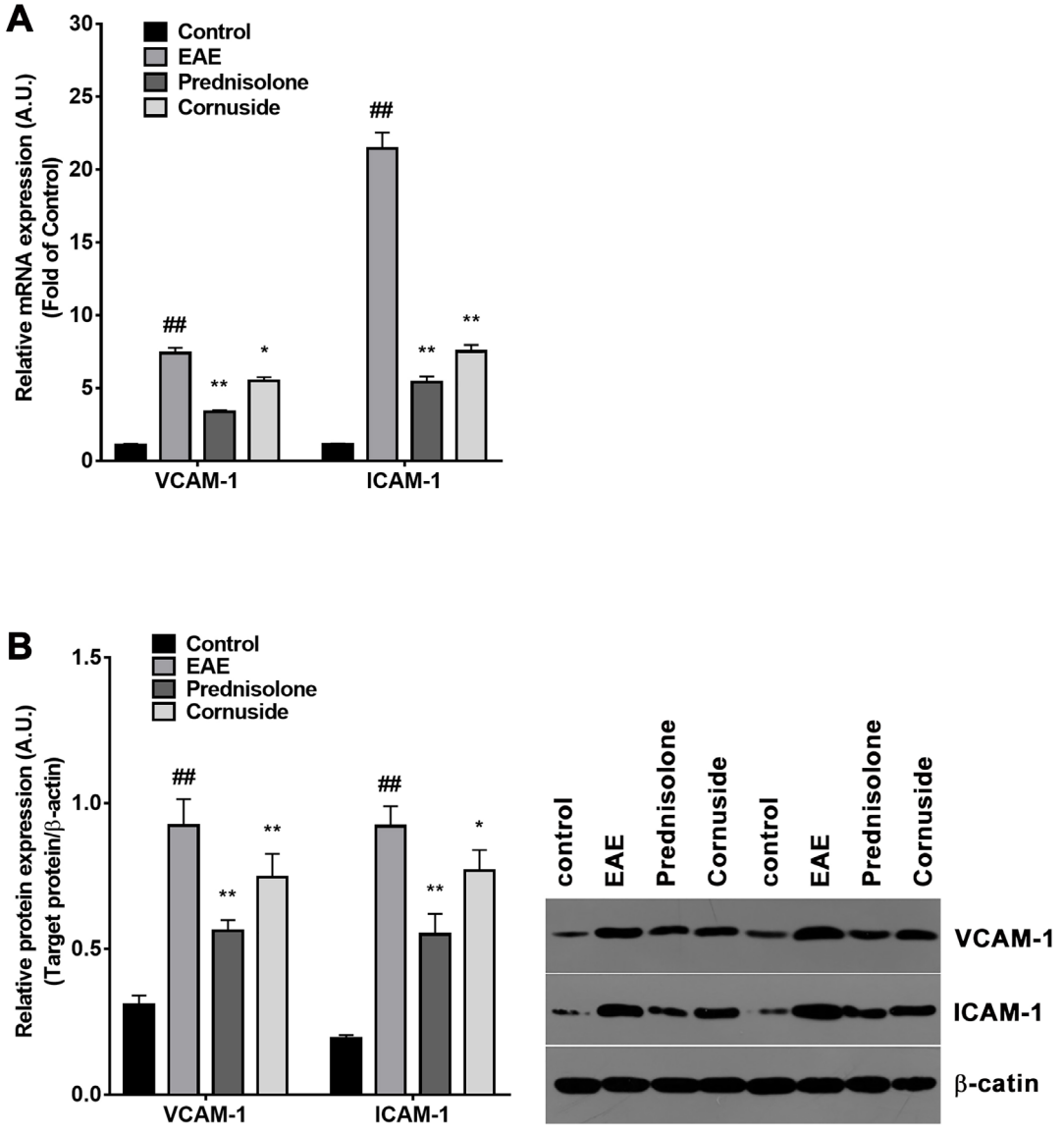

FIGURE 3 - Cornuside inhibited mRNA and protein expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in the spinal cord (SC). VCAM-1 and ICAM-1 mRNA expressions in the SC were analyzed using real-time quantitative PCR, with 18s rRNA as the normalization standard (A). VCAM-1 and ICAM-1 protein expressions in the SC were detected via western blotting (B), and representative blots are shown at the right panel of quantified data. A.U. means arbitrary units. $^{*}P<0.05$, $^{**}P<0.01$ for the cornuside or prednisolone group versus the EAE group, $^{\#\#}P<0.01$ for the EAE group versus the control group.

Cornuside inhibited TNF-α mRNA expression in the spinal cord

Studies have shown that TNF-α could induce VCAM-1 and ICAM-1 expressions in the human umbilical vein (Ismail *et al.*, 2018; Kang *et al.*, 2007). Therefore, we studied the effect of cornuside on the TNF-α mRNA expression in the SC of EAE rats. Compared to the control group, the SC TNF-α mRNA expression was significantly increased in the EAE group rats. On the other hand, both cornuside and prednisolone had a markedly reduced TNF-α mRNA expression (Figure 4). This indicates that cornuside could inhibit CNS TNF-α mRNA expression and that the effects of cornuside on SC VCAM-1 and ICAM-1 expressions could be related to the altered TNF-α expression.

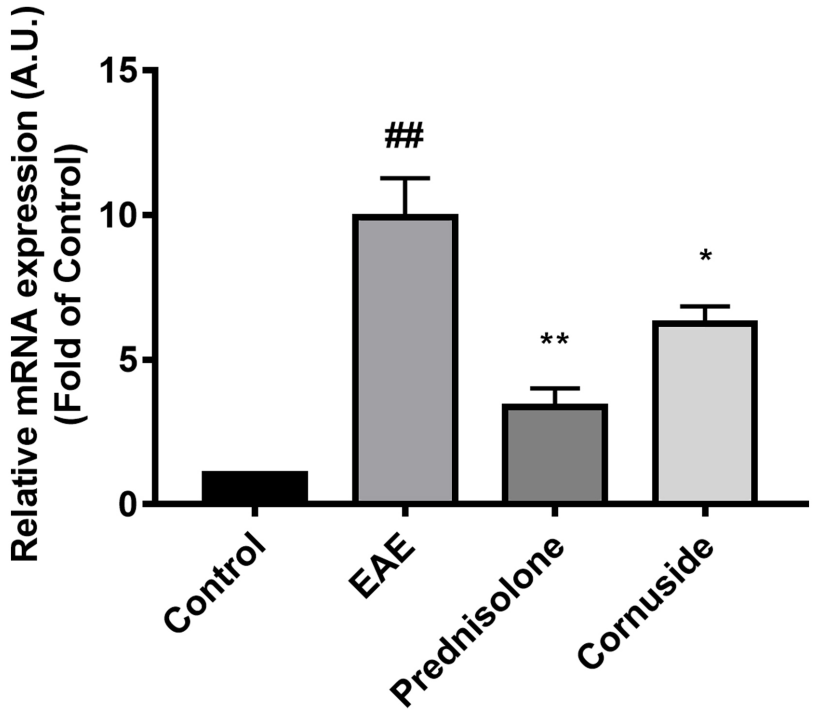

FIGURE 4 - Cornuside inhibited tumor necrosis factor-α (TNF-α) mRNA expression in the spinal cord (SC). SC TNF-α mRNA expressions were analyzed using real-time quantitative PCR, with 18s rRNA as the normalization standard. A.U. means arbitrary units. $^{*}P<0.05$, $^{**}P<0.01$ for the cornuside or prednisolone group versus the EAE group, $^{\#\#}P<0.01$ for the EAE group versus the control group.

DISCUSSION

The primary findings of this study showed that in the rat EAE model, cornuside improved the neurological function score and symptoms of EAE. This might be associated with the fact that, at least partially, cornuside inhibited $CD4^+$ T lymphocyte infiltration and VCAM-1 and ICAM-1 expressions in the SC.

Currently, corticosteroids are the mainstay treatment for MS during the acute attack stage period, thus we chose prednisolone as the positive control drug for EAE. (Tomkinson *et al.*, 2020). Additionally, the results showed that cornuside treatment had the same trend as prednisolone treatment in improving EAE symptoms, as well as in the inhibition of $CD4^+$ T lymphocyte infiltration and SC VCAM-1 and ICAM-1 expressions.

Cornus officinalis, known in China as "Shanzhuyu", is a member of the Cornaceae family, which contains various chemical constituents, including iridoid glycosides and alkaloids, and has been reported to possess multiple

beneficial effects, such as immunoregulatory, anti-oxidation, and anti-inflammatory actions (Czerwinska, Melzig, 2018; Huang *et al*., 2018; Ji *et al*., 2019).

Regarding the association between cornuside and EAE, studies have found that cornel iridoid glycosides can significantly improve EAE symptoms (Qu *et al*., 2019; Yin *et al*., 2014). Moreover, Yin *et al.* (2014) have found decreased incidence, neurological deficit, body weight loss, and, more importantly, less $CD3^+$ T cell infiltration into the SC after cornel iridoid glycoside treatment in the rat EAE model.

$CD4^+$ T cells play a major role in the pathogenesis of MS and EAE through the transfusion of myelin-specific $CD4^+$ T lymphocytes into normal mice (Hemmer, Kerschensteiner, Korn, 2015). These lymphocytes are reactivated by the CNS antigen-presenting cells through cell-cell contact and pro-inflammatory cytokine secretion. This secretion of proinflammatory cytokines can induce a large number of immune cells to enter, which in turn will activate in the CNS and secrete inflammatory factors that lead to nerve tissue damage (Rostami, Ciric, 2013). Therefore, $CD4^+$ T lymphocyte infiltration into the CNS through the BBB is the key to EAE pathogenesis, and blocking this step can inhibit the disease's pathological process. In this study, we demonstrated that cornuside could improve the clinical symptoms of EAE rats, and that the beneficial effects of cornuside on EAE might be associated with the inhibition of $CD4^+$ T lymphocytes.

VCAM-1 and ICAM-1, which belong to the immunoglobulin superfamily, can bind to ligand integrin α4β1, and mediate leukocyte recruitment and lymphocyte homing. They are highly expressed in EAE, playing an important role in mediating T lymphocyte entry into the CNS through the BBB (Abadier *et al*., 2015; Cook-Mills, Marchese, Abdala-Valencia, 2011; Steiner *et al*., 2010; Wu *et al*., 2016). Additionally, cornuside has been reported to inhibit VCAM-1 and ICAM-1 expression in umbilical vein in vitro (Kang *et al*., 2007). Our present study further demonstrated this previous finding that cornuside inhibited SC VCAM-1 and ICAM-1 expressions and $CD4^+$ T lymphocyte infiltration in EAE rats, thereby improving EAE symptoms.

Considering the important role of TNF-α in inducing expression of adhesion molecules (*i.e.* VCAM-1, ICAM-1) in inflammation (Chandrasekharan *et al*., 2007; Zelova, Hosek, 2013), we also studied the effect of cornuside on SC TNF-α mRNA expression of EAE rats. Our results suggested that the inhibitory effect of cornuside on SC VCAM-1 and ICAM-1 expressions may be related to its inhibitory effect on TNF-α expression.

It has also been reported that cornuside could inhibit pro-inflammatory cytokine release from human mast cells (Li *et al*., 2016). Additionally, it can inhibit lipopolysaccharide-induced inflammatory mediator secretion by macrophages, including TNF-α, IL-6, IL-1β, nitric oxide, and prostaglandin E2, while simultaneously up-regulating IL-10 serum levels in experimental sepsis rats (Choi *et al*., 2011; Jiang *et al*., 2009). Thus, the ameliorating effects of cornuside on EAE symptoms may also be related to its anti-inflammatory action.

In conclusion, the present study confirmed that cornuside could reduce the neurological function score of EAE rats and improve EAE symptoms. This may be due to the inhibited TNF-α expression and, consequently, VCAM-1 and ICAM-1 in the SC, leading to inhibited $CD4^+$ T lymphocyte infiltration. This also points to the possible therapeutic potential of cornuside in neuroinflammatory diseases, which is worthy of further investigation.

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