The protective effect of berberine against neuronal damage by inhibiting matrix metalloproteinase-9 and laminin degradation in experimental autoimmune encephalomyelitis

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Objective: This study aims to assess the protective effect of berberine against neuronal damage in the brain parenchyma of mice with experimental autoimmune encephalomyelitis (EAE).

Methods: EAE was induced in female C57 BL/6 mice with myelin oligodendrocyte glycoprotein 35–55 amino acid peptide. The berberine treatment was initiated on the day of disease onset and administered daily until the mice were sacrificed. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay, gelatin gel, and gelatin in situ zymography were analysed in this study.

Results: Berberine reduced the TUNEL-positive neuronal cells of EAE mice. Gelatin gel and gelatin in situ zymography showed up-regulation of gelatinase activity, which was mainly located in neurons and colocalized with remarkable laminin degradation in EAE mice. Berberine significantly inhibited gelatinase activity and reduced the laminin degradation in EAE mice.

Discussion: Our data suggest that berberine could provide protection against neuronal damage in EAE by inhibiting gelatinase activity and reducing laminin degradation. These findings provide further support that berberine can be a potential therapeutic agent for multiple sclerosis.

Keywords: Berberine, Apoptosis, Matrix metalloproteinase, Laminin, Neuroprotection, Experimental autoimmune encephalomyelitis

Introduction
Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). An increasing number of reports have focused on the neurodegenerative aspects of the disease pathogenesis, which is the major cause of irreversible neurological disability in MS patients and experimental autoimmune encephalomyelitis (EAE, an established model of MS) animals. It has been found that diffuse axonal injury and neuronal damage occur in the early stage of the disease, accumulate with disease progression, and compromise motor and sensory deficit.

Although anti-inflammatory and immunoregulatory therapies are effective in slowing down the progression of MS, disease-modifying agents, targeted at episodic immune-mediated inflammation, presumably cannot sufficiently reduce the axonal injury of MS pathology. Hence, it is necessary to develop effective therapeutic strategies for the disease focusing on the inhibition of axonal degeneration and neuronal damage.

Berberine (Fig. 1) is an isoquinoline derivative alkaloid isolated from many medicinal herbs, such as Hydrastis canadensis (goldenseal), Cortex phellodendri (Huangbai), and Rhizoma coptidis (Huanglian). It is frequently utilized in proprietary Chinese herbal drugs to treat inflammation, especially in the oral cavity. Berberine exhibits its anti-inflammatory effects by inhibiting the production of tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), and monocyte chemoattractant protein 1 (MCP-1),...
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Figure 1: Chemical structural formula of berberine.

suppressing cyclooxygenase-2 (COX-2) expression, retarding prostaglandin E2 (PGE2) production and exude formation, and down-regulating the expression of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) through mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) signaling pathways. Besides its anti-inflammatory effects, many other pharmacological benefits of berberine, including anti-oxidation, anti-tumor, and neuroprotective effects also have been reported. Berberine has potent neuroprotective effects against ischemic damage via the reduction of N-methyl-D-aspartate-receptor activity, and can suppress hippocampal CA1 neuronal apoptosis and increase the cell survival rate by blocking potassium channels under anoxic/ischemic injury. However, there are few reports about the neuroprotective effects of berberine against neuronal damage in MS and EAE.

Matrix metalloproteinases (MMPs), especially MMP-9, are important cytokines in inflammation, immunoregulation, and neurodegeneration in the CNS. In EAE/MS, MMP-9 activity is associated with short duration relapsing and active disease, and the severity of disease is significantly attenuated in mice deficient in the type MMP-9. Laminins, which participate in neuronal development, survival, and regeneration, are major components of the extracellular matrix and substrates for MMPs. The destruction of laminins around nerve cells by MMP-9 plays an important role in causing neuronal apoptosis. In MS, the ability of peripheral blood lymphocytes in degrading laminin may correlate with the disease activity. In EAE, laminin can selectively inhibit T lymphocyte extravasation into the brain and affect disease susceptibility and severity. 

Our previous study has found that berberine could attenuate the clinical severity of EAE in C57 BL/6 mice by reducing the permeability of the blood–brain barrier (BBB), and decreasing the activity of MMP-9 and inflammatory infiltration. The goal of the current study is to further assess the protective effect of berberine against neuronal damage in EAE mice. Neuronal apoptosis, gelatinase activity, and degradation of laminin in the brain parenchyma of EAE are evaluated. Our results demonstrate that berberine can significantly decrease neuronal apoptosis, inhibit gelatinase activity that is mainly located in neurons, and reduce the laminin degradation in EAE mice.

Materials and Methods

Animals and regents
Six to eight-week-old female C57 BL/6 mice weighing 16–18 g were obtained from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). Experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Bioethics Committee of Sun Yat-sen University. Berberine and complete Freund’s adjuvant (CFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by CL, Bio-Scientific Co., Ltd (Xi’an, China). Amino acid sequences were confirmed by amino acid analysis and mass spectrometry. The purity of the peptide was greater than 95%. Mycobacterium tuberculosis H37RA was purchased from Difco (Detroit, MI, USA). Pertussis toxin (PTX) was purchased from Alexis Corp (San Diego, CA, USA).

Induction and evaluation of EAE

Animals were housed 6/cage with a 12-h light/dark cycle, at 22–23°C and allowed free access to food and tap water. The induction of EAE was performed as described previously. Briefly, mice were immunized subcutaneously in the flanks with 0.2 mg of MOG35–55 peptide per animal emulsified in CFA containing 500 μg of Mycobacterium tuberculosis H37RA. Immediately thereafter, and again 48 h later, the mice received an intraperitoneal injection of 300 ng of PTX in 0.1 mL of phosphate buffered saline (PBS). An additional injection of MOG 35–55 peptide in CFA was administered 7 days later. The animals were examined daily for disability. Clinical scores were defined as follows: 0, no signs; 1, loss of tail tonicity; 2, flaccid tail; 3, ataxia and/or paresis of hindlimbs; 4, complete paralysis of hindlimbs; 5, moribund or death. The maximum disease score was achieved by each mouse over the course of the entire experiment. The overall disease burden of each mouse was represented as the cumulative disease severity, which was the sum of the disability scores obtained daily over the course of the 35-day experiment.

Treatment of mice
Mice were randomly assigned to three groups: control mice, PBS-treated EAE mice, and berberine-treated EAE mice. Each group had seven animals and every experiment was repeated three times. The dose of berberine was chosen on the basis of our previous report. Berberine was dissolved in PBS and an intragastric administration via per os (p.o.) using a 20 ga straight oral gavage needle for EAE mice at a...
dose of 30 mg/kg was given. PBS-treated EAE mice were given intragastric administration with PBS only. Treatment was initiated at the disease onset and administered daily until mice were sacrificed on day 35 post immunization (p.i.). To evaluate the treatment effects, the brains of differently treated EAE mice were subjected to different assay.

**Immunofluorescence and fluorescein-linked terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)**

To detect DNA strand breaks, TUNEL was measured using an *in situ* cell death detection kit, POD (Roche, Switzerland). Briefly, sections were incubated with fluorescein isothiocyanate (FITC)-linked TUNEL (Roche, Switzerland) and afterwards incubated with anti-neuron specific enolase (NSE) antibody (1:100; Sigma-Aldrich, USA) at 4°C overnight followed by goat anti-rabbit IgG/TRITC secondary antibody (1:300; Zhongshanjinqiao, China) for 2 h at room temperature. Images were acquired with an epifluorescence microscope (Nikon, Japan).

**Gelatin gel zymography**

Gelatinase activities of MMP-2 and MMP-9 in the brains of mice were identified as described previously.31,33 Briefly, brain tissues were homogenized in lysis buffer including protease inhibitors under ice-cold conditions. Supernatants were collected for analysis after centrifugation. The total protein concentrations were determined by Bradford assay. Equal amounts of prepared protein samples (20 µg) were loaded and separated by 10% Tris-glycine gel with 0.1% gelatin as substrate. After electrophoretic separation, the gel was renaturated and then incubated with developing buffer at 37°C for 48 h. After developing, the gel was stained with 0.4% Coomassie Blue for 1 h and then destained appropriately. The bands were quantified by densitometric analysis with Quantity One software.

**In situ zymography**

*In situ* zymography was performed to localize enzymatic activity on brain sections.34 Fresh brain slices (15 µm) of each group were incubated with reaction solution containing FITC-labeled gelatin (Molecular Probes, Eugene, OR, USA). To test the inhibitory effect of berberine on gelatinase activity in EAE mice, fresh brain sections were incubated with reaction solution containing FITC-labeled gelatin. *In situ* gelatinase activity was revealed by the appearance of fluorescent brain constituents. To identify the cellular sources of *in situ* gelatinase activity, we performed immunohistochemistry on the same brain section after incubation for gelatin *in situ* zymography. Sections were successively incubated with anti-NSE antibody (1:100; Sigma-Aldrich, USA), anti-glial fibrillary acidic protein (GFAP) antibody (1:300; Cell Signaling Technology, USA), anti-CD-45 antibody (1:100; Millipore, USA), anti-MMP-9 antibodies (1:50; Abcam, England) or anti-MMP-2 antibody (1:100; Thermo Scientific, USA) at 4°C overnight followed by goat anti-mouse IgG/TRITC secondary antibody (1:300; Zhongshanjinqiao, China) or goat anti-rabbit IgG/TRITC secondary antibody (1:300; Zhongshanjinqiao, China) for 2 h at room temperature, respectively. Images were acquired with an epifluorescence microscope (Nikon, Japan).

**Immunohistochemistry**

To access the expression of laminin in EAE, deeply anesthetized animals were perfused transcardially with ice-cold PBS, pH 7.4, followed with 4% ice-cold paraformaldehyde in PBS, pH 7.4. The brains were post-fixed overnight in the same fixative at 4°C, and cryoprotected in 15% and 30% sucrose solutions. Frozen sections (10 µm thick) were prepared using a cryostat. After blocking with PBS containing 0.2% Triton X-100 and 3% normal goat serum, sections were incubated overnight at 4°C with rabbit anti-laminin antibody (1:60; Abcam, UK) in PBS 0.2% Triton X-100 and 2% normal goat serum. The sections were washed with PBS, and incubated with secondary antibody solutions (goat-anti-rabbit IgG/FITC, 1:300, Zhongshanjinqiao, China) for 2 h. The sections were washed in PBS and the coverslips were mounted on slides with 90% glycerol. To examine the expression of MMP-9, frozen sections (10 µm thick) were prepared using a cryostat. After blocking with PBS containing 0.2% Triton X-100 and 3% normal goat serum, sections were incubated overnight at 4°C with anti-MMP-9 antibody (1:50; Abcam, UK) in PBS 0.2% Triton X-100 and 2% normal goat serum. The sections were washed with PBS, and incubated with secondary antibody solutions (goat-anti-rabbit IgG/TRITC or goat-anti-mouse IgG/TRITC, 1:300, Zhongshanjinqiao, China) for 1 h. Negative control sections received identical treatment except for the primary antibody. Reaction products showing FITC signal (green color) and TRITC signal (red color) were visualized by fluorescence microscopy (Nikon, Japan).

**Quantification of gelatin in situ zymography and laminin expression**

To measure fluorescent intensities of gelatin *in situ* zymography and laminin expression, quantification of fluorescent intensities were performed using an image analysing software (Image-Pro Plus 6.0). In each brain section, fluorescence was measured and presented as the percentage of fluorescent intensity of gelatin *in situ* zymography and laminin expression.

**Statistical analysis**

Data were expressed as means ± S.E.M. and analysed by SPSS 13.0 software. To evaluate the berberine...
effect, an ANOVA with Bonferroni post hoc test or a Student’s t-test was used. P values less than 0.05 were considered statistically significant.

The control mice showed very weak expression of TUNEL (Figs. 2 and 3A). TUNEL-positive cells were markedly present in EAE mice, berberine administration reduced TUNEL-positive cells (Fig. 3D and G). To determine the TUNEL-positive cellular sources involved, double staining was performed. According to the NSE expression, TUNEL staining was very weak in neurons of control mice (Fig. 3A–C), and TUNEL staining was located mainly in neurons of PBS-treated EAE mice (Fig. 3D–F). Berberine administration significantly reduced TUNEL-positive neuronal cells (Fig. 3G–I). Quantification of the number of TUNEL-positive cells and NSE-positive/TUNEL-positive cells in PBS-treated and berberine-treated mice is shown in Fig. 3J.

Berberine inhibited the activity of MMP-9 but not MMP-2 in the brains of EAE mice

The brains of control mice, PBS-, and berberine-treated EAE mice were obtained on day 20 p.i. (n=7). The expressions of MMP-2 and MMP-9 in the brains of differently treated mice were detected by gelatin gel (Fig. 4). There was a marked increase in MMP-2 and MMP-9 activity in the brains of PBS-treated EAE mice compared with control mice (P<0.01 for MMP-2 and MMP-9, respectively). The MMP-9 activity in the brains of berberine-treated EAE mice was significantly reduced when compared with PBS-treated EAE mice (P<0.05). There was no significant difference in MMP-2 expression between the PBS- and berberine-treated EAE mice.
Berberine reduced gelatinase activity and laminin degradation in the brains of EAE mice

The control mice showed weak gelatinase activity (Fig. 5A). Increased green-colored FITC signal representing gelatinase activity was clearly observed in the brains of PBS-treated EAE mice (Fig. 5B). Berberine-treated animals showed decreased gelatinase activity (Fig. 5C). In laminin immunostaining, a positive signal was also shown as green-colored FITC fluorescence (Fig. 5D–F). Laminin was degraded markedly in PBS-treated EAE mice (Fig. 5E). Berberine treatment reduced laminin degradation following EAE (Fig. 5F). In quantification of fluorescent intensity for gelatinase activity and laminin expression, berberine treatment significantly reduced the increased gelatinase activity and laminin degradation compared with PBS-treated EAE mice (Fig. 5G and H).

Gelatinase activity mainly located in neurons of EAE mice

To determine the cellular sources involved, sequential immunohistochemistry was performed on the brain sections after gelatin in situ zymography was completed. According to the NSE expression, gelatinase activity was located mainly in neurons (Fig. 6C). In laminin immunostaining, a positive signal was also shown as green-colored FITC fluorescence (Fig. 6D–F). Laminin was degraded markedly in PBS-treated EAE mice (Fig. 6E). Berberine treatment reduced laminin degradation following EAE (Fig. 6F). In quantification of fluorescent intensity for gelatinase activity and laminin expression, berberine treatment significantly reduced the increased gelatinase activity and laminin degradation compared with PBS-treated EAE mice (Fig. 5G and H).

Gelatinase activity mainly related to MMP-9 in EAE mice

Although both MMP-2 and MMP-9 may contribute to gelatinase activity, our double staining data showed that gelatinase activity was mainly related to the expression of MMP-9 (Fig. 7A–C), not MMP-2 (Fig. 7D–F).

Berberine reduced degradation of laminin by MMP-9 in EAE mice

Our double staining data showed that the increased activity of MMP-9 in EAE was colocalized with
laminin degradation (Fig. 8A–C). Berberine administration reduced the down-regulation of laminin activity simultaneously accompanied with the reduced fluorescent signal of MMP-9 activity (Fig. 8D–I). These results provided evidence for a link between decreased laminin immunoreactivity with increased MMP-9 activity.

Discussion

Berberine, an isoquinoline alkaloid of natural herb medicines with many pharmacological benefits, has been used in Chinese traditional medicine for the treatment of various infectious disorders for more than 3000 years. Recently, berberine has been found to regulate the differentiation of Th1 and Th17 cells and ameliorate the severity of EAE.35 Our laboratory has also found that berberine can attenuate the clinical severity of EAE by reducing the permeability of BBB, decreasing the activity of MMP-9 and the inflammatory infiltration.31 In the current study, the clinical parameter of EAE after oral berberine administration (Fig. 2) is similar to our previous results,31 and we further assess the protective effect of berberine against neuronal damage in the brains of EAE mice.

In our experiment, DNA degradation, a generally accepted feature of apoptosis, is detected in the brain parenchyma cells of EAE mice. Double labeling of TUNEL and NSE immunostaining shows that TUNEL staining mainly localizes in neurons of brain parenchyma. This is coincident with previous reports that neuronal damage is a prominent feature in the spinal cord and brain parenchyma of MS/EAE.36–40 Berberine administration effectively reduces TUNEL-positive neuronal cells in EAE mice. According to our results, we observe that not all TUNEL-positive cells are NSE-positive cells (neuronal cells). It may be that the apoptotic cells include the other cells in the brain of EAE. T cells, B cells, macrophages and microglia all undergo apoptosis in the CNS.41 Apoptosis of inflammatory cells also occurs in the brain of chronic relapsing EAE, especially during relapses.41 Bonetti et al.42 reported that apoptosis of CD4 T cells and macrophages/microglia occurred in EAE. There are some experimental data pointing to an important role for caspases in the OLGs apoptosis associated with autoimmune demyelination.43,44 In acute MS, 14–40% of OLGs die by apoptosis as measured by the TUNEL method.45 Barnett and Prineas proposed that OLG apoptosis is the initial event in new lesion formation and the primary cause of inflammation in MS.46 So, TUNEL-positive cells may include the above mentioned cells.

The mechanisms of neuronal damage in EAE have not been clearly elucidated, but MMPs, especially MMP-9 activity, play an important role. It is well known that MMP-9 is associated with the development and/or pathogenesis of MS as well as EAE,21 and causes more axonal injury than MMP-2.47 In our results, we find that both MMP-9 and MMP-2 activities increase in brain parenchyma, and berberine inhibits the activity of MMP-9 but not MMP-2 in gelatin zymography, which coincides with our previous report using Western blot assay.31 According to gelatin in situ zymography, we find increased gelatinase activity localizes in neurons of EAE mice. Berberine reduces gelatinase activity in neurons of EAE mice. Although both MMP-2 and MMP-9 may contribute to gelatinase activity in situ, our results show that MMP-9 is the dominant gelatinase in EAE, which coincides with previous studies.34,48–50

Figure 7 Double labeling of gelatinase activity in situ and MMP-9 and MMP-2 immunostaining in PBS-treated EAE mice. Gelatinase activity (A, green color) colocalized with MMP-9 staining of neurons (B, red color; C, merge). MMP-2 staining (E, red color) did not colocalize with gelatinase activity (D, green color). MMP-2 signals (E, red color) contacted with gelatinase activity signals (D, green color; F, merge). Scale bar = 100 μm.

Figure 8 Double labeling of laminin and MMP-9 immunostaining in differently treated mice. In the control mice, the expression of laminin was strong (A), but MMP-9 expression was very weak (B). Compared with the control mice, the fluorescent signal of laminin degradation (D) simultaneously accompanied with the up-regulating fluorescent signal of MMP-9 expression (E) in PBS-treated EAE (F, merge). However, berberine administration reduced the down-regulation of laminin activity (G) simultaneously accompanied with the down-regulating fluorescent signal of MMP-9 expression (H) (I, merge). Scale bar = 100 μm.
study also showed that MMP-9-null mice exhibited marked decrease in the severity of EAE and gelat
inase activity. For these reasons, we suggest that most of the gelatinase activity in neurons of EAE may be attributed to MMP-9 and not MMP-2.

The cellular source of MMP-9 detectable in EAE remains controversial because extracellular molecules in the brain may be derived from blood, and previous investigations showed that infiltrating macrophages, T cells, endothelial cells, and microglia were the main source of MMP-9. We here demonstrate that neuronal cells are the major location of MMP-9 in EAE, which is similar to the studies of cerebral ischemia, cortical spreading depression, and HIV-1 gp120-induced injury to striatum. Since MMP-9 functions as a protease after being secreted from cells, the location of MMP-9 immunoreactivity does not necessarily reflect the cells releasing MMP-9. It is unknown whether MMP-9 released from other cells is actively taken up by neurons or MMP-9 passively diffuses preferentially into injured neurons through the damaged plasmalemma. There may be more complicated interactions between neurons and the immune system. The neuronal localization of gelatinase activity in our study shows that the neuronal apoptosis is associated with the increased activity of MMP-9 in neurons of EAE. Berberine directly decreases gelatinase activity and participates in a suppressive mechanism of neuronal damage in EAE.

It has been reported that the destruction of the extracellular matrix around nerve cells by MMP-9 plays an important role in causing neuronal apoptosis. Matrix metalloproteinase-9 degrades the extracellular matrix protein laminin and this degradation induces neuronal apoptosis. Matrix proteins such as laminin are also widely disseminated throughout the brain parenchyma, and loss of parenchymal laminin may affect cell–matrix interactions and cell survival. It is conceivable that MMP-mediated matrix protein degradation may disrupt cell–matrix interactions and further contribute to neuronal cell death. In our study, increased gelatinase activity in EAE is colocalized with laminin degradation. Berberine administration reduces the down-regulation of laminin activity simultaneously accompanied with the reduced fluorescent signal of gelatinase activity. These results are similar to a recent research that berberine inhibited gelatinase activity directly in gelatin in situ zymography and reduced neuronal damage following global ischemia. Although EAE and global ischemia are different diseases with different pathogenesis, the inflammation mechanism may play a critical role in neuronal damage during the acute period of the two diseases. Berberine also may play a role in neuroprotection by influencing the relationship between MMP-9 and laminin with the BBB. The BBB opening is mediated by MMPs in neuroinflammation. Infiltrating inflammatory cells such as neutrophils, which contain latent MMP-9, bring it into the brain during inflammation. Matrix metalloproteinases open the BBB by attacking proteins in the basal lamina, including laminin. Disruption of the basal lamina may further lead to T lymphocyte extravasation into the brain and be sufficient to open the BBB, or it may expose tight junction proteins to MMP-9 and other proteases produced in the brain. The blockage to reduction of laminin due to decrease in MMP-9 expression by berberine administration in EAE probably links to restoration of the BBB, with its consequences, particularly the decrease in the number of infiltrating inflammatory cells and cytokines/chemokines expression.

Overall, our results suggest that berberine could provide a neuroprotective effect against neuronal damage in the brain following EAE by inhibiting gelatinase activity and reducing laminin degradation. These findings further support that berberine can be a potential therapeutic agent for MS.

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Conflict of Interest
All authors declare that there are no conflicts of interest.

References


