Inhibitory effect of matrine on blood-brain barrier disruption for the treatment of experimental autoimmune encephalomyelitis.

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Research Article

Inhibitory Effect of Matrine on Blood-Brain Barrier Disruption for the Treatment of Experimental Autoimmune Encephalomyelitis

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Dysfunction of the blood-brain barrier (BBB) is a primary characteristic of experimental autoimmune encephalomyelitis (EAE), an experimental model of multiple sclerosis (MS). Matrine (MAT), a quinolizidine alkaloid derived from the herb Radix Sophorae Flave, has been recently found to suppress clinical EAE and CNS inflammation. However, whether this effect of MAT is through protecting the integrity and function of the BBB is not known. In the present study, we show that MAT treatment had a therapeutic effect comparable to dexamethasone (DEX) in EAE rats, with reduced Evans Blue extravasation, increased expression of collagen IV, the major component of the basement membrane, and the structure of tight junction (TJ) adaptor protein Zonula occludens-1 (ZO-1). Furthermore, MAT treatment attenuated expression of matrix metalloproteinase-9 and -2 (MMP-9/-2), while it increased the expression of tissue inhibitors of metalloproteinase-1 and -2 (TIMP-1/-2). Our findings demonstrate that MAT reduces BBB leakage by strengthening basement membrane, inhibiting activities of MMP-2 and -9, and upregulating their inhibitors. Taken together, our results identify a novel mechanism underlying the effect of MAT, a natural compound that could be a novel therapy for MS.

1. Introduction

Multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), are T-cell-mediated inflammatory diseases characterized by lymphocyte infiltration, demyelination, and axonal injury [1, 2]. Although MS pathology is not fully understood, blood-brain barrier (BBB) dysfunction plays an essential role in the pathogenesis of this disease. In both MS and EAE, proinflammatory cells and toxic molecules migrate into the brain via the damaged BBB, resulting in cerebral edema, demyelination, and neural cell death [3, 4].

The BBB is composed of basement membrane, interendothelial tight junctions (TJs), and perivascular astrocytes [5]. The basement membrane, which is composed of two distinct types, namely, endothelial basement membrane and parenchymal basement membrane, is a tight assembly of specialized extracellular matrix molecules [6]. This membrane, together with the endothelial cell monolayer, forms a structural barrier that selectively filters blood elements [6, 7]. Collagen IV comprises 90% of total protein in the basement membrane and plays a decisive role in maintaining the structural integrity of the vessel wall [8, 9]. Collagen IV, as a major component of the cerebral microvascular basal lamina, is widely used as a marker to determine the extent of destruction of the basement membrane.

TJs, composed of large multiprotein complexes, seal the gaps between biological barriers [4]. Altered distribution or loss of TJs is frequently seen in ischemic cerebral microvessels, resulting in diminished BBB integrity [10]. Zonula occludens-1 (ZO-1) is the primary cytoplasmic protein associated with TJs, which links the C-terminal ends of occludin and claudins to the underlying actin cytoskeleton [7]. A decrease in ZO-1 expression results in increased BBB...
permeability [11]. In addition, disease severity during the acute phase of EAE is directly associated with the extent of BBB permeability [12].

It has been shown that BBB disruption is accompanied by excessive expression of matrix metalloproteinases (MMPs) [13]. MMPs, including MMP-9 and MMP-2, belong to a class of zinc-bound proteases, whose functions include induction of inflammation, cleavage of myelin proteins, activation or degradation of disease-modifying cytokines, and direct damage to CNS cells [14]. Abnormal increases in MMP-9 and MMP-2 in endothelial cells may collectively impair endothelial barrier function by degrading the vascular basement membrane and TJ's [10, 14, 15]. Furthermore, MMP-9 and MMP-2 are upregulated in the CNS of rat models of EAE [16].

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of MMPs. TIMP-1 controls MMP-9 activity through high affinity, noncovalent binding to the MMP catalytic domain, whereas MMP-2 is bound by TIMP-2 [17]. It has been shown that TIMP-1 deficiency enhances disease severity during EAE [18]. Under normal physiological conditions, there is a constant balance between MMP and TIMP activity, which is essential in maintaining the physiological functions of the organism [19]. In contrast, an imbalance in MMP/TIMP ratio is found in various pathological conditions in humans, such as cancer, rheumatoid arthritis, and vascular diseases [20]. For example, the serum MMP-9/TIMP-1 ratio in relapsing-remitting MS patients correlates with development of the disease [17]. An imbalance between MMP-2 and TIMP-2 caused by radiation plays a role in the pathogenesis of brain injury [21].

Currently, treatment of MS is limited to immunomodulatory or immunosuppressive therapy, which is not always successful and often has severe side effects [22]. Hence, the search for more effective and more tolerable compounds is of great importance. Matrine (MAT) is a natural alkaloid component extracted from the herb Radix Sophorae Flaves, with a MW of 258.43 (C_{18}H_{23}N_{2}O, Figure 1(a)). It has been reported that MAT suppressed immune activities of T cells, B cells, and macrophages [23]. Matrine has long been used for the treatment of viral hepatitis, cardiac arrhythmia, and skin inflammation, without known side effects [24, 25]. While MAT suppressed development of EAE, its mechanism for neuroprotection has not been elucidated. The purpose of this study was to determine whether MAT treatment inhibits BBB disruption by reducing BBB leakage, strengthening the basement membrane, enhancing TJ's, and regulating the balance between MMPs and TIMPs during disease progression of EAE.

2. Materials and Methods

2.1. Animals. Female, 6-7 week-old Wistar rats were purchased from Shanghai Xipuer-Bikai Experimental Animal Company, China, and housed in the aseptic laboratory of the Experimental Animal Center of Henan, China. All efforts were made to minimize the numbers of animals used and to ensure minimal suffering.

2.2. EAE Induction and Treatment. EAE was induced as described previously [23] with only minor modifications. Spinal cord homogenate of guinea pig (Experimental Animal Center of Hebei) weighing 300–350 g was emulsified with the same volume of complete Freund's adjuvant (CFA) (Sigma, USA) containing 6 mg/mL Bacillus Calmette-Guérin vaccine (Shanghai Institute of Biological Products, China). Each rat received a subcutaneous injection of 0.5 mL emulsion divided among 5 sites draining into the nape and back. All procedures were approved by the Bioethics Committee of Zhengzhou University.

Immunized rats were randomly divided into four groups (n = 16 each group) for different treatments. Briefly, MAT (Jiangsu Chia Tai Tianqing Pharmaceutical Co., Jiangsu, China) was dissolved in normal saline and injected intraperitoneally (i.p.) daily at two doses: low (150 mg/kg; MAT-L) and high (250 mg/kg; MAT-H), the dosage calculated at 6.7 mL/kg, from day 1 until day 17 after immunization (p.i.). Dexamethasone (DEX) (Henan Hongrun Pharmaceutical Co., Henan, China), as the positive control drug, was dissolved in normal saline (6.7 mL/kg) and injected (i.p.) daily, from day 1 until day 17 p.i. at 1 mg/kg. Immune rats that received the same amount of normal saline only i.p. served as a vehicle control, and 16 nonimmunized naive rats that received the same amount of normal saline i.p. served as the naive group.

2.3. Clinical Scoring and Weight. Rats were monitored and weighed daily by two independent observers to evaluate clinical scores of EAE after immunization. Neurological signs were assessed as follows [23]: 0 = no clinical score; 1 = loss of tail tone; 2 = hind limb weakness; 3 = hind limb paralysis; 4 = forelimb paralysis; 5 = moribund or death.

2.4. Histopathological Evaluation. On day 17 p.i., two independent observers randomly selected 8 rats from each group. When animals were sacrificed, sera and spinal cords were collected after extensive perfusion. The lumbar enlargement of spinal cords was embedded in paraffin. After embedding, 2-3 μm thick sections were prepared and stained with hematoxylin-eosin (HE) for inflammatory infiltration and chromotrope 2R-brilliant green (C-2R-B) for demyelination. Histopathological examination was performed and scored in a blinded fashion as follows [22]: for inflammation: 0, no inflammatory cells; 1, a few scattered inflammatory cells; 2, organization of inflammatory infiltrates around blood vessels; 3, extensive perivascular cuffing with extension into adjacent parenchyma, or parenchyma; for demyelination: 0, none; 1, rare foci; 2, a few areas of demyelination; 3, large (confluent) areas of demyelination.

2.5. Evaluation of BBB Leakage. BBB leakage was assessed using Evans Blue (EB) dye as previously described [26]. On day 17 p.i., the 8 rats remaining in each group were anesthetized. EB dye (2%; 4 mL/kg; Sigma, St. Louis, MO, USA) was injected slowly into the tail vein and was allowed to circulate for 60 minutes. When the rats were sacrificed, brains were removed and immediately weighed. The EB
Figure 1: MAT reduces clinical signs of EAE. (a) Structure of MAT. (b) Effect-versus-time curves of MAT effects on EAE. Clinical EAE was scored daily after immunization according to a 0–5 scale. (c) The mean maximum clinical scores are expressed as mean ± SD (n = 16 each group). △△p < 0.01, compared to naive group; *p < 0.05, compared to vehicle-treated group.

dye was extracted in 2.5 mL of PBS, and 2.5 mL of 60% trichloroacetic acid was added. The mixture was then vortexed and centrifuged for 40 min at 4000 rpm, and the amount of EB dye in supernatants was determined at 610 nm by spectrophotometry and quantified to μg/g brain tissue.

2.6. RT-PCR Analysis of Collagen IV and ZO-1 mRNAs. The cervical spinal cords were harvested on day 17 p.i. and were prepared for analysis of collagen IV and ZO-1 mRNA using real-time polymerase chain reaction (PCR). Total cellular RNA from these tissues was isolated using TRIzol reagent (Beijing TransGen Biotech Co., Beijing, China) following the standard protocol. cDNA synthesis was performed by reverse transcription using a Promega reverse transcription kit. The cDNA copy number for each gene was determined using standard curves of the corresponding PCR product. Primers for collagen IV were 5’-GGCCCCCTGCTGAAGCCTTGT-3’ (forward) and 5’-GTTCCCCGAGCACCTTAG-3’ (reverse), which produced a 306 bp PCR product. Primers for ZO-1 were 5’-CCATCTTTGGACCAGTTGCTG-3’ (forward) and 5’-TAATGCCCGAGCTCCGATG-3’ (reverse), which produced a 372 bp PCR product. Gene expression was normalized to expression of the endogenous housekeeping gene β-actin. The β-actin primers were 5’-CCCTGAGCTTGAAGGCCAAC-3’ (forward) and 5’-TGCCACAGGATTCATACC-3’ (reverse), which produced a 564 bp PCR product. To determine the relative quantification of target gene expression, we used the gel imaging analysis system (Dalian Jingmai Biotech Co., Liaoning, China).

2.7. ELISA Analysis of MMP-9 and TIMP-1. Serum collected on day 17 p.i. was assayed for concentrations of MMP-9 and TIMP-1 by ELISA following the manufacturer’s instructions (R & D Systems, USA). Samples were quantified by comparison with the standard curves of MMP-9 and TIMP-1 (0–200 ng/mL).

2.8. Immunohistochemical Analysis of MMP-2 and TIMP-2. Paraffin-embedded tissue of spinal cords from each group was cut into 5 μm thick sections. Immunohistochemistry was performed on these slices, using anti-rat antibodies for MMP-2 and TIMP-2 (all from Beijing TransGen Biotech Co.; Beijing, China). Sections were rinsed and incubated in nonbiotinylated goat anti-rabbit IgG secondary antibody. The chromophore product was developed using a Simple Stain DAB solution (Beijing TransGen Biotech Co., Beijing, China). The integral optical density (IOD) of positive cells in a restricted area was determined to represent the expressions of MMP-2 and TIMP-2 using Biosens Digital Imaging System v1.6.

2.9. Statistical Analysis. All data are presented as mean ± SD. Statistical analysis was performed with SPSS 16.0 (SPSS,
TABLE 1: Mean maximum clinical scores and body weight on day 17 p.i.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g) (mean ± SD) (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive control</td>
<td>224.38 ± 13.99</td>
</tr>
<tr>
<td>Vehicle-treated group</td>
<td>154.63 ± 10.83ΔΔ</td>
</tr>
<tr>
<td>MAT-L</td>
<td>173.56 ± 11.03**E</td>
</tr>
<tr>
<td>MAT-H</td>
<td>182.31 ± 10.12**E</td>
</tr>
<tr>
<td>DEX</td>
<td>145.68 ± 9.89</td>
</tr>
</tbody>
</table>

ΔΔP < 0.01, compared to the naive group; *P < 0.05 and **P < 0.01, compared to vehicle-treated group; △△P < 0.01, compared to DEX group; δP < 0.05, comparison between MAT-L group and MAT-H group.

Chicago, USA). Multiple comparisons were performed using the Kruskal-Wallis test, or ANOVA, followed by the LSD-t test, as appropriate. A P value less than 0.05 was considered significant.

3. Results

3.1. MAT Treatment Alleviates Clinical Severity of EAE. In the vehicle-treated group, clinical decline typically started on day 10 p.i., while EAE onset for MAT-treated rats occurred on day 12 p.i. (low dose) and 13 p.i. (high dose) (Figure 1(b)). Compared to the vehicle-treated group, both MAT-treated groups exhibited significantly lower mean maximum clinical scores (Figure 1(c)) and body weight loss (Table 1). Treatment with DEX also delayed disease progression and reduced clinical scores compared to the vehicle-treated group (P < 0.05). There was no significant difference between animals treated with DEX and the two different doses of MAT.

3.2. Effect of MAT Treatment on CNS Histopathology. To assess EAE neuropathology, lumbar enlargements of spinal cord samples were examined using H&E and myelin staining (Figure 2). Perivascular cuffing with mononuclear cells and infiltration into CNS parenchyma were observed in the spinal cord of rats in the vehicle-treated group (Figure 2), while the extent of cellular infiltration was significantly decreased in the MAT-treated groups (both P < 0.01). Treatment with DEX showed a stronger inhibition in cellular infiltration than MAT-treated groups (both P < 0.01). Cellular infiltration was not observed in the naive group. Moreover, as shown in Figure 2, large areas of demyelination were observed in the vehicle-treated group, while MAT- and DEX-treated groups exhibited only a few areas of demyelination. No significant difference in demyelination was observed between MAT-H and DEX groups.

3.3. MAT Inhibits Evans Blue (EB) Leakage through the BBB. Destruction of the BBB is one of the important features of MS and EAE. We quantified the extravasation of EB dye into the brain as an indicator of BBB permeability. EB extravasation into the brain of vehicle-treated EAE rats was significantly higher than in naive brain (P < 0.01) (Figure 3). The content of EB was significantly decreased in MAT- and DEX-treated groups compared to the vehicle-treated group (all P < 0.01), while a more profound decrease in EB content was observed in rats treated with MAT-H and DEX than with MAT-L (P < 0.05). No significant difference was observed between those treated with high doses of MAT and DEX.

3.4. MAT Protects Collagen IV and ZO-1 mRNA Expression. To determine the basement membrane disruption, we evaluated mRNA expression of collagen IV, a major component of the cerebral microvascular basement membrane, using RT-PCR analysis. As shown in Figure 4(a), the brightest band was exhibited in the naive group, while the faintest band was in the vehicle-treated group, consistent with quantitative analysis (Figure 4(c)). A dose-dependent increase in collagen IV expression was observed in MAT-treated groups. While the MAT-L-treated group showed lower collagen IV expression than the DEX group (P < 0.01), there was no significant difference between MAT-H and DEX groups.

We also assessed ZO-1 mRNA expression to determine TJ disruption. A similar pattern of mRNA expression of ZO-1 was observed for collagen IV (Figures 4(b) and 4(c)).

3.5. MAT Adjusts the Balance between MMP-9 and TIMP-1 in Serum. Serum concentration of MMP-9 was measured by ELISA. The amount of MMP-9 was dramatically increased in the vehicle-treated group compared to the naive group (P < 0.01) (Figure 5). MAT treatment largely reduced MMP-9 content compared to the vehicle-treated group (both P < 0.01), and the effect was dose dependent. Furthermore, a significantly lower amount of MMP-9 was observed in the DEX-treated group than in the MAT-treated groups (P < 0.01).

We also measured serum concentrations of TIMP-1 in the different groups. Figure 5 shows a significant decrease in TIMP-1 concentration in the vehicle-treated group compared to the naive group (P < 0.01). TIMP-1 serum levels in the MAT-treated groups were significantly higher than in the vehicle-treated group (both P < 0.01). While a low dose of MAT induced a lower serum TIMP-1 level than DEX (P < 0.01), a high dose of MAT increased the serum TIMP-1 level to a greater extent than DEX (P < 0.01).

3.6. MAT Regulates the Balance between MMP-2 and TIMP-2 in the CNS. To determine the MMP-2/TIMP-2 balance in the CNS, their expression in the spinal cord was measured by immunohistochemistry. As shown in Figure 6, MMP-2 expression was significantly increased in the vehicle-treated group over the naive group (P < 0.01). TIMP-2 serum levels in the vehicle-treated group were significantly higher than in the naive group (both P < 0.01). While the differences in MMP-2 expression between vehicle-treated and the two MAT-treated groups were significant (both P < 0.01). Furthermore, the effect of DEX in decreasing MMP-2 content was stronger than low-dose MAT (P < 0.05), but there was no significant difference compared with the MAT-H group.

We then measured TIMP-2 content using the same method. As shown in Figure 6, TIMP-2 expression in the vehicle-treated group was significantly lower than in the naive group (P < 0.01) and MAT-treated groups (P < 0.05–0.01). A significantly lower amount was obtained in the MAT-L group than in the MAT-H group (P < 0.05). While the
MAT-L-treated group showed lower TIMP-2 expression than the DEX-treated group ($P < 0.05$), there was no significant difference between MAT-H and DEX groups.

4. Discussion

Although administration of MAT reduced CNS inflammatory infiltration and demyelination, the effect of this natural compound on the BBB has not yet been studied. In the present study, we provide evidence that the therapeutic effect of MAT is, at least partially, through its strengthening of BBB integrity, its protection of the basement membrane as well as TJ proteins, and its ability to regulate the balance between MMPs and TIMPs in both the periphery and CNS.

BBB destruction has been implicated in many CNS diseases, such as MS and stroke [4, 11]. In MS patients and in the EAE model, breakdown of the BBB is an early critical event, which is associated with the influx of inflammatory cells and, ultimately, with a poor outcome [3]. We thus quantified the extravasation of EB dye into the brain as an indicator of BBB permeability. Indeed, our study showed that EB leakage was markedly increased in the EAE model, which was associated with a decrease in collagen IV and ZO-1 expression in vehicle-treated rats compared to naive rats. MAT treatment largely...
and significantly decreased EB content in the brain compared with the vehicle group. Together, these results demonstrate that BBB integrity is compromised during the development of EAE and that MAT treatment protects the BBB.

BBB integrity depends on adequate structural support from the basement membranes, and collagen IV comprises up to 90% of total protein in the basement membrane [7]. In addition, collagen IV is critical for cell signaling by the interaction with various receptors and adhesion molecules, and its expression is therefore a marker of barrier damage and impairment [27]. A study by Lee et al. clearly shows that degradation of collagen IV has a role in the pathogenesis of BBB destruction and brain injury [28]. To date, it is not known whether decreased collagen IV contributes to BBB damage caused by EAE. We have shown in the present study that expression of collagen IV mRNA was significantly decreased in the vehicle-treated group compared to the naive group, and a dose-dependent increase of collagen IV mRNA expression was observed in MAT-treated groups. These results indicate that collagen IV degradation plays an important role in BBB disruption and that MAT treatment effectively preserves the content of collagen IV.

In the BBB, TJs are composed of large multimeric complexes that mediate tight intercellular contacts among adjacent cells and play a critical role in maintaining BBB function by improving the barrier function at the endothelial level [29]. Previous publications have reported varying degrees of TJ pathology in EAE [30, 31]. Bennett et al. found that relocation of ZO-1, which is a multidomain polypeptide required for the assembly of TJs [32], precedes disease onset and correlates with CNS infiltration in EAE [33]. Similar to these observations, our study found a decrease in ZO-1 mRNA expression in vehicle-treated group compared to the naive group and that ZO-1 mRNA levels were significantly improved with MAT treatment in a dose-dependent manner. These results are consistent with preserved collagen IV levels and indicate a protected BBB basement membrane.

In order to further study the mechanism of BBB protection induced by MAT treatment, we evaluated the activity and balance of MMPs and TIMPs. MMPs, a group of zinc-containing endopeptidases, cleave most components of the basement membrane including fibronectin, laminin, proteoglycans, and collagen IV [34, 35]. It has been found that focal MMP-2 and MMP-9 activity is closely associated with the infiltrating T cells penetrating through the parenchymal basement membrane [5, 6]. Upregulation of MMP-2 and MMP-9 resulted in the degradation of TJs after focal ischemia/reperfusion, which can be reversed by MMP inhibition [36, 37]. In addition, a selective upregulation of MMP-9 in MS disease activity has been described [17]. Similarly, a significant increase in MMP-2 expression in the central canal of the cervical spinal cord is a sign of inflammation in acute EAE [38]. Furthermore, among the MMP family, MMP-9, together with MMP-2, is a member of the collagenase IV family, which has been implicated in the degradation of constituents of the basement membranes [15]. Targeting MMPs and chemokines has been considered an important therapeutic approach, alone or in combination with current medications, in enhancing their effect in neurological disorders such as MS [39]. In the present study, significant upregulation of MMP-9 in serum was observed in the vehicle-treated group compared to the naive group, and MAT treatment reduced the levels of MMP-9. Further, the loss of collagen IV and ZO-1 was reduced by blocking MMP-9 and MMP-2 in EAE. These studies suggest that overexpression of MMP-9 and MMP-2 in EAE could have been a causative agent in the reduced intensity of collagen IV and ZO-1; MAT treatment inhibition of MMP-9 and MMP-2 levels will preserve levels of collagen IV and ZO-1 and will thus be beneficial for BBB integrity.

The active forms of all MMPs are inhibited by a family of specific inhibitors, tissue inhibitors of metalloproteinases (TIMPs) [18, 19]. MMP-9 and MMP-2 are preferentially inhibited by TIMP-1 and TIMP-2, respectively, [17]. It has been found that normal homeostasis in the CNS requires a balance between MMPs and TIMPs, while an imbalance between these molecules is often associated with CNS pathology [40]. For example, radiation-induced brain injury is associated with an increased ratio between MMP-2 and TIMP-2 [21], and a significant increase in the MMP-9/TIMP-1 ratio also correlates with MS activity [17]. In our study, an imbalance between MMP-9 and TIMP-1 was observed in the vehicle-treated EAE group, with upregulation of MMP-9 and downregulation of TIMP-1; this was also the case for balance between MMP-2 and TIMP-2. We could speculate that overexpression of MMP-9/-2 in EAE was counterbalanced by MAT-mediated increase in TIMP-1/-2 expression, thus constituting a steady balance of MMPs and TIMPs that can be attributed to improved BBB function.

To further investigate the therapeutic effects of MAT on EAE, we compared MAT with DEX, a glucocorticoid, which was chosen as the positive control drug because of its strong ability to suppress inflammation. While MAT at a low dose showed a weaker effect than DEX, a comparable effect was observed between DEX and MAT-H groups, with a stronger effect of MAT in improving the TIMP-1 content in the serum. We believe that MAT would prove to be superior to DEX, whose long-term use carries with it the risk...
of side effects common to systemic glucocorticoids occurring over a relatively prolonged period. These side effects include hyperglycemia, hypertension, negative calcium balance, osteoporosis, weight gain, or even immunodeficiency [41, 42]. The extensive use of DEX in the treatment of severe acute respiratory syndrome (SARS) has often resulted in hypocortisolism and osteonecrosis of the femoral head, causing patients to lose the ability to work [43]. In contrast, patients with hepatitis B who used MAT for a long time showed significant therapeutic effect and good tolerance, with only minor side effects such as infrequent, transient dizziness and nausea. Whether long-term use of MAT would be safer and have fewer side effects than DEX needs further investigation.

In summary, our study demonstrates that improving BBB integrity is one of the mechanisms of MAT action in EAE therapy. This effect is at least partially through inhibiting activities of MMP-2/-9 and protecting the basement membranes and tight junction proteins, thus improving BBB integrity. As a result, inflammatory infiltration into the CNS is largely reduced, thereby protecting CNS tissues from proinflammatory cell/mediator-induced damage. While the process of immune cell extravasation is partially an endothelial cell-mediated process [44], whether MAT reduces this pathway of immune cell infiltration is not yet

**Figure 4:** Collagen IV and ZO-1 mRNA expression. Spinal cords were harvested from treated and untreated EAE rats at day 17 p.i.; mRNA expression of collagen IV (a) and ZO-1 (b) was determined by RT-PCR, and an example of bands is shown. (c) Quantitative analysis. Results are expressed as mean ± SD (n = 8 each group). △△P < 0.01, compared to naive group; **P < 0.01, compared to vehicle group; ##P < 0.01, compared to DEX group; ⋄⋄P < 0.01, comparison between MAT-L and MAT-H groups.

**Figure 5:** Serum concentration of MMP-9 and TIMP-1. At day 17 p.i., sera were harvested from treated and nontreated EAE rats, with sera from naive rats serving as control. MMP-9 and TIMP-1 production was determined by ELISA. Results are expressed as mean ± SD (n = 8 each group). △△P < 0.01, compared to naive group; **P < 0.01, compared to vehicle group; ##P < 0.01, compared to the DEX group; ⋄⋄P < 0.01, comparison between MAT-L and MAT-H groups.
known. Nevertheless, results from the present study, together with the suppressive effect of MAT on Th1/Th17 cells [23] and its safety, suggest that MAT could qualify as an effective, alternative medication in MS therapy and that further study to test this possibility is warranted.

**Conflict of Interests**

None of the authors have a conflict of interests with any trademark mentioned in this paper.

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