Article

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Rapamycin Plays an Anti-Epileptic Role by Restoring Blood-Brain Barrier Dysfunction, Balancing T Cell Subsets and Inhibiting Neuronal Apoptosis

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Background: Rapamycin (RAP), as a Mammalian Target of Rapamycin (mTOR) inhibitor, has a certain antiepileptic effect. The blood-brain barrier (BBB), neuroinflammation, lymphocyte immune cells, and neuronal apoptosis play an obligatory role in the course of a seizure. The aim of this study is to probe whether the antiepileptic mechanism of RAP involves the blood-brain barrier, neuroinflammation, lymphocytes, and neuronal apoptosis.

Methods: First, we established a rat epilepsy model by injecting lithium chloride and pilocarpine into the rats (intraperitoneal injection). Then the epileptic rats were treated with different doses of RAP (1 mg/kg.d, 2 mg/kg.d, 4 mg/kg.d). Peripheral blood, brain tissue, and temporal lobe tissue were collected. The levels of blood-brain barrier-related proteins and inflammatory cytokines in the peripheral blood of rats were measured by enzyme-linked immunosorbent assay (ELISA). The effect of RAP on T cell subsets in epileptic rats was analyzed by flow cytometry. The apoptosis of neurons and glial cells in the temporal lobe of rats was analyzed by immunohistochemistry.

Results: This study found that RAP reduces the levels of BBB-interrelated proteins (matrix metallopeptidase 9 (MMP-9), MMP-2, tissue inhibitor of metalloproteinases 1 (TIMP-1), TIMP-2) and inflammatory cytokines (interleukin-2 (IL-2), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α)) in epileptic rats compared to the model group ($p < 0.05$). RAP increases the level of total T cells ($CD3^+CD45^+$) and T helper cells ($CD3^+CD4^+$), decreases the level of cytotoxic T lymphocytes ($CD3^+CD8^+$), and inhibits the apoptosis of neurons and glial cells in the temporal lobe compared to the model group ($p < 0.05$).

Conclusions: The antiepileptic mechanism of RAP may be to restore BBB dysfunction, reduce the inflammatory response, balance T cell subsets, and inhibit neuronal and glial cell apoptosis in temporal lobe epilepsy lesions.

Keywords: rapamycin; epilepsy; BBB; inflammatory cytokines; T cell subsets; neurons; glia

Introduction

Epilepsy is a common neurological disease. It is mainly characterized by sudden disturbance of consciousness and limb convulsions [1]. The epileptic seizures are caused by abnormal electrical activity in the brain's neurons, and currently, artificial neurons are used to simulate human brain biological neurons for the diagnosis of epilepsy [2]. According to the latest data, about 70 million people have epilepsy globally, and 30% of them develop intractable epilepsy, which can lead to listlessness, confusion, and even suicidal tendencies [3]. As many as one-third of people with epilepsy develop persistent drug resistance that cannot be adequately treated with antiepileptic drugs [4]. Therefore, there is an urgent need to develop a treatment that can prevent seizures in patients at high risk of epilepsy.

In recent years, rapamycin (RAP), a Mammalian Target of Rapamycin (mTOR) pathway inhibitor, has been demonstrated to be effective as a drug for the treatment of epilepsy [5]. RAP therapy has been also shown to play a neuroprotective role, but the detailed mechanism of RAP antiepilepsy is still unknown.

Blood-brain barrier (BBB) dysfunction is important in seizure onset and seizure development [6,7]. Increased BBB permeability plays a vital role in brain disorders, resulting in an influx of extracellular matrix, as well as endogenous and exogenous substances, causing local inflammation and angiogenesis [8]. Prolonged BBB destruction can lead to epilepsy, including chronic recurrent seizures [9]. A growing number of testimonies have indicated the fundamental function of neuroinflammation in the development of epilepsy, and inflammatory cytokines are key contributors to the inflammatory response in the brain [10,11].

There is further evidence that seizures are closely related to the body's immunity, especially T cell immunity [12]. In addition, T lymphocyte subsets are the basis for

maintaining cellular immune function, including $CD4^+$ and $CD8^+$ T cells [13]. The apoptosis of neuronal cells and glial cells plays an irreplaceable role in epilepsy [14,15].

We hypothesized that the antiepileptic effects of RAP may be mediated by the BBB, neuroinflammation, T lymphocyte subsets, temporal lobe neurons, and glial cells. Hence, we researched the role of RAP in regulating BBB-related proteins, inflammatory cytokines, T cell subsets, and neuronal and glial cell apoptosis in epileptic rats.

Materials and Methods

Experimental Animals

In this work, we selected 50 SPF-grade male SD rats, aged 8 weeks, as the research animals, and 40 of them were randomly selected to be injected with lithium chloride (120 mg/kg) and pilocarpine (20 mg/kg) to establish a rat model of epilepsy (intraperitoneal injection). Using the Racine scoring criteria, rats with three or more episodes of 4 points were considered to have been successfully established as animal models [16]. We anesthetized the rats by injecting pentobarbital sodium (60 mg/kg) and euthanized them. This study was approved by the Laboratory Animal Management and Ethics Committee of Xiamen University (approval no. XMULAC20220008).

Treatment with RAP

Ten rats without epilepsy were used as the control group (n = 10). We divided rats with epilepsy into four groups (n = 10 in each group). Ten epileptic rats without RAP treatment were used as the model group. The other groups of epileptic rats were treated with RAP injection. RAP stock solution was prepared with 100% ethanol and stored at 20 ℃. The control group and model group were treated with normal saline. According to intervention dosages of 1 mg/kg.d, 2 mg/kg.d, and 4 mg/kg.d, rats receiving RAP were divided into low-dose (n = 10), middle-dose (n = 10), and high-dose (n = 10) groups. Rats with epilepsy that received RAP were treated for 8 weeks. After 8 weeks, peripheral blood and temporal lobe tissues of rats were collected.

Enzyme-Linked Immunosorbent Assay (ELISA) [17]

We analyzed four BBB-associated proteins (matrix metallopeptidase 9 (MMP-9) [EK3M09], MMP-2 [EK1M02], tissue inhibitor of metalloproteinases 1 (TIMP-1) [EK3138], TIMP-2 [EK1283]) and inflammatory cytokines (interleukin-2 (IL-2) [EK302], IL-10 [EK310], interferon-γ (IFN-γ) [EK380], tumor necrosis factor-α (TNF-α) [EK382]) in peripheral blood using a double-antibody sandwich assay. ELISA test kits were purchased from Hangzhou Multi Sciences Biotechnology Co., LTD. (Hangzhou, China). Data were acquired using a Multiskan Go microplate reader (muLISKANMK3, Thermo, Massachusetts, USA).

Flow Cytometry

Total T cells ($CD3^+CD45^+$), T helper cells ($CD3^+CD4^+$), and cytotoxic T lymphocytes ($CD3^+CD8^+$) were treated with the corresponding antibodies (CD3-BV510, CD45-PE-CY7, CD8-BV421, CD4-PerCP-Cy5.5) and were measured by flow cytometer (CytoFLEX, Beckman Coulter, Chaska, MN, USA). All antibodies were purchased from Invitrogen (CA, USA). Each 1-mL sample of peripheral blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes for cell counting analysis. 50 µL of whole blood per tube was incubated with monoclonal antibodies for 30 min shielded from light. Erythrocytes were lysed in a buffer containing 0.8% NH_4Cl and 0.1% $KHCO_3$. The cells were suspended in 200 mL Phosphate-Buffered Saline (PBS) to detect T-lymphocyte subsets.

Histological Examination

We fixed temporal lobe tissue in phosphate buffer containing 10% paraformaldehyde (G1128, Servicebio, Wuhan, China); the tissue was dehydrated, embedded in paraffin, and sectioned with a thickness of about 5–7 µm. Subsequently, staining was performed using hematoxylin and eosin (H&E; G1003, Servicebio, Wuhan, China) as well as Nissl (G1430, Solarbio, Beijing, China) staining reagents. The observation was performed with a light microscope (Nikon Eclipse E100, Nikon, Tokyo, Japan) and the images were used for histomorphological analysis.

Immunohistochemistry

The assessment of apoptosis in the white and gray matter regions of the cerebral cortex was conducted by detecting the presence of caspase-3 using immunohistochemistry (IHC). The temporal lobe tissue section was subjected to IHC reactions. Rabbit polyclonal antibody against active caspase-3 (1:500, 66470-2-Ig, Proteintech, Wuhan, China) and an image detection system (Nikon Eclipse E100, Nikon, Tokyo, Japan) were used to determine apoptotic cells. Other reagents used in ICH experiments were purchased from Servicebio (Wuhan, China).

Statistical Analysis

We used GraphPad Prime software 8.0 (San Diego, CA, USA, https://www.graphpad-prism.cn/) for statistical analysis. We tested mean differences between the two groups using *t*-tests. One-way analysis of variance was used to test mean differences across multiple groups. Data were presented as mean ± standard deviation (SD). *p*-values < 0.05 were considered statistically significant.

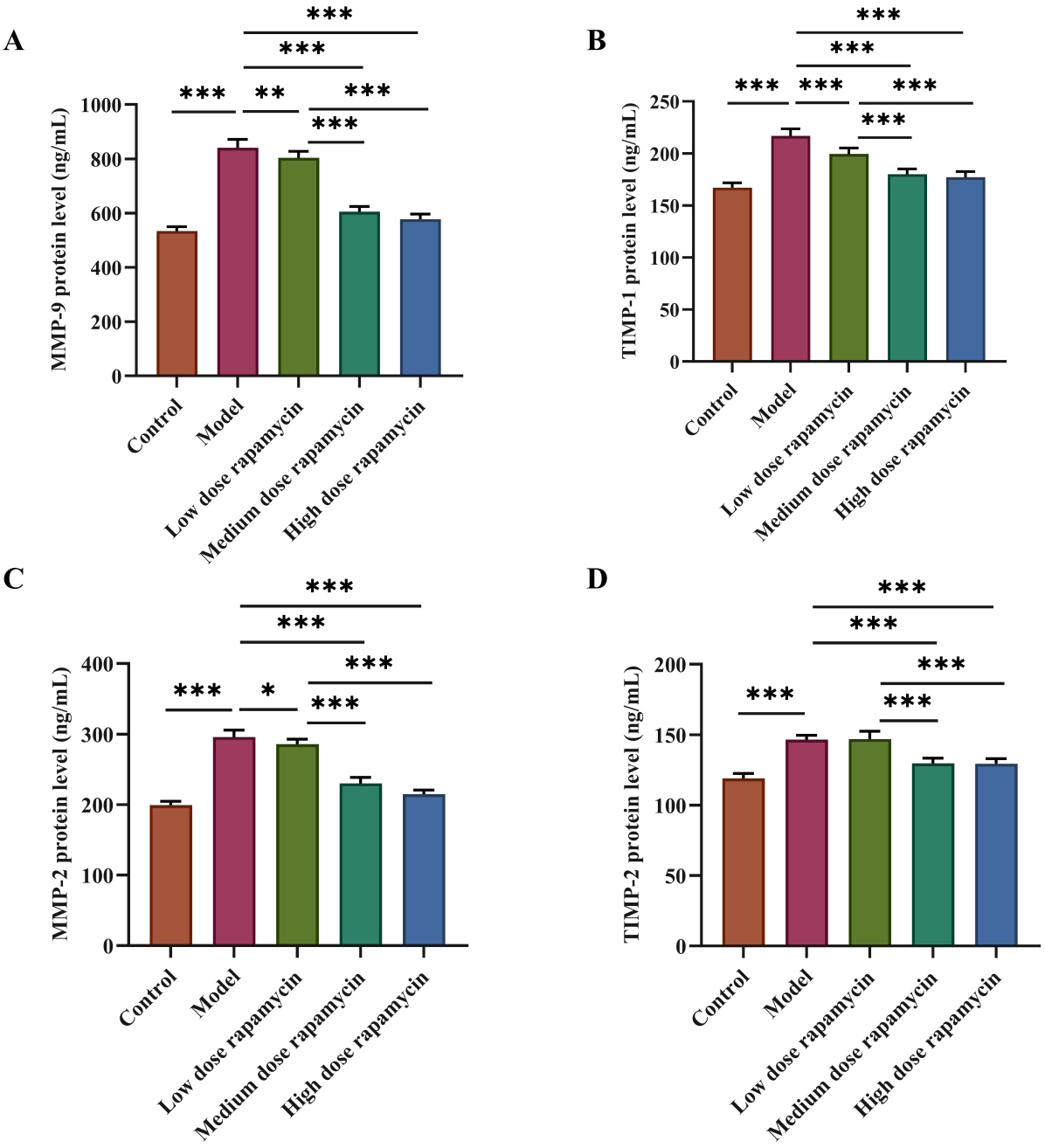

Fig. 1. Changes in blood-brain barrier-related factors in epileptic rats induced by rapamycin (RAP). (A) Mean matrix metallopeptidase 9 (MMP-9) protein level by group. (B) Mean tissue inhibitor of metalloproteinases 1 (TIMP-1) protein level by group. (C) Mean MMP-2 protein level by group. (D) Mean TIMP-2 protein level by group (n = 10 for all groups). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Results

RAP Reduced the Levels of BBB-Related Proteins in the Serum of Epileptic Rats

We further investigated the role of RAP on BBB in rats with epilepsy by measuring the serum levels of BBB permeability-related factors (MMP-9, MMP-2) and restoration-related factors (TIMP-1, TIMP-2). As indicated in Fig. 1A–D, the levels of MMP-9, MMP-2, TIMP-1, and TIMP-2 were higher in the model group than those in the control group ($p < 0.01$). RAP treatment reduced the serum levels of MMP-9, MMP-2, TIMP-1, and TIMP-2, compared to the model group ($p < 0.05$; Fig. 1A–D). The levels of MMP-9, MMP-2, TIMP-1, and TIMP-2 in the medium-dose and the high-dose RAP groups were significantly lower than those in the low-dose RAP group ($p < 0.001$; Fig. 1A–D). Nevertheless, there were no significant differences between the medium-dose RAP treatment group and the high-dose RAP treatment group.

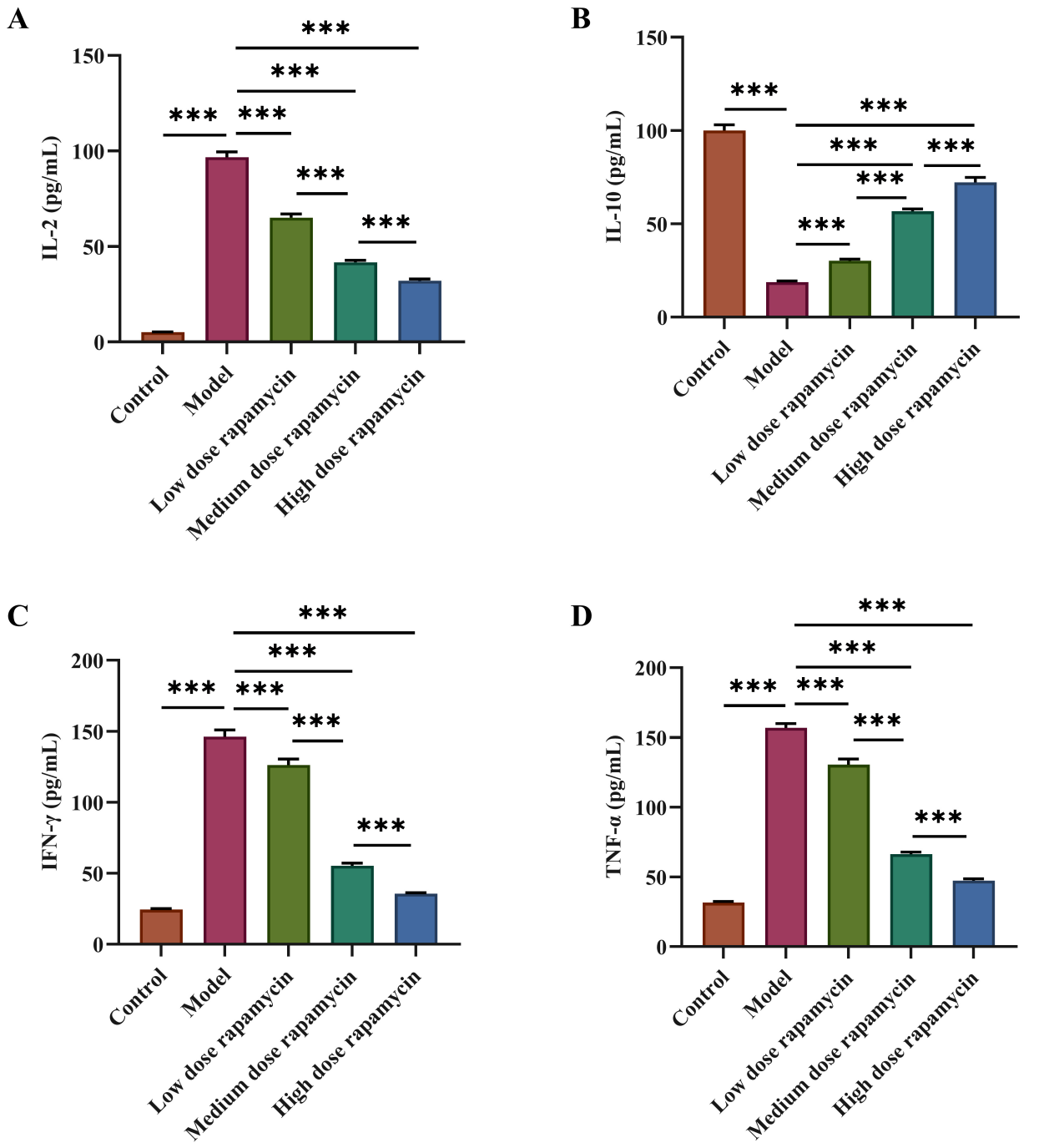

Fig. 2. Role of RAP on the levels of inflammatory cytokines in rats. (A) Mean interleukin-2 (IL-2) level by group. (B) Mean IL-10 level by group. (C) Mean interferon-γ (IFN-γ) level by group. (D) Mean tumor necrosis factor-α (TNF-α) level by group (n = 10 for all groups). ***$p < 0.001$.

RAP Reduced the Levels of Inflammatory Cytokines in the Peripheral Blood of Rats with Epilepsy

Next, we further explored the effects of RAP on neuroinflammation by measuring the levels of inflammatory cytokines *in vivo*. Our data revealed that the levels of IL-2, IFN-γ, and TNF-α in the model group were higher than those in the control group ($p < 0.001$; Fig. 2A,C,D). As seen in Fig. 2A,C,D, the levels of IL-2, IFN-γ, and TNF-α were significantly lower in the RAP treatment groups than in the model group ($p < 0.001$). The level of IL-10 in the model group was significantly lower than that of the control group ($p < 0.001$; Fig. 2B). The levels of IL-10 in the RAP treatment groups were significantly higher than those in the model group ($p < 0.001$; Fig. 2B). The levels of IL-2, IFN-γ, and TNF-α decreased with increasing RAP dose; whereas the level of IL-10 increased with increasing RAP dose.

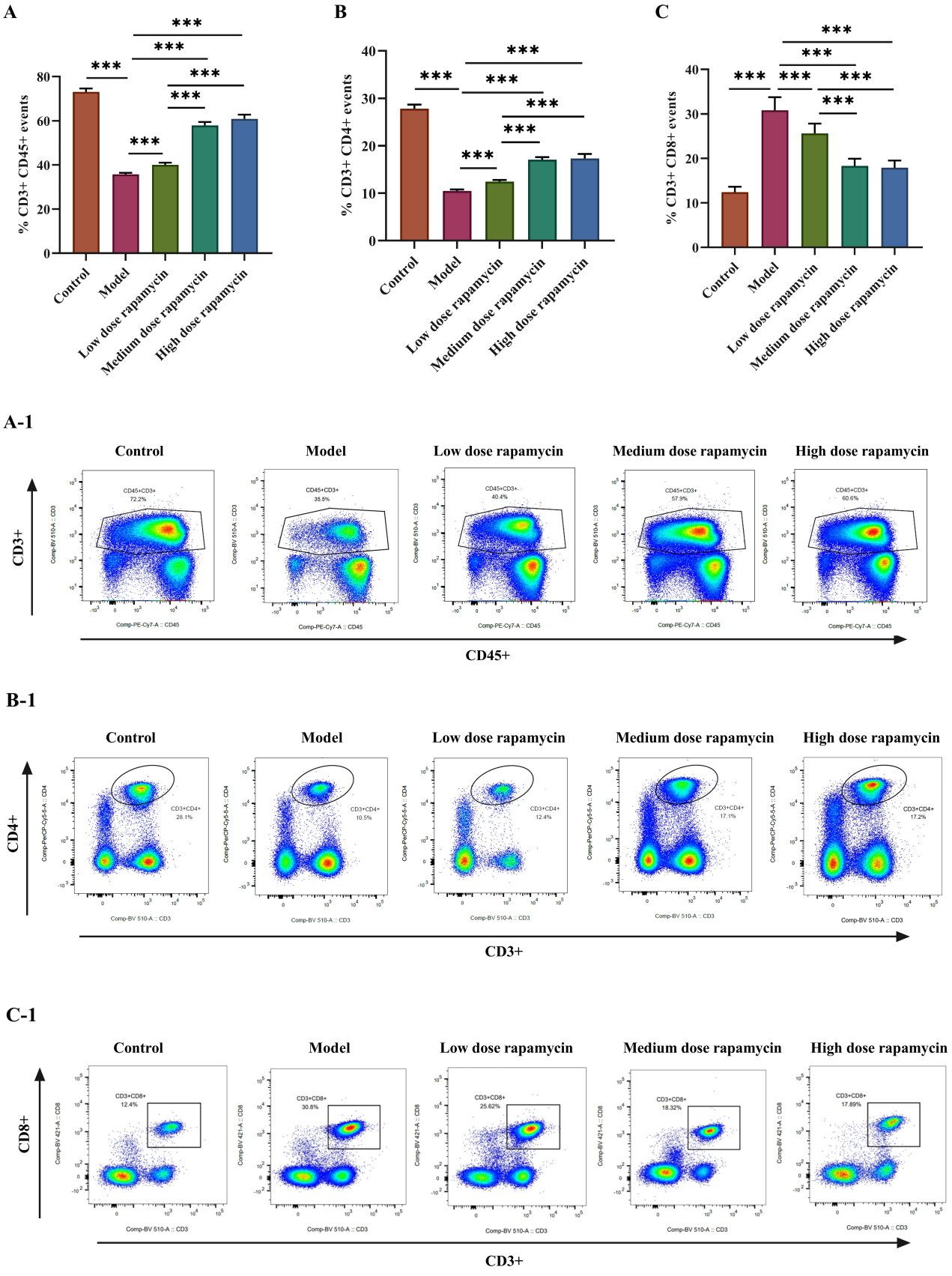

Fig. 3. The variation in T lymphocyte subsets of rats *in vivo*. (A,A-1) The level of total T cells ($CD3^+CD45^+$) was measured by flow cytometry. (B,B-1) The level of T helper cells ($CD3^+CD4^+$) was measured by flow cytometry. (C,C-1) The level of cytotoxic T lymphocytes ($CD3^+CD8^+$) was measured by flow cytometry (n = 10 for all groups). ***$p < 0.001$.

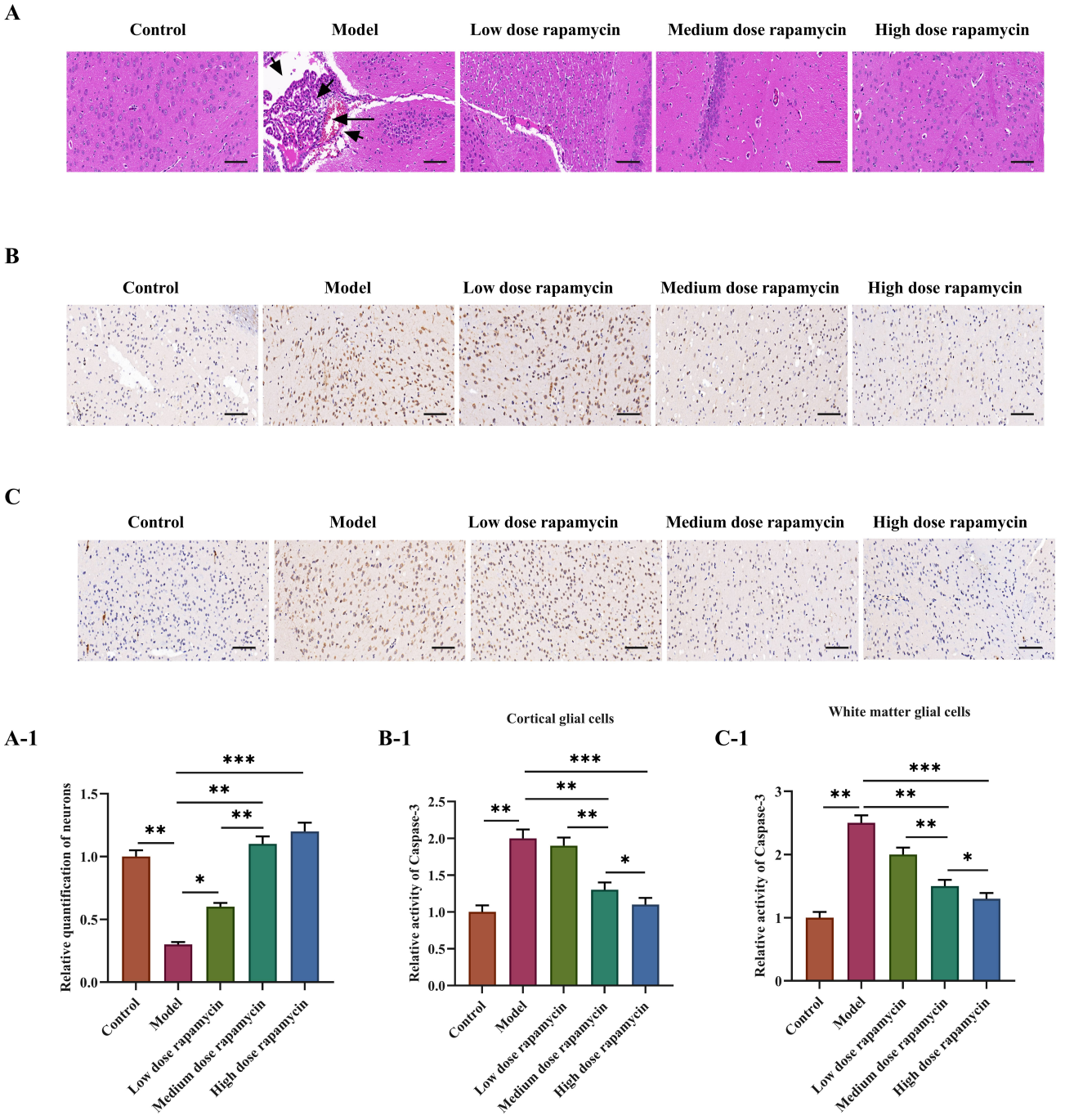

Fig. 4. Structural changes in temporal lobe epileptic foci and studies of apoptosis in temporal lobe epileptic foci. (A&A-1) The effect of RAP treatment on rat cortical neurons was evaluated by Nissl staining. Chromatin breakdown and vacuolization of neuronal cytoplasm are observed where the arrows point. (B&B-1) Caspase-3 activity in cortical cells was detected by light microscopy (the nuclei were stained brown). (C&C-1) Caspase-3 activity in white matter cells was detected by light microscopy (the nuclei were stained brown) (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Scale bar: 50 µm.

Effects of RAP on T Lymphocyte Subsets in Epileptic Rats in Vivo

We determined the levels of T lymphocytes, including total T cells ($CD3^+CD45^+$), T helper cells ($CD3^+CD4^+$), and cytotoxic T lymphocytes ($CD3^+CD8^+$) in the peripheral blood of the control group, the model group, and RAP-treated groups by flow cytometry. Fig. 3A-1,B-1,C-1 illustrates representative flow cytometry plots of ($CD3^+CD45^+$)/($CD3^+CD4^+$)/($CD3^+CD8^+$). We found a decrease in the mean number of total T cells and T helper cells, and an increase in the number of cytotoxic T lymphocytes, in the model group compared to the control group ($p < 0.001$; Fig. 3A–C). In contrast with the model

group, RAP treatment significantly increased mean levels of ($CD3^+CD45^+$) and ($CD3^+CD4^+$) cells, and significantly decreased the levels of ($CD3^+CD8^+$) cells in epileptic rats ($p < 0.001$; Fig. 3A–C). In contrast with the low-dose group, the medium-dose and high-dose groups had higher levels of total T cells and T helper cells, and lower levels of cytotoxic T lymphocytes ($p < 0.001$; Fig. 3A–C). There was no significant difference in peripheral blood T cell subsets between medium-dose RAP and high-dose RAP treatment groups ($p > 0.05$; Fig. 3A–C).

RAP Protected Cortical Neurons and Inhibited Apoptosis of the Cells in White and Gray Matter Areas Involved in Temporal Lobe Epilepsy

We assessed the effect of RAP on cortical neurons in epileptic rats by Nissl staining and H&E staining. As seen in Fig. 4, we observed reactive and destructive changes associated with neuronal loss and temporal lobe epilepsy in the model group. The number of neurons obviously reduced in the model group ($p < 0.05$). We observed chromatin breakdown and vacuolization of neuronal cytoplasm, and neuronal cells exhibited dystrophic and atrophic phenomena. More caspase-3 was present in the nucleus of both cortical cells and white matter cells in the model group, which is a marker of early apoptosis of cells ($p < 0.05$, $p < 0.01$, respectively; Fig. 4B,C). Compared with the model group, RAP improved the structural changes of temporal lobe epileptic foci, effectively protecting neurons and inhibiting the apoptosis of both cortical cells and white matter cells in epileptic rats ($p < 0.05$, $p < 0.01$, respectively; Fig. 4A–C).

Discussion

The mTOR target pathway has been listed as a drug target for the treatment of seizures [18]. In this study, a rat model of epilepsy was established, and whose blood and brain tissues were collected to evaluate the neuroprotective mechanism of RAP treatment on epilepsy.

BBB dysfunction is a common feature during seizures [19,20]. It may be caused by a variety of mechanisms, including inflammatory responses and angiogenesis [21–23]. Previous studies have shown that manipulating the BBB can reduce seizures [24]. In our work, we further confirmed that seizures disrupt the BBB in rats and induce BBB dysfunction. We also observed that RAP can reduce the serum levels of MMP-2, TIMP-1, MMP-9, and TIMP-2 in epileptic rats. MMPs are proteolytic enzymes that function to degrade Extracellular Matrix (ECM) and add BBB permeability [25]. TIMPs act as antagonists of MMPs; they restrain immoderate cell death and cell injury [26]. It has been demonstrated that higher levels of TIMPs are connected with BBB dysfunction, suggesting a neuroprotective role of TIMPs in epilepsy [27]. Meanwhile, the equilibrium between MMPs and TIMPs is significant for the health of the central nervous system [28]. Our results suggested that RAP may improve epilepsy by repairing BBB dysfunction, which is in keeping with the data of former reports [29].

Neuroinflammation is considered to be a major factor in the progression of epilepsy [30]. We probed the effects of RAP on neuroinflammatory cytokines. Studies have shown that seizures are associated with increased levels of inflammatory cytokines, especially IL-2, IFN-γ, and TNF-α [31]. Our results confirmed that RAP exerts antiepileptic effects by inhibiting the levels of neuroinflammatory cytokines IL-2, IFN-γ, and TNF-α. This is in keeping with the conclusion of a previous study, which also demonstrated that RAP inhibits neuroinflammatory processes in the epileptic brain by inhibiting the levels of inflammatory cytokines [32].

T lymphocyte subsets are the basis for maintaining the cellular immune function of the body. More and more evidence has further shown that seizures are closely related to the body's immunity, especially T cell immunity [33]. The levels of total T cells and T helper cells in epileptic rats were reduced, and the level of cytotoxic T lymphocytes was increased. RAP treatment improved the imbalance of T lymphocyte subsets. The results of Chu *et al*. [34] demonstrated that RAP reduces disease activity in patients with SLE by regulating the balance between helper T cells and regulatory T cells. The above results showed that RAP can suppress the progression of the disease by regulating the balance of T cell subsets.

Previous studies have found cell apoptosis and inflammation in the temporal lobe of patients with epilepsy [14,35]. In our work, we evaluated the role of RAP on the apoptosis of glial cells and neurons in the epileptic lesion tissue of rats. When stimulated, caspase-3 is activated and translocated to the nucleus [36]. Related experiments have shown that the presence of caspase-3 in neurons is increased in epileptic lesion tissue after seizures [37]. It has been shown that apoptosis of glial cells is involved in the process of neuronal apoptosis and the occurrence of epilepsy [38]. We found that RAP exerts a neuroprotective effect by inhibiting neuronal apoptosis and caspase-3 levels in glial cells.

Conclusions

In summary, this study demonstrated that RAP has antiepileptic properties, and its antiepileptic mechanisms may include the restoration of BBB function, reduction of neuroinflammation, regulation of the balance of T cell subsets, and inhibition of apoptosis of neurons and glial cells.

Availability of Data and Materials

Data to support the findings of this study is available on reasonable request from the corresponding author.

Author Contributions

PSX, SBZ, and JSH contributed to the concept and designed the research study. HWZ, RTF and JLZ performed the research. JFW and MXY provided help and advice on the experiments. PSX and JSH contributed to the analysis and interpretation of the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

This study was approved by the Laboratory Animal Management and Ethics Committee of Xiamen University (approval no. XMULAC20220008).

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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