

## Original Article

# Effects of ulinastatin on the neurological function of the SLE mouse model with secondary lupus encephalopathy and the mechanism

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**Abstract:** Objectives: We observe the effect of ulinastatin on the mRNA and protein expression of chemotactic factor CXCL12 and its receptor CXCR4 in brain tissue of SLE mice with secondary lupus encephalopathy, and we preliminarily investigated the protective effect of ulinastatin on the neurological function of this mouse model. Methods: Ninety SLE mice were divided into three groups: blank control group (normal saline injected i.p.); ulinastatin group (ulinastatin injected i.p.); prednisone group (prednisone injected i.p.). Mouse serum was collected at the intermediate phase (the second month) and final phase (the fourth month) of medicine intervention for detection of cytokines (TNF- $\alpha$  and TGF- $\beta$ ) by ELISA. Mice were sacrificed after four-month drug intervention and brain tissue was harvested for pathological analysis. Apoptosis was detected by TUNEL method. The mRNA and protein expression levels of CXCL12 and its receptor CXCR4 were analyzed by RT-PCR and Western blot, respectively. The mortality of each group was analyzed before sacrifice. Results: Ulinastatin relieved cerebral bleeding, inflammation, edema, etc., and improved memory of model mice. Ulinastatin down-regulated the expression levels of CXCL12 and its receptor CXCR4 in brain tissue, down-regulated serum TNF- $\alpha$ , up-regulated serum TGF- $\beta$  and reduced neuronal apoptosis. Moreover, ulinastatin significantly decreased the mortality of mice compared with the prednisone and blank control group. Conclusions: Ulinastatin affects the progression of secondary lupus encephalopathy and exerts protective effects on the neurological function of CNS-SLE mice. The regulation of the expression levels of CXCL12 and its receptor CXCR4 may be one of the mechanisms.

**Keywords:** Ulinastatin, lupus encephalopathy, mouse, neurological function, CXCL12, CXCR4, interventional mechanism

## Introduction

Systemic lupus erythematosus (SLE) is a multiple-autoimmune disease caused by dysfunction of cellular and humoral immunity, which commonly implicates various tissues. Secondary lupus encephalopathy of SLE mice is an autoimmune disease with multiple systems involved systemically, which is also the most severe complication of SLE with relatively complex pathogenesis and an annually increased incidence [1-3]. Central nervous system impairment is the clinical omen of critically exacerbating SLE and one of the primary causes of lupus crisis-induced death; however, the exact pathogenesis has not been clarified [4, 5]. SLE has a variety of clinical symptoms which could occur

at any phase of SLE progression in an alternating process of onsets and alleviation. Moreover, a majority of patients suffer poor prognosis [6, 7].

The investigation on the pathogenesis remains limited. Studies reported that ulinastatin was an endogenous protective substance in the human body with extensive bioactivities. It can alleviate pathological injury caused by cerebral damage. Many researches have shown that ulinastatin could display remarkable therapeutic effects on serious brain, heart and lung impairment [8, 9]. Studies reported that prednisone, as the first-line drug against SLE, could inhibit the secretion of IL-6 by mouse lupus-like splenocytes cultured in vitro and reduce the

production of autoantibodies in lupus-like mice. In this study, we aimed to observe the effects of ulinastatin on the mRNA and protein expression of chemotactic factor CXCL12 and its receptor CXCR4 in brain tissue of SLE mice with secondary lupus encephalopathy in contrast to prednisone, and we preliminarily investigated the protective effect of ulinastatin on the neurological function of this mouse model.

### Material and methods

#### Materials

**Design:** Randomized controlled trials. **Time and place:** The experiment was carried out in the Animal Experiment Center of Hebei Medical University from July 2013 to November 2015. **Experimental animals:** Ninety male MRL/lpr SLE mice aged four months and weighing 38 to 40 g were provided by the Animal Experiment Center of Hebei Medical University (license: SCXK(Ji)2012-0005). These animals were kept under SPF-graded conditions in the Animal Experiment Center of Hebei Medical University, receiving a regular diet of mouse chow and allowed free access to water.

**Materials and sources:** Ulinastatin (Guangdong Techpool Co., Ltd; approval number: H1999-0133); prednisolone acetate injection (Zhejiang Xianju Pharma Co., Ltd; approval number: H20023134); EDTA (Tianjin first chemical reagent factory); TUNNEL kit (Roche); mRNA extraction kit, Taq enzyme (MBI, USA); PCR kit (Dalian Takara Bio Co., Ltd); primers of CXCL12, CXCR4 and GAPDH (synthesized by Beijing SBS Genetech Co., Ltd); anti-CXCL12, CXCR4 monoclonal antibody (SANTA); rabbit anti-mouse GAPDH polyclonal antibody and HRP-labelled goat anti-rabbit IgG antibody (Santa Cruz, USA); Morris water maze DMS-2 system (Shanghai Institute of Materia Medica of China Academy of Sciences); water maze analysis software (NOLDUS, Netherlands); BrdU antibody (Sigma, USA); CO<sub>2</sub> incubator (Thermo Forma); clean bench (Suzhou Antai Airtech Co., Ltd); fluorescence inverted microscope (Leica, Germany); gel imaging system (Kodak, Japan).

#### Methods

**Grouping and administration:** Ninety male MRL/lpr SLE mice were divided into three groups based on the random number table with 30 mice in each group, blank control group (i.p.

injection with 30 mL/(kg·d<sup>-1</sup>) normal saline), ulinastatin group (i.p. injection with 50000 U/(kg·d<sup>-1</sup>) ulinastatin), and prednisone group (i.p. injection with 1 mg/(kg·d<sup>-1</sup>) prednisone). Administration continued for four months in an alternating circle of intraperitoneal injection (15 d) and intervals (15 d). There was no attrition or death during the treatment.

**Detection of learning and memory behavior of mice:** The CNS-SLE mice of each group were placed into the YLS-3T step-down recorder to detect their learning and memory behavior (step-down method). After adaptation for 60 s, mice were subjected to electric shock (25 V), and the normal response was to jump to the platform to avoid harmful stimulation. The time spent in the first seeking of the platform (response time) and the times of electric shocks within 300 s (error times) were recorded. The above-mentioned test was repeated 24 h later, and the time until the first jump-off (latent phase) and error times were recorded as the memory result.

**Detection of serum TNF- $\alpha$  and TGF- $\beta$ :** Five mice were randomly selected from each group and 3 mL blood was collected from each mouse at the intermediate phase (the second month) and final (the fourth month) phase of intervention, respectively: mice received anesthesia by i.p. injection with 1.2 g/kg urethane (200 g/L) and were then fixed at supine position for blood collection (1.5 mL) by cardiac puncture. After standing for half an hour, the harvested blood was centrifuged at 3700 r/min for 10 min at normal temperature. Then the serum was collected in aliquots (100  $\mu$ L/EP tube) and stored in the -80°C refrigerator. The expression levels of serum TNF- $\alpha$  and TGF- $\beta$  were detected by ELISA strictly according to the instructions. The ELISA plate was coated with anti-rat TNF- $\alpha$  or TGF- $\beta$  McAb. After TNF- $\alpha$  or TGF- $\beta$  in the standard substance and samples combined with the coated antibody, biotinylated anti-rat TNF- $\alpha$  or TGF- $\beta$  antibody was added to form the immune complex. Then HRP-labelled Strepta-TGF- $\beta$  idin was added to bond with biotin. After the enzyme substrate TMB was added, blue color was developed. Sulfuric acid was finally added to end the reaction and the system turned yellow. OD values were measured at 450 nm, and the standard curve was built based on the positive correlation between the concentrations of TNF- $\alpha$  or TGF- $\beta$  and OD val-

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**Table 1.** Analysis on learning and memory behavior of the rats in each group

Genes	Primer sequence (5'-3')	Length of product (bp)	Temperature
CXCL12	Upstream: 5'-GTC AGC CTG AGC TAC CGA-3	103 bp	55 °C
	Downstream: 5'-GAA GGG CAC AGT TTG GAG-3		
CXCR4	Upstream: 5'-GGC TGA CCT CCT CTT TGT-3	137 bp	55 °C
	Downstream: 5'-GTT TCC TTG GCC TTT GAC-3		
GAPDH	Upstream: 5'-ACA GCC GCA TCT TCT TGT GCA GTG-3'	203 bp	55 °C
	Downstream: 5'-GGC CTT GAC TGT GCC GTT GAA TTT-3'		

ues. And the concentrations of TNF- $\alpha$  or TGF- $\beta$  in each group were calculated based on the standard curve (0, 50, 100, 200, 500, 800 ng/mL TNF- $\alpha$ /TGF- $\beta$  corresponding to OD values).

*Mortality of CNS-SLE mice of each group:* The natural mortality of CNS-SLE mice in each group was recorded during treatment. The survival rate chart was then established with half a month as a time node.

*Pathological examination:* After sacrifice, tissue was harvested and subjected to fixation with 10% neutral formalin, paraffin embedding, routine sectioning at 4  $\mu$ m, baking in the 80 °C oven and HE staining. The sections were stained with hematoxylin for 5 min and then washed with running water. Hydrochloric acid alcohol differentiation was then performed for 10 s, followed by washing with running water for another 10 min. After stained with eosin for 7 min, the sections were again washed with running water, followed by dehydration with ethanol gradients, cleaning with xylene and sealing with neutral balsam. Then the sections were examined under the microscope.

*TUNEL staining:* According to the instructions, the sections of different groups were respectively subjected to fixing with 4% paraformaldehyde, blocking with 3% H<sub>2</sub>O<sub>2</sub>, incubating with DIG-dUDP, incubating with anti-digoxin antibody at 1:100, DAB color developing, dehydrating with ethanol gradients, cleaning with xylene and sealing with neutral balsam. PBS was adopted in place of the primary antibody as the negative control. The sections were observed and photographed under the microscope. Three sections were selected from each group for observation. Ten 400  $\times$  view fields were picked from the cell-abundant region of each section. Apoptotic hepatocytes and total hepatocytes were counted, and apoptosis index (AI) was calculated based on the following formula:

AI = (apoptotic cell count/total cell count)  $\times$  100%.

*RT-PCR detection:* Brain tissue of mice (50 mg) was collected and grinded in liquid nitrogen, which was then added into 1 mL Trizol reagent for RNA extraction. The ultraviolet spectrometry was employed to measure RNA concentrations. Then RNA was reversely transcribed into cDNA according to the instructions of MMLV kit, and cDNA was subjected to RT-PCR analysis. Total RNA of tissue was extracted based on Trizol instructions. Then total RNA was used as the template and Oligo (dT) as the primer to reversely transcribe mRNA into cDNA by RT-PCR two-step kit. The sequences of the primers are shown in **Table 1**. The reaction conditions are as follows: GAPDH, denaturation at 94 °C, 25 circles (94 °C for 45 s, 58 °C for 30 s, 72 °C for 1 min), extension at 72 °C; CXCL12, denaturation at 94 °C for 5 min, 25 circles (94 °C for 45 s, 55 °C for 30 s, 72 °C for 1 min), extension at 72 °C; CXCR4, denaturation at 94 °C, 25 circles (94 °C for 45 s, 53 °C for 30 s, 72 °C for 1 min), extension at 72 °C. The UV-light gel imaging and analyzing system was employed for photographing, and the grey values of amplified fragments were measured. The expression levels of CXCL12 and CXCR4 were shown with the ratios of their grey values to the grey value of GAPDH fragment. The analysis was carried out with FR200 image analyzing system (see **Table 1**).

*Western blot:* After extraction, the remained RT-PCR products were centrifuged at 1500 r/min for 30 min to collect the supernatant as crude protein, and the protein concentration was measured by Bradford assay. Then the samples were subjected to separation through 5% spacer gel at 40 V for 1 h and 10% separation gel at 60 V for 3.5 h, followed by wet transfer at 14 V for 14 h. Then the 37 °C shaker was used for blocking for 2 h. After transfer, the primary antibody was added for overnight incubation.

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**Table 2.** Analysis on learning and memory behavior of the rats in each group

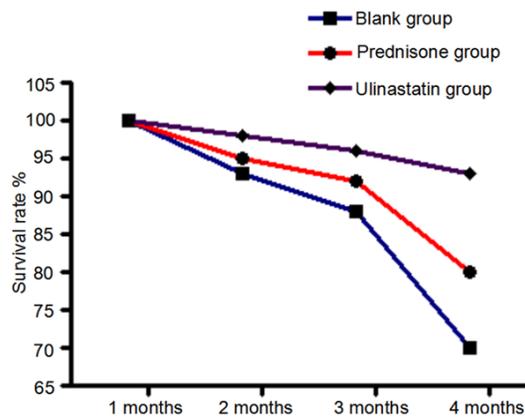
Group	Learning capacity		Memory capacity	
	Response time/s	Error times/times	Latent phase/s	Error times/times
Blank	42.00 ± 16.00*	1.40 ± 1.23*	59.00 ± 10.17*	1.20 ± 0.48*
Prednisone	34.60 ± 4.62#	1.00 ± 0.48#	83.20 ± 17.26#	0.80 ± 0.77#
Ulinastatin	39.10 ± 6.70	0.77 ± 0.68	89.77 ± 15.31	0.77 ± 0.68

Note: \*P<0.05; #P<0.05.

**Table 3.** Expression levels of TNF-α and TGF-β of each group ( $\bar{x} \pm s$ )

Group	Drug intervention for 2 month		Drug intervention for 4 month	
	TNF-α	TGF-β	TNF-α	TGF-β
Blank	9.1 ± 0.54*	7.55 ± 0.28*	8.41 ± 0.21*	7.43 ± 0.41*
Prednisone	7.52 ± 0.36#	10.04 ± 0.31#	6.54 ± 0.31#	12.21 ± 0.31#
Ulinastatin	7.96 ± 0.36	9.34 ± 0.21	7.33 ± 1.24	9.38 ± 0.22

Note: \*P<0.05; #P<0.05.



**Figure 1.** Survival rate curves of each group.

tion at 4°C: rabbit anti-mouse CXCL12 antibody or rabbit anti-mouse CXCR4 antibody (1:800); rabbit anti-mouse GAPDH (1:5000). After washing with TBST four times (each for 5 min), goat anti-rabbit antibody (1:700) was added for incubation in the 37°C shaker for 1.5 h, followed by washing with TBST four times (each for 5 min). Then, dimethylbenzidine (DAB) was adopted for color development. Three paralleled tests were set. Quantity one software was used for image analyzing. The results were shown and analyzed with ratios of the grey values of the target bands to the grey values of β-actin.

Designer, executor and estimator: experimental designer was the first author, intervention executor the second and estimator the third. Formal training was carried out. Double-blinded method was used for assessment.

### Statistics

All experimental data were presented as mean ± standard deviation ( $\bar{x} \pm s$ ). And data were statistically processed with SPSS 15.0 software. Intergroup comparison was carried out by one-way ANOVA. P<0.05 indicated statistical significance.

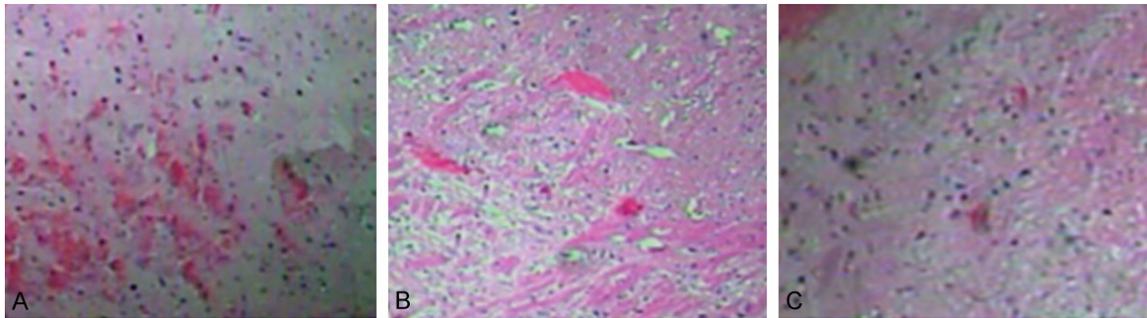
### Results

#### Analysis of learning and memory behavior

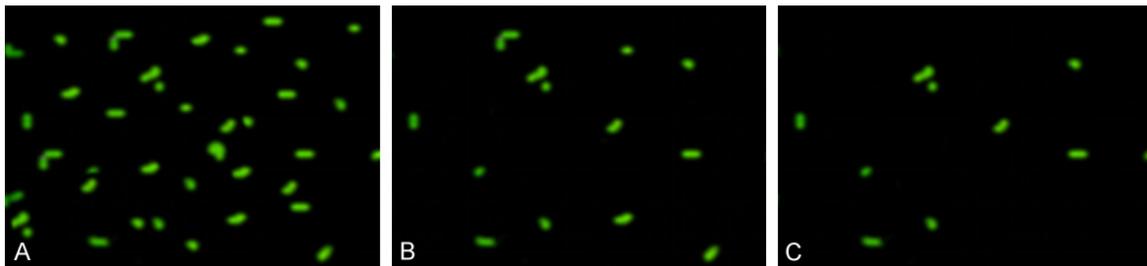
For learning capability, the three groups showed no significant difference in terms of response time and error times. The memory test showed that the ulinastatin and prednisone group had remarkably longer latent phases before leaping off the platform compared with the blank control group (P<0.05), while no significant difference was found in terms of error times (see **Table 2**). This indicated that mice had improved memory holding capacity after treatment.

#### Detection of serum TNF-α and TGF-β

The expression levels of pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine TGF-β in rat serum were detected by double-antibody sandwich ELISA, and the results showed that the ulinastatin and prednisone group had lower serum TNF-α levels and higher serum TGF-β levels compared with the blank control group at the middle (the second month) and the final (the fourth month) phase of drug intervention, with the changes of prednisone



**Figure 2.** Pathological analysis and apoptosis detection of mouse brain tissue.



**Figure 3.** Apoptotic cell count of brain tissue of each group.

group more significant than those of the ulinastatin group ( $P < 0.05$ ). See **Table 3**.

### *Survival rate curves of CNS-SLE mice*

At the fourth month of the experiment (final phase of drug intervention), the blank control group had 21 mice survived (with a survival rate of 70.0%), the prednisone group had 24 mice survived (with a survival rate of 80.0%, higher than that of the control group), and the ulinastatin group had 28 mice survived (with a survival rate of 93.3%), which indicated that ulinastatin could markedly improve the prognosis of CNS-SLE mice compared with the prednisone group. See **Figure 1**.

### *Pathological analysis and apoptosis detection of mouse brain tissue*

The blank control group had abundant inflammatory factors, hemorrhagic spots and edema in brain tissue, with normal neuron hyperplasia, while the prednisone and ulinastatin group had a much lower degree of these pathological features or even none of these, with active neuron hyperplasia (**Figure 2**). Positive cells (apoptotic cells) were seen with green fluorescence under the fluorescent microscope. Compared with the blank control group ( $27.54 \pm 4.38$  cells/high power lens), the prednisone

group ( $13.23 \pm 3.46$  cells/high power lens) and ulinastatin group ( $14.48 \pm 3.76$  cells/high power lens) had substantially lower levels of apoptotic cells in brain tissue with significant difference ( $P < 0.05$ ). See **Figure 3**.

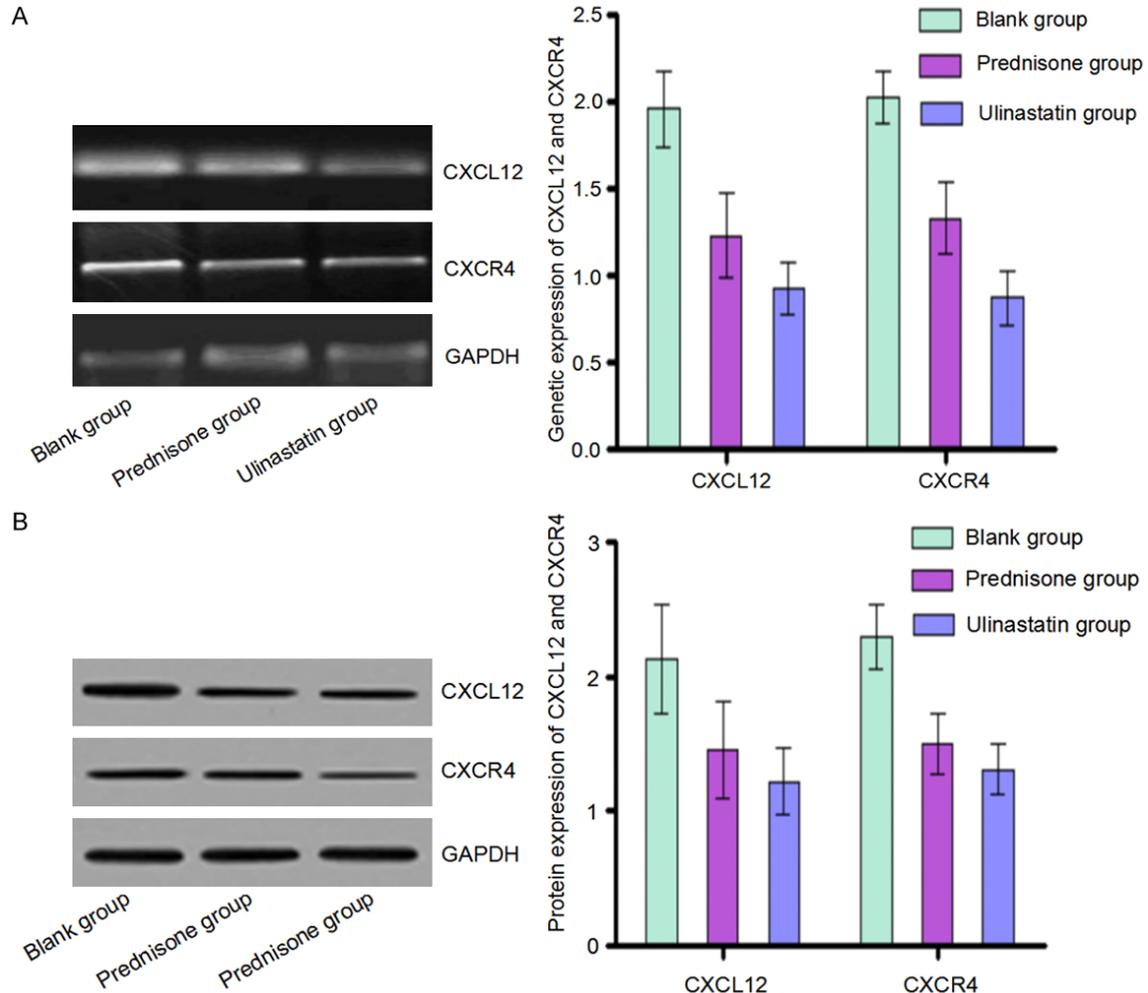
### *Genetic and protein expression of CXCL12 and CXCR4 in mouse brain tissue*

The three groups all had positive genetic and protein expression of CXCL12 and CXCR4 in brain tissue. The genetic and protein expression levels of CXCL12 and CXCR4 of the prednisone and ulinastatin group were lower than those of the blank control group, with significant difference ( $P < 0.05$ ); the ulinastatin group had lower genetic and protein expression levels of CXCL12 and CXCR4 compared with the prednisone group, with significant difference ( $P < 0.05$ ). These results indicated ulinastatin inhibited the progression of secondary lupus encephalopathy in SLE mice through down-regulating the expression levels of CXCL12 and its receptor CXCR4 and exerted protective effects on the neurological function in CNS-SLE mice. See **Figure 4**.

## **Discussion**

Secondary lupus encephalopathy of SLE mice usually leads to poor prognosis, declining life

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**Figure 4.** A: Genetic expression of CXCL12 and CXCR4 in brain tissue; B: Protein expression of CXCL12 and CXCR4 in brain tissue.

quality and constant threats to life [10, 11]. Lupus encephalopathy has a relatively complicated pathogenesis which has been indicated by various studies to be related to vasculitis with immune complex deposition, damage from phospholipid antibody, anti-neuronal antibody and anti-brain histone antibody response, anti-ribosome P protein antibody diseases, hypoproteinemia, etc. [12-15]. Furthermore, due to the fact that lupus encephalopathy has different clinical symptoms and it can be affected by genetic and environmental factors of patients, there is no uniform standard of diagnosis and differentiation by now [16, 17]. Clinical treatments primarily include immunosuppressor therapy, hormone therapy and symptomatic treatment, etc., but none of these can realize complete healing [18-20]. In this study, the efficacy of ulinastatin in secondary lupus encephalopathy of SLE mice was investigated, with the hope that curative effects can be realized and the disease progression can be suspended [21-23].

Ulinastatin is an enzyme inhibitor with a broad spectrum, which inhibits activities of multiple enzymes such as hyaluronidase, trypsin, plasmin, elastinase, etc., exhibits antishock effect, inhibits inflammatory factors and scavenges free radicals. Studies reported that ulinastatin was a glycoprotein extracted from fresh urine of males and it could exert supportive therapeutic effects on shock, operation injury, chemoradiotherapy response of tumor, etc. [24-26]. Some studies suggested that there were several possible working mechanisms of ulinastatin, including inhibiting proteolysis hyperfunction, improving immunity and renal func-

tion, relieving brain cell edema, disturbance of water and electrolyte, clouding of consciousness, hemorrhage of digestive tract, imbalance of metabolism, and infection after cerebral injury [27-29].

Literature [30] reported that mice were an ideal model for SLE secondary lupus encephalopathy studies. Therefore, mice were selected as the subjects and superiority of the mouse model was also verified in our study. In this research, HE staining showed that ulinastatin alleviated cerebral hemorrhage, inflammation and edema of model mice. The learning and memory behavior test showed that ulinastatin could improve memory capacity of mice, which probably relates to the improvement of neuron activity of mice. RT-PCR and Western blot showed down-regulated expression of CXCL12 and its receptor CXCR4 in brain tissue after ulinastatin intervention. TUNEL calculation showed significantly reduced the neuron apoptosis rate.

In this study, prednisone group had a remarkably longer latent phase before leaping off the platform compared with the blank control group ( $P < 0.05$ ), which indicated that prednisone treatment could also improve memory holding capacity of mice. Prednisone group showed down-regulated TNF- $\alpha$  and up-regulated TGF- $\beta$  in serum compared with the blank control group, which indicated that prednisone treatment could decrease pro-inflammatory cytokine expression and increase anti-inflammatory cytokine expression. The intergroup comparison of survival rates showed that the prednisone and ulinastatin group had remarkably higher survival rates compared with the blank control group, but the survival rate of the prednisone group was markedly lower than that of the ulinastatin group. Moreover, the ulinastatin group had lower genetic and protein expression levels of CXCL12 and CXCR4 compared with the prednisone group, which indicated that ulinastatin inhibited the progression of secondary lupus encephalopathy in SLE mice through down-regulating the expression levels of CXCL12 and its receptor CXCR4 and it was much more effective than prednisone. Therefore, it was speculated that prednisone and ulinastatin both could exert protective effects on the neurological function in CNS-SLE mice, and they worked in coordination to display anti-inflammatory, anti-coagulation and immunoreg-

ulatory effects and to effectively treat SLE and lung injury complicated.

In conclusion, SLE secondary lupus encephalopathy is a challenge to modern clinical medicine. In this study, we found that prednisone and ulinastatin both could exert desirable therapeutic effects, and ulinastatin could contribute to the treatment of lupus encephalopathy, reduction of complications and improvement of prognosis. Our study provides experimental basis for the treatment of SLE secondary lupus encephalopathy. However, the risk factors triggering lupus encephalopathy remain unclear, which require further investigation.

### Disclosure of conflict of interest

None.

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