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Original Article

Expression of matrix metalloproteinase-9 in mice with tuberculous meningitis and its significance

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Abstract: Objective: To analyze the characteristics of the expression of matrix metalloproteinase-9 (MMP-9) in the pathophysiological process of tuberculous meningitis in mice and its significance. Methods: Sixteen mice injected intracerebroventricularly with the H37RV suspension of M. tuberculosis were employed as the model group, while another 16 mice injected with 0. 9% sodium chloride solution of an equal volume served as the control group. Thirty days later, all mice were sacrificed by decapitation, and the culture of M. tuberculosis from the brain tissue and the pathological observation were performed, respectively. The activity of MMP-9 was detected by gelatin zymography, the permeability and the moisture content of the blood brain barrier were detected, and the immunofluorescence staining of MMP-9, the glial fibrillary acidic protein (GFAP) and the integrin αM (OX-42) were performed. The *t* test was used to compare the differences between the two groups. Results: Every mouse in the model group was injected with (1.271±0.111) × 10^6 colony-forming units (cfu), and 30 days later, the amount of M. Tuberculosis in the brain tissue homogenate was (4.900±1.407) × 10^4 cfu/mL. The hematoxylin and eosin (HE) staining showed subarachnoid and cerebroventricular dilatation, and infiltration of a large number of inflammatory cells. The cumulative absorbance (A) value of the MMP-9 band in the brain tissue of a mouse was 47821±19932 in the model group and 10082±3544 in the control group, respectively, with the difference being statistically significant (t=3.728, P=0.010). The content of Evans blue (EB) in the brain tissue of a mouse was (11.8±3.6) μg/g in the model group and (4.7±3.4) μg/g in the control group, with the difference being statistically significant (t=2.887, P=0.028). The moisture content density of the brain tissue of a mouse was 0.849±0.035 in the model group and 0.775±0.037 in the control group, and difference was statistically significant (t=2.925, P=0.026). The immunofluorescence staining showed that MMP-9, GFAP and OX-42 were highly expressed in the brain tissues of infected mice after infection, and that MMP-9 was significantly overlapped with both GFAP and OX-42. Conclusions: The activity of MMP-9 is enhanced in the brain tissues of mice with tuberculous meningitis, and it is involved in the damage of the blood brain barrier, tissue edema and the exudation of inflammatory cells. In addition, the microglia-astrocytes network is involved in the secretion of MMP-9.

Keywords: Tuberculous meningitis, MMP-9, mice, blood brain barrier, microglia, astrocytes

Introduction

As a serious infection of the central nervous system (CNS) caused by the invasion of M. tuberculosis, tuberculous meningitis occurs frequently secondary to tuberculosis and accounts for 5% to 15% of extrapulmonary tuberculosis. The morbidity of tuberculous meningitis stands at 20% to 50%, while 20% to 30% of survivors suffer from permanent neurological sequelae [1]. Studies found that the expression of MMP-9 was abnormally elevated during the onset of CNS infections, suggesting that MMP-9 may play an important role in the pathogenesis of CNS infections [2-5]. In addition, microglia and astrocytes were closely associated with the secretion of MMP-9 [6]. In the present study, we aimed to explore the role

of MMP-9 in tuberculous meningitis and the relevant mechanism of action by studying the characteristics of the pathophysiological changes, changes in the activity of MMP-9 and its association with microglia and astrocytes after the mice were intracranially infected with M. tuberculosis.

Materials and methods

Instruments and devices

The electronic balance was purchased from Shanghai Hengping Scientific Instrument Co., Ltd, and the stereotaxic apparatus for the exclusive use in mice (ST-51730) was purchased from Stoelting, US. The oven and the incubator were purchased from Yiheng Science Instrument Co., Ltd, Shanghai, and the refrigerated centrifuge and the microinjector were purchased from Shanghai Anting Scientific Instrument Factory. The ultra-low temperature freezer was purchased from the Japanese company Sanyo, the electron microscope was purchased from the US Company Laica and the fluorescence confocal microscope was purchased from the Japanese company Olympus.

Culture medium

The 7H9 and 7H10 culture media (Difco, US.) were taken, dissolved in distilled water, supplemented with glycerol and Tween (No Tween for the 7H10 medium), placed under high pressure at 121°C for 10 min, and added with sterile oleic acid-albumin-glucose-catalase (OADC) before use.

Experimental animals

Four to six-week old C57BL/6 female mice of SPF grade weighing 14 to 16 g were purchased from Beijing Vital River Laboratory Animal Co.[Animal License No.: SCXK (Beijing) 2007-0001], housed in the SPF-grade negative pressure infectious experimental animal facility with the temperature at (21±2)°C and humidity at (55±15)%.

Bacterial strains

The laboratory standard strains of M. tuberculosis H37Rv after the recovery of virulence in mice in vivo were inoculated into the 7H9 culture medium, and cultured on the thermostatic shaker at 37°C until the logarithmic growth phase. Then the medium was filtered, centrifuged, resuspended in 0.9% sodium chloride solution (containing 0.05% Tween 80), aliquoted and preserved at -80°C for use. The Absorbance (A) value was 0.21, and the suspension was diluted at 1:10. Then it was cultured on the 7H10 plate medium for four weeks, and the detection showed that the actual number of colonies was (1.271±0.111) × 10^8 (cfu)/mL.

Establishment of tuberculous meningitis models

Intraperitoneal anesthesia with 2% sodium pentobarbital (Sigma, P3761, USA) at the proportion of 0.005 ml/g, fixation and scalp cutting were performed on each mouse, followed by the puncture of a microinjector (10 μL) into its lateral ventricle by 3 mm at 0.5 mm behind the bregma and 1 mm laterally, slow injection of bacterial suspension (10 μL), suturing of the scalp and insulation. Two to four hours later, mouse woke. A total of 16 mice were employed as the model group, and 16 mice intracerebroventrically injected with 0.9% sodium chloride solution served as the control group. At 30 d after M. tuberculosis infection, all mice were sacrificed by cervical dislocation, and their brains were harvested after decapitation.

Culture of M. tuberculosis from the brain tissue

Four mice were selected from the model group and the control group, respectively, and their cerebral hemispheres were harvested under sterile conditions and weighed. Sterile 0.9% sodium chloride solutions (containing 0.05% Tween 80) were added in a proportion of 2 mL/g, followed by the procedures of homogenization and of the 10-fold, 100-fold and 1000-fold dilution with the sterile 0.9% sodium chloride solution. Three bacterial suspensions (100 μL for each) at the above-mentioned dilutions were drawn, inoculated into the 7H10 medium, smeared evenly and placed in the incubator at 37°C. Observation of the colonies growth and colony counting were performed after the routine culture for 4 weeks.

Pathological observations

Four mice were selected from the model group and the control group, respectively. Their cerebral hemispheres were harvested, quickly

placed in the paraformaldehyde with a volume fraction of 0.04 for fixation for 48 h, dehydrated, embedded in paraffin, stained with HE and observed under a light microscope.

Immunofluorescence detection

The sections embedded in paraffin were taken, baked, dewaxed, hydrated, washed with PBS (0.01 mol/L, pH 7.2) and made into slices. The citrate retrieval solution (0.01 mol/L, pH 6.0) was adopted for antigen retrieval at high pressure for 2 min, with the retrieval solution cooling down naturally. The slices were then washed with PBS (0.01 mol/L, pH 7.4), added with a non-immune goat serum blocking buffer containing 0.3% Triton X-100 (Fuzhou Maixin Biotech. Co., Ltd.) dropwise and placed at room temperature for 1 h, which were added dropwise with MMP-9 antibody as the primary antibody (ab76003, diluted at 1:20), placed overnight at 4°C, and washed with PBS. Then they were added dropwise with fluorescently labeled secondary antibody (ab150078, diluted at 1:20) at room temperature for 1 h, and washed with PBS. After that, they were added dropwise with collagen fibers acidic proteins (GFAP, glial cytoplasmic protein, mAb # 3670, diluted at 1:300) or integrin αM (OX-42, microglia cell membrane protein, ab78457, diluted at 1: 200) as the primary antibody at 37°C for 1 h, and washed with PBS. Then they were added dropwise with the fluorescently labeled secondary antibody (ab150113, diluted at 1:200), placed at room temperature for 1 h, washed with PBS, stained with 4', 6-diamidino-2-phenylindole (DAPI, a nuclear stain) for 2 min, and washed with PBS. The sections were then mounted and observed under a fluorescence confocal microscope and photographed.

Detection of MMP-9 in the brain tissue using gelatin zymography

Four mice were selected from the model group and the control group, respectively, and their cerebral hemispheres were harvested and homogenized at low temperatures. The supernatant was collected by centrifugation, filtered and sterilized using a low protein adsorption pinhole filter (Millipore Corporation, USA). The total protein in each sample was detected using the bicinchoninic acid (BCA) assay, and then underwent the procedure of 10% SDS-PAGE using gelatin (lg/L). Next, the gel was rinsed and the incubating medium was incubated at room temperature for 4 h. The medium was then stained with Coomassie brilliant blue R-250 for 2 h, decolored for 1 h (zymography kit P1700, PPLYGEN gene technology Co., Ltd), and the bands were analyzed using the laser scanning densitometry analysis.

Detection of the permeability of the blood brain barrier in the brain tissue

Different concentrations of Evans blue (EB) formamide solutions were prepared and incubated in the constant temperature water bath (54°C) for 48 h. The spectrophotometer (λ=610 nm) was utilized to measure their A values, with the values of the EB concentrations and those of A linearly regressed so as to obtain a standard curve and the regression equation. Four mice from the model group and the control group were selected, respectively, and injected with 4% EB (0.004 ml/g) via the tail vein, with bulbar conjunctiva and limbs turning blue indicating a successful injection. 2 h later, the mice underwent such procedures as intraperitoneal anesthesia with 2% pentobarbital sodium, fixation, chest-opening, cutting of the right atrial appendage and perfusion of 0.9% sodium chloride solution in the left ventricle until the colorless liquid flowed from the right atrium. Then they were decapitated to harvest their right cerebral hemispheres, which were weighed, supplemented with formamide (10 mL/g) and incubated at 54°C for 24 h. The formamide solution containing EB was filtered to detect the value of A, and the content of EB in the formamide solution was obtained according to the standard curve and the regression equation. The content of EB in the brain tissue was obtained after calculation [7].

Detection of moisture content of the brain tissue

Four mice were selected from the model group and the control group, respectively, and their cerebral hemispheres were harvested. The hemispheres were then placed on the precision electronic balance for weighing the wet weight. Then they were placed in the constant temperature oven at 60°C and had their dry weight weighed on the precision electronic balance. Density of the brain moisture content = (wet weight-dry weight)/wet weight.

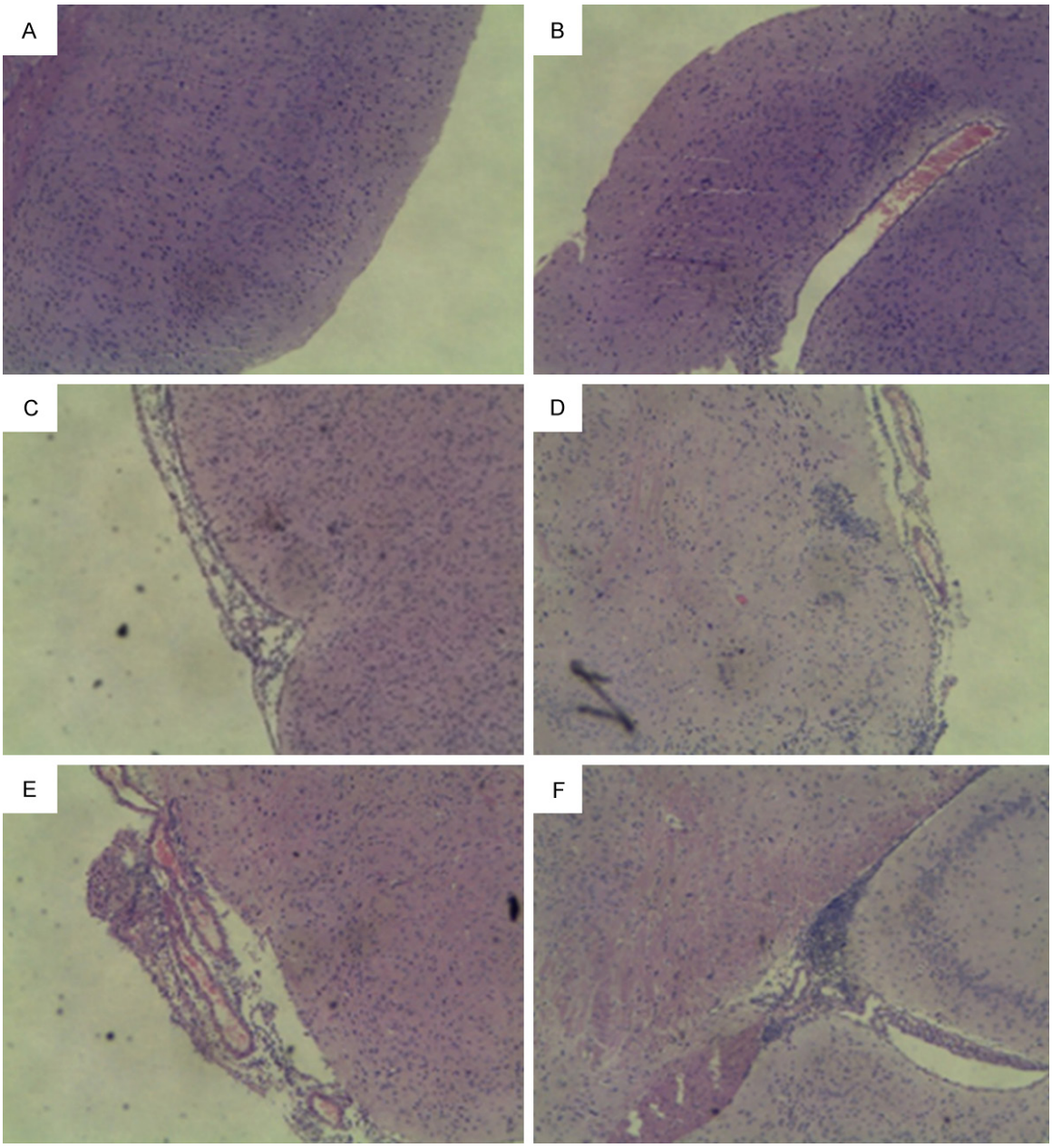

Figure 1. Pathological observation of the brain tissues of mice infected with M. tuberculosis (HE staining, × 40). Notes: A: No meningeal thickening, or dilation of blood vessels, or infiltration of inflammatory cells in the subarachnoid space in the control group; B: Mild cerebroventricular dilatation and congestion, yet no infiltration of inflammatory cells in the control group; C: Meningeal thickening, significant subarachnoid dilation and infiltration of a large number of inflammatory cells in the model group; D: Vascular dilation and congestion in the subarachnoid space, infiltration of inflammatory cells in the vessel wall, and inflammatory nodules near the cortex area in the pia mater in the model group; E: Vascular dilation and congestion in the subarachnoid space, infiltration of perivascular inflammatory cells and the formation of granulomas nearly in the model group; F: Cerebroventricular dilatation and infiltration of a large number of inflammatory cells in the cerebroventricular cavity and in the blood vessel wall in the model group.

Statistical method

The SPSS 19.0 statistical software was utilized. The data were subjected to the normal distribution and the homogeneity of variance tests. The measurement data were expressed as $\bar{x} \pm s$, and the *t* test was performed on the independent samples in the two groups. The value

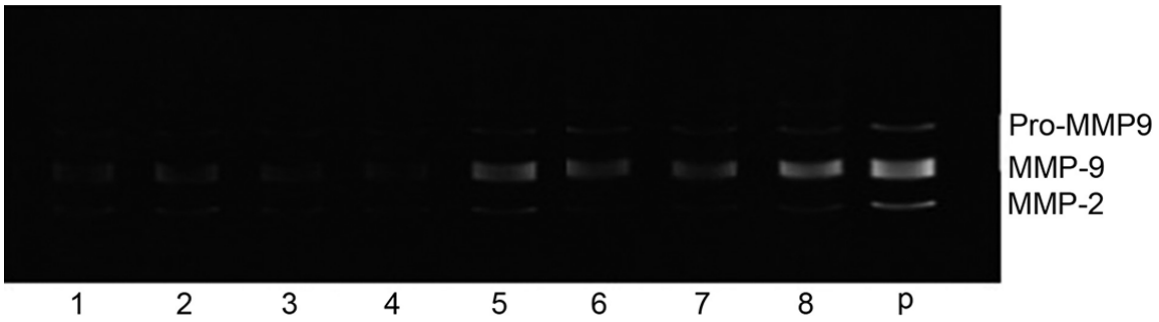

Figure 2. Gel images of detection of MMP-9 in the brain tissues of mice with tuberculous meningitis using gelatin zymography. Note: MMP stands for matrix metalloprotease. P. positive control; 1-4: control group; 5-8: model group.

of $P<0.05$ was considered statistically significant.

Results

Establishment of tuberculous meningitis mice models

Each mouse in the model group was injected with H37RV at (1.271±0.111) × 10^6 cfu, and all mice grew normally without growth delay after the infection. No exudate or purulent discharge was observed through naked eyes in the brain tissues of mice in the model group, and meningitis and infiltration of a large number of subarachnoid, intracerebroventricular and perivascular inflammatory cells were seen under the microscope (**Figure 1**). M. Tuberculosis culture in the brain tissue homogenate in the model group was tested positive, with the amount of bacteria being (4.900±1.407) × 10^4 cfu/mL. No bacterial colony was found in the brain tissue homogenate in the control group, and no abnormalities were seen through the pathological examinations.

Detection of MMP-9 in the brain tissue using gelatin zymography

As is shown in **Figure 2**, the band of MMP-9 in the model group was significantly brighter than that in the control group. The cumulative absorbance (A) value of the band was 47821± 19932 in the model group and 10082±3544 in the control group, and the difference was statistically different (t=3.728, P=0.010), indicating that the activity of MMP-9 in the brain tissues of mice was significantly enhanced after the infection by M. tuberculosis (**Figure 2**).

Detection of the permeability of the blood brain barrier

The standard curve regression equation of EB was y=14.285x-5.603 (y: EB concentration, x: A value, R^2=0.915, F=53.929, P=0.001). The EB content in the brain tissues of mice was (11.8±3.6) μg/g in the model group and (4.7±3.4) μg/g in the control group, and the difference was statistically significant (t=2.887, P=0.028), indicating that the blood brain barrier of mice was damaged and its permeability was increased after the infection with M. tuberculosis.

Detection of moisture content of the brain tissue

The specific gravity of moisture content in the brain tissues of mice was 0.849±0.035 in the model and 0.775±0.037 in the control group, and the difference was statistically significant (t=2.925, P=0.026), indicating that edema occurred in the brain tissue of mice after the infection.

Immunofluorescence detection of the brain tissue

As is shown in **Figures 3** and **4**, the fluorescence intensity of MMP-9, GFAP and OX-42 in the brain tissue of mice in the model mice brain were increased. The number of GFAP-labeled astrocytes was increased, with significant cell synapses. There was an increase in the number of OX-42-labeled microglias, which were round and had larger volumes. All these showed that the astrocytes and microglia were activated. MMP-9 and GFAP as well as OX-42 were overlapping in the same cells, respectively after the

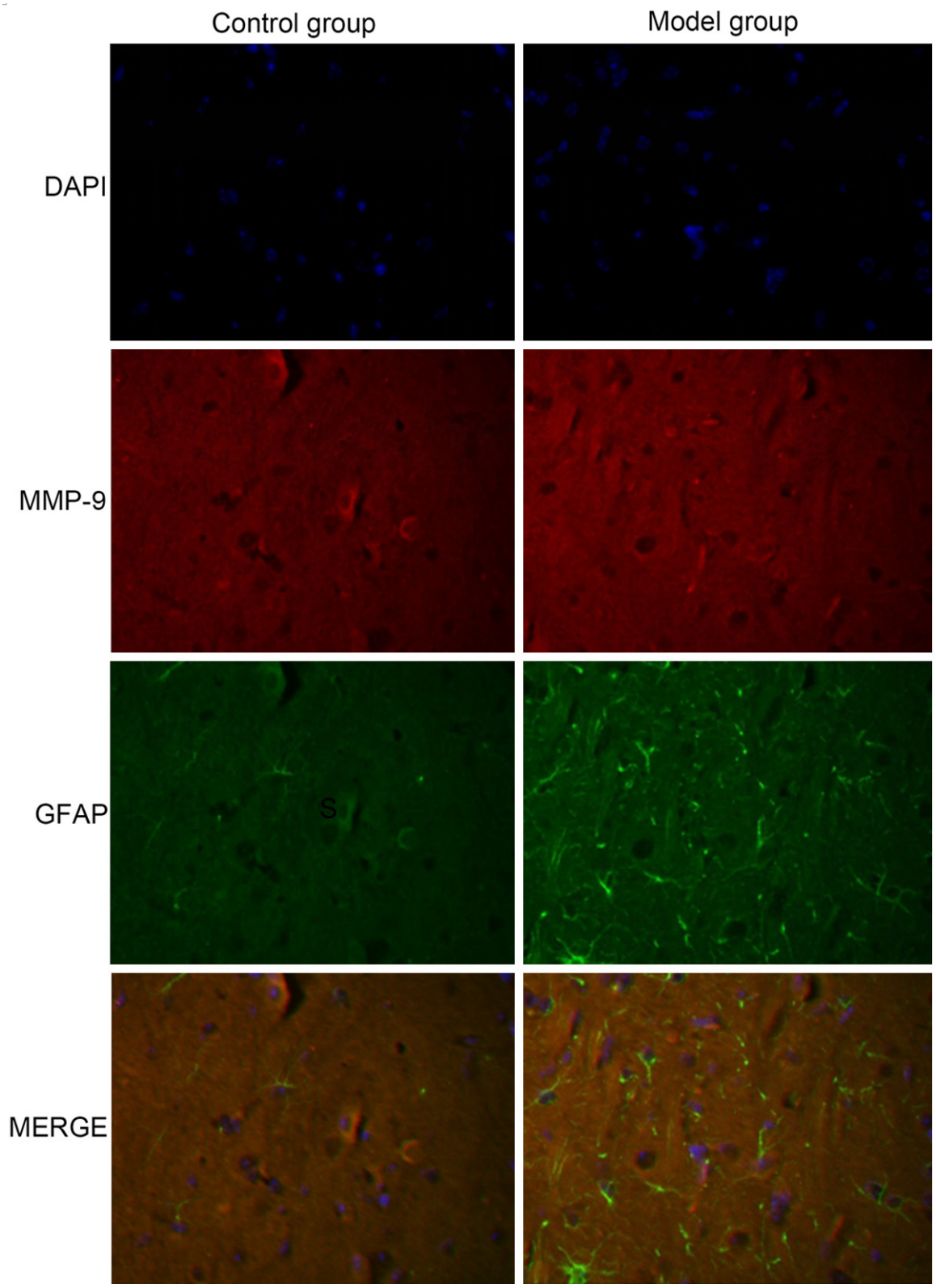

Figure 3. Fluorescent double staining images of MMP-9 and GFAP in the brain tissues of mice with tuberculous meningitis (×400). Notes: DAPI stands, 4', 6-diamidino-2-phenylindole, a nucleus stain; MMP-9, matrix metalloproteinase-9; GFAP, glial fibrillary acidic protein, the plasma protein in astrocytes; MERGE represents overlapping of the three images of DAPI, MMP-9 and GFAP. The arrows indicate typified astrocytes expressing MMP-9 and GFAP simultaneously.

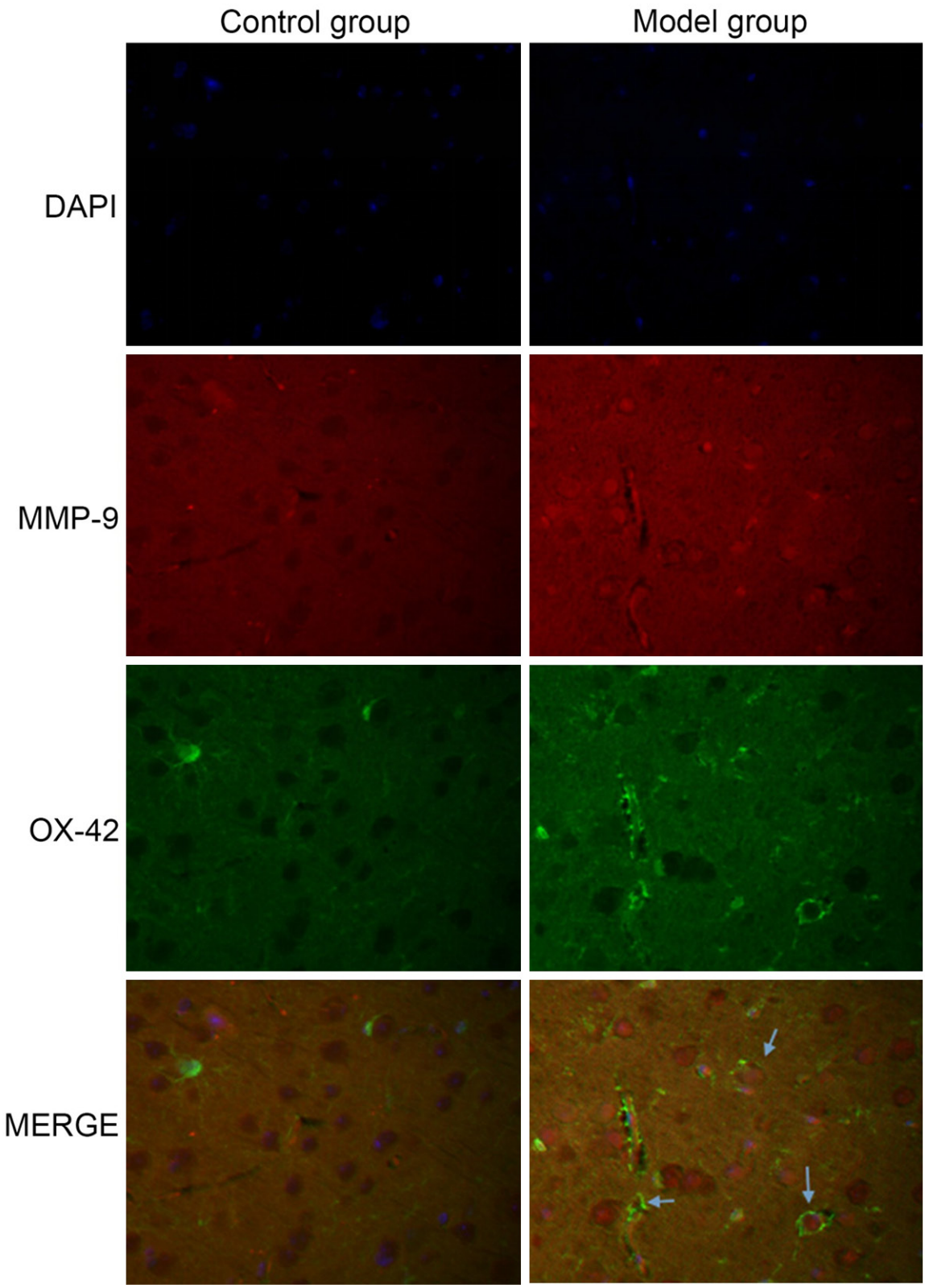

Figure 4. Fluorescent double staining images of MMP-9 and OX-42 in the brain tissues of mice with tuberculous meningitis (×400). Notes: DAPI stands, 4', 6-diamidino-2-phenylindole, a nucleus stain; MMP-9, matrix metalloproteinase-9; OX-42, integrin αM, the membrane protein of microglia. MERGE represents overlapping of the three images of DAPI, MMP-9 and OX-42. The arrows indicate typified microglia expressing MMP-9 and OX-42 simultaneously.

overlapping of the staining images, suggesting that after the infection stimulation, MMP-9 was highly expressed in both astrocytes and microglia (**Figures 3** and **4**).

Discussion

The histopathological changes of tuberculous meningitis in humans mainly include leukocyte immune responses and infiltration of proteins (cytokines and chemokines, etc.), resulting in the subarachnoid, cerebroventricular and perivascular inflammatory responses. Liana Tsenova et al. [8] successfully established meningitis models by injecting M. tuberculosis into the cisterna magnas of white rabbits, and Gijs Th. J. van Well et al. [9] also successfully established the C57BL/6 mice models with tuberculous meningitis by the same means. Rosanna Mazzolla et al. [10, 11] injected M. tuberculosis directly into the cerebroventricle through the puncture at some site next to the anterior fontanelle to establish the tuberculous meningitis models. Therefore, it is feasible to establish tuberculous meningitis animal models by the intracerebroventricular injection of M. tuberculosis. In this study, M. Tuberculosis were cultured from the brain tissue homogenates of all infected mice, and the pathological manifestations were the infiltration of a large number of inflammatory cells in the pia mater, choroid and perivascularly, significant dilation of cavum subarachnoidale, as well as the formation of inflammatory nodules, which proved the successful establishment of tuberculous meningitis models.

In previous studies on tuberculous meningitis patients, the expression of MMP-9 was found to be increased in patients' cerebrospinal fluid, while its expression level was significantly decreased after the treatment [12]. Studies found that when brains were infected with bacteria, viruses and fungi, the expression of MMP-9 was significantly higher in the brain tissue or the cerebrospinal fluid [3, 13-17]. Roland Nau et al. [15] used Streptococcus pneumoniae to establish intracranial infection in mice with MMP-9 gene deficiency, and the immunity of mice to pathogens was changed instantly and their ability to clear Streptococcus in the blood circulation was declined. After the activity of MMP-9 was inhibited using GM6001 by Leib et al. [13], the expression level of TNF-α in the cerebrospinal fluid of experimental animals, which had been identified as a key factor in the progression of tuberculous meningitis [17, 18], was decreased. At the cellular level, studies found that after the stimulation of M. tuberculosis infection, the expression of MMP-9 in the culture medium of microglia and astrocytes was increased [6, 19]. All these data suggest that MMP-9, as part of the immune system of organisms, is involved in the inflammatory defense responses of after the invasion of M. Tuberculosis into the brain tissue. In this experiment, the activity of MMP-9 in the brain tissue homogenate of mice in the model group was higher than that in the control group, with significant infiltration of inflammatory cells in the brain tissue, elevated water content and EB content, suggesting that MMP-9 is involved in the destruction of the blood brain barrier, promotion of penetration of leukocytes and fluids in blood vessels into the brain tissue and the activation of the body's immune response against M. Tuberculosis in tuberculous meningitis. However, the oversensitive immune responses also cause tissue edema, vasculitic reactions, and even lead to such severe tissue damages as vascular occlusion, cerebral ischemia and nerve palsy, etc.

Monocyte-phagocyte infection is critical in the immune pathological process of tuberculous meningitis. As immune cells with the monocyte-phagocyte function in the brain tissue, microglia is the most important cells in the early-stage tuberculous meningitis and involved in the regulation of the secretion of cytokines and chemokines in immune responses. M. Tuberculosis was utilized to infect astrocytes and microglia, respectively, and the results showed that 76% of microglias were infected with M. Tuberculosis, while only 15% of astrocytes were infected with M. Tuberculosis. In addition, the infected microglia secreted cytokines and chemokines in large amounts, such as IL-1β, IL-6, TNF-α, human monocyte chemoattractant protein-1 (CCL-2), recombinant human chemokines CCL5 (CCL-5), interleukin-8 (CXCL-8) and recombinant human interferon-inducible protein-10 (CXCL-10), while astrocytes only secreted a small amount of CXCL-10 [20]. However, the pathological examination of the brain tissue of tuberculous meningitis patients by James et al. [6] found that MMP-9 was primarily expressed in astrocytes, and also highly expressed in microglia. This experiment also found that after the M. tuberculosis infection, the number and

activity of astrocytes and microglias in the brain tissues of mice were increased, and that the cellular localizations of MMP-9 were mainly astrocytes and microglias. In addition, James also found that the direct infection of astrocytes with M. tuberculosis could not stimulate the gene expression and the secretion of MMP-9, and the expression and secretion of MMP-9 were elevated only when the conditioned medium was used to stimulate human astrocyte cell line after M. tuberculosis stimulated monocytes. The results of previous experiments also confirmed that M. tuberculosis count on monocytes to upregulate the expression and secretion of MMP-9 in astrocytes, that MMP-9 and inflammatory factors were upregulated when the conditioned medium was used to restimulate glioma cells (U251) after M. tuberculosis stimulated monocytes. Moreover, MMP-9 and inflammatory factors were not upregulated when the conditioned medium of unstimulated monocytes was used to stimulate U251 cells, and no MMP-9 secretion elevation was observed when M. Tuberculosis-stimulated conditioned medium was used to infect U251 cells [21]. All these data suggest that the microglia-astrocyte network plays an important role in the secretion of MMP-9.

It is now clear that MMP-9 is involved in the pathological process of tuberculous meningitis by disrupting the blood brain barrier and the microglia-astrocyte network regulates the generation of MMP-9. However, how microglias activate and regulate the secretion of MMP-9 in astrocytes remains unclear. In the preliminary experiment, the comparison between CoMTB and CoMCON showed that the expression of NF-κB p65 was increased, indicating that the medium under the CoMTB condition may upregulate the expression and secretion of the MMP-9 gene by activating the NF-κB signaling pathway, and the activated NF-κB further induced the expressions of TNF-α, IL-1β and IL-6 genes at the transcriptional level. Furthermore, such inflammatory factors as TNF-α, IL-1β and IL-6 secreted in large amounts, together with the elevated MMP-9, caused the pathological lesions of tuberculous meningitis [21].

Although M. Tuberculosis infection in mice in the experiment progressed into meningitis, all mice survived and no such abnormal clinical manifestations as the loss of appetite, growth delay, reduced activities, sports injuries and convulsions occurred. In 1933, Rich and McCordock put forward that human tuberculosis in tuberculous meningitis was caused by the distribution of M. Tuberculosis into the cavum subarachnoidale and the cerebral ventricles due to the ulceration of the submeningeal Rich nodule [22], and therefore, M. tuberculosis injected intracranially directly experienced a different natural progression process in mice from in humans after all. In addition, the species difference between mice and humans may also lead to the differences in tolerance and immune responses to M. tuberculosis, and the virulence of H37RV was weaker than that of clinical strains. The limitations of specimen collection time in this study led to the differences between tuberculous meningitis in experimental models and humans.

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Disclosure of conflict of interest

None.

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