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·Original Article·

Fingolimod alters inflammatory mediators and vascular permeability in intracerebral hemorrhage

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ABSTRACT

Intracerebral hemorrhage (ICH) leads to high rates of death and disability. The pronounced inflammatory reactions that rapidly follow ICH contribute to disease progression. Our recent clinical trial demonstrated that oral administration of an immune modulator fingolimod restrained secondary injury derived from initial hematoma, but the mechanisms remain unknown. In this study, we aim to investigate the effects of fingolimod on inflammatory mediators and vascular permeability in the clinical trial of oral fingolimod for intracerebral hemorrhage (ICH). The results showed that fingolimod decreased the numbers of circulating $CD4^+$ T, $CD8^+$ T, $CD19^+$ B, NK, and NKT cells and they recovered quickly after the drug was stopped. The plasma ICAM level was decreased and IL-10 was increased by fingolimod. Interestingly, fingolimod protected vascular permeability as indicated by a decreased plasma level of MMP9 and the reduced rT1%. In conclusion, modulation of systemic inflammation by fingolimod demonstrates that it is an effective therapeutic agent for ICH. Fingolimod may prevent perihematomal edema enlargement by protecting vascular permeability.

Keywords: fingolimod; inflammatory mediator; vascular permeability; intracerebral hemorrhage

INTRODUCTION

Intracerebral hemorrhage (ICH) accounts for 10%–15% of all strokes worldwide and affects more than 2 million people each year[1, 2]. Its rate of incidence is 24.6 per 100 000 per year and this is expected to double by 2050 due to the increasing elderly population[3-5]. ICH is particularly severe as it is associated with a mortality rate of 30%–50%[6]. The high rate of mortality and morbidity likely results from a lack of proven medical treatment[1, 7]. Both the initial hematoma and the secondary perihematomal edema (PHE) contribute to the high mortality and morbidity. Most patients are able to survive the primary injury caused by the initial hematoma, but suffer severe neurological deficits caused by the secondary PHE injury. Increasing evidence has shown that inflammation plays a critical role in ICH-induced injury in the acute stage and the inflammatory cascades contribute to the formation of edema that surrounds hematomas, exacerbate the mass effect, and amplify the cell death process *via* secondary ischemia[7]. Although progress has been made in identifying the roles of inflammatory signaling molecules, cells, and proteins in the initiation

and progression of post-ICH inflammation-mediated brain injury[5], no therapies had been directed toward altering the inflammatory and edematous processes of this disease until our oral fingolimod trial in ICH[8].

Fingolimod (FTY720) was the first FDA-approved oral drug for multiple sclerosis treatment; it depletes circulating lymphocytes by preventing their migration from lymph nodes to the circulating pool[9], thus possibly preventing their infiltration of the central nervous system (CNS). In our recent trial, oral fingolimod in ICH (2-arm proof-of-concept clinical trial: Fingolimod for the treatment of intracerebral hemorrhage)[8] was found to effectively reduce the secondary injury caused by the initial hematoma. In particular, fingolimod administration within 72 h of disease onset was shown to be safe, reduces PHE, decreases circulating $CD4^+$ T, $CD8^+$ T, and $CD19^+$ B cells, attenuates the neurologic deficits, promotes recovery, and has therapeutic potential for preventing secondary brain injury in ICH patients. However, the mechanisms contributing to the reduction of PHE need further study. Post-cerebral hemorrhage inflammation, as characterized by elevated plasma levels of inflammatory mediators including MMP9, is correlated with both ICH severity and poor clinical outcome[5, 6, 10]. In addition, as inflammation may play quite different roles in the early (injury) and delayed phases (neuroregeneration) after ICH, the purpose of this study was to further determine the impact of fingolimod on the dynamic changes in plasma levels of inflammatory mediators and on lesion vascular permeability.

MATERIALS AND METHODS

Participants

This study was a two-arm, evaluator-blinded clinical trial of oral fingolimod for ICH patients at a dose of 0.5 mg per day for 3 consecutive days, the initial administration being within 1 h after the baseline computed tomographic (CT) scan and no later than 72 h after the onset of symptoms. Twenty-three ICH patients with well-matched clinical characteristics, hematoma location, and volume as defined by CT, were assigned into control and fingolimod groups, the former receiving standard management for ICH and the latter receiving standard management plus fingolimod treatment. Standard management included general support, monitoring of blood pressure and intracranial pressure, and administration of Nimodipine to prevent cerebral vasospasm. Blood samples were collected from the fingolimod group before treatment (baseline), a mean time of 20 h from symptom onset, as well as 1, 3, and 7 days (T-0, T-1, T-3, T-7) after the first dose. Blood samples in the control group were collected 4 days after the onset of symptoms (O-4) which approximately paralleled the time point T-3 of the fingolimod group. This trial protocol and supporting documentation were approved by the Tianjin Medical University General Hospital Review Board.

Immune Cell Subpopulation Analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood of patients at different time points after fingolimod treatment using a lymphocyte-separating medium (Mediatech, Manassas, VA). The isolated PBMCs were subdivided into different tubes and stained with the fluorescence-conjugated mouse anti-human monoclonal antibodies CD4-FTIC, CD8-PE, CD19-PE, CD3-Percp-cy5.5, CD56-PE, and CD11c-FITC (BD Biosciences, Franklin Lakes, NJ). Data were acquired using FACSCalibur (Becton Dickinson, San Jose, CA) and analyzed with FlowJo 7.6 software. The absolute numbers of $CD4^+$ T, $CD8^+$ T, $CD19^+$ B, $CD3^-CD56^+$ NK, $CD3^+CD56^+$ NKT, and $CD11c^+$ DC cells per unit volume were compared between the groups.

Plasma Levels of Cytokines and MMP9 Detection

The plasma levels of intracellular adhesion molecule (ICAM), IL-6, IL-21, IL-10, and MMP9 were measured using enzyme-linked immunosorbent assay (ELISA) (R&D System, Minneapolis, MN) according to the manufacturer's instructions. Optical densities were measured at 450 nm and 570 nm.

Neuroimaging

MRI was performed using a 3.0T GE system and included high-resolution anatomical images acquired from sagittal 3D T1 images by a brain volume (BRAVO) sequence (number of slabs = 191; TR = 8.16 ms; TE = 3.18 ms; flip angle = 12°; slice thickness = 1 mm; within-plane resolution 1 mm^3) before and after contrast injection (immediately post-contrast, 15 min on average). All image analysis was performed using MatLab software. The

overall goal was to obtain subtraction maps, where the T1-MRIs of the series before contrast were subtracted from those of later series after contrast. The subtraction maps depict the spatial distribution of contrast accumulation in the tissue. In order to increase the sensitivity to small changes, it is essential to perform image correction for intensity variations for whole-body registration. Therefore, rT1% was calculated for quantitative analysis: rT1% = (mean signal intensity of a region of the lesion – mean signal intensity of the contralateral homologous normal brain area) / mean signal intensity of the contralateral homologous normal brain area). The lesion was outlined and signal intensity measured independently and blindly by two neuroradiologists using ImageJ 1.38. We analyzed the intraclass correlation coefficient between the two data sets (P <0.05, r = 0.905) and the average values are presented.

Statistical Analysis

Continuous variables are presented as mean ± SD. Comparisons for repeated measures parameters (lymphocyte counts and levels of inflammatory mediators) within the fingolimod-treated group were analyzed by repeated measures ANOVA with *post hoc* Bonferroni pairwise analysis. Student's *t*-test was used to compare the continuous variables in the fingolimod-treated and control groups. All the analyses were performed in Graphpad Prism 5 software. Statistical significance was defined as P <0.05.

RESULTS

Fingolimod Dynamically Decreased Circulating Immune Cells in the Acute Stage

In this study, we found a reduction of NK and NKT cells per milliliter from (0.27 ± 0.24) ×10^6 and (0.08 ± 0.07) ×10^6 at T-0 to (0.14 ± 0.08) ×10^6 (P = 0.01) and (0.02 ± 0.01) ×10^6 (P = 0.02), respectively at T-3, 48% and 75% reductions from baseline. Compared to the control group (O-4), the fingolimod-treated group at T-3 also showed marked reductions in $CD3^-CD56^+$ NK (P <0.01) and $CD3^+CD56^+$ NKT (P <0.01) (Fig. 1). No significant changes of $CD11c^+$ cells were found. Further examination of the dynamic changes during treatment showed that all except the dendritic cells showed a further decrease at T-3, that is, 3 days after the first dose of fingolimod. Then all returned to baseline at T-7 (Fig. 1). With circulating immune cells decreased, neutrophils in the peripheral blood (leukocyte subsets were assessed by standard laboratory differential white blood cell counts) were decreased at T-3 compared to the control (4.6 ± 0.6 *vs* 7.5 ±1.2, 10^6/mL, P <0.001).

Fingolimod Differentially Affects Systemic Cytokine Secretion in the Acute Stage

During fingolimod treatment, the plasma concentration of ICAM-1 was notably lower in ICH patients at T-3 compared to the baseline (P <0.05) and the control group (P <0.01), and then returned to baseline at T-7. However, no significant changes were found in the plasma levels of proinflammatory IL-6 and IL-21 after fingolimod treatment (Fig. 2). Interestingly, the plasma level of the anti-inflammatory cytokine IL-10 was increased at day 3 after the first dose of fingolimod (P <0.05) (Fig. 2).

Reduction of Vascular Permeability in Fingolimod-Treated ICH Patients

The presence of parenchymal enhancement on CET1-WI is a generally accepted indicator of contrast medium leakage across the disrupted blood-brain barrier (BBB). Here, we further evaluated the vascular permeability based on the CET1-WI images collected in previous clinical trials[8]. We found that parenchymal enhancement of the PHE was much greater in the control group (rT1%, 40.6 ± 3.0) than in the fingolimod group (20.5 ± 6.7) on day 7 after onset (P <0.001, Fig. 3A, B).

MMP9 is used as a plasma indicator of BBB disruption. In this study, we found that MMP9 was notably lower with fingolimod treatment 1 day after the first dosage than baseline (P = 0.03) and the control group (P <0.01). This effect was sustained until 3 days after the first dosage (P = 0.01 compared with baseline). The plasma MMP9 level returned to baseline at day 7 after onset (Fig. 3C).

DISCUSSION

PHE is closely associated with post-stroke inflammation[7]. Inflammation is an important host-defense response to brain injury after ICH. The activation of innate immunity following ICH results in microglial activation, perihematomal inflammation reactions, infiltration of blood-derived inflammatory cells including leukocytes, macrophages, and

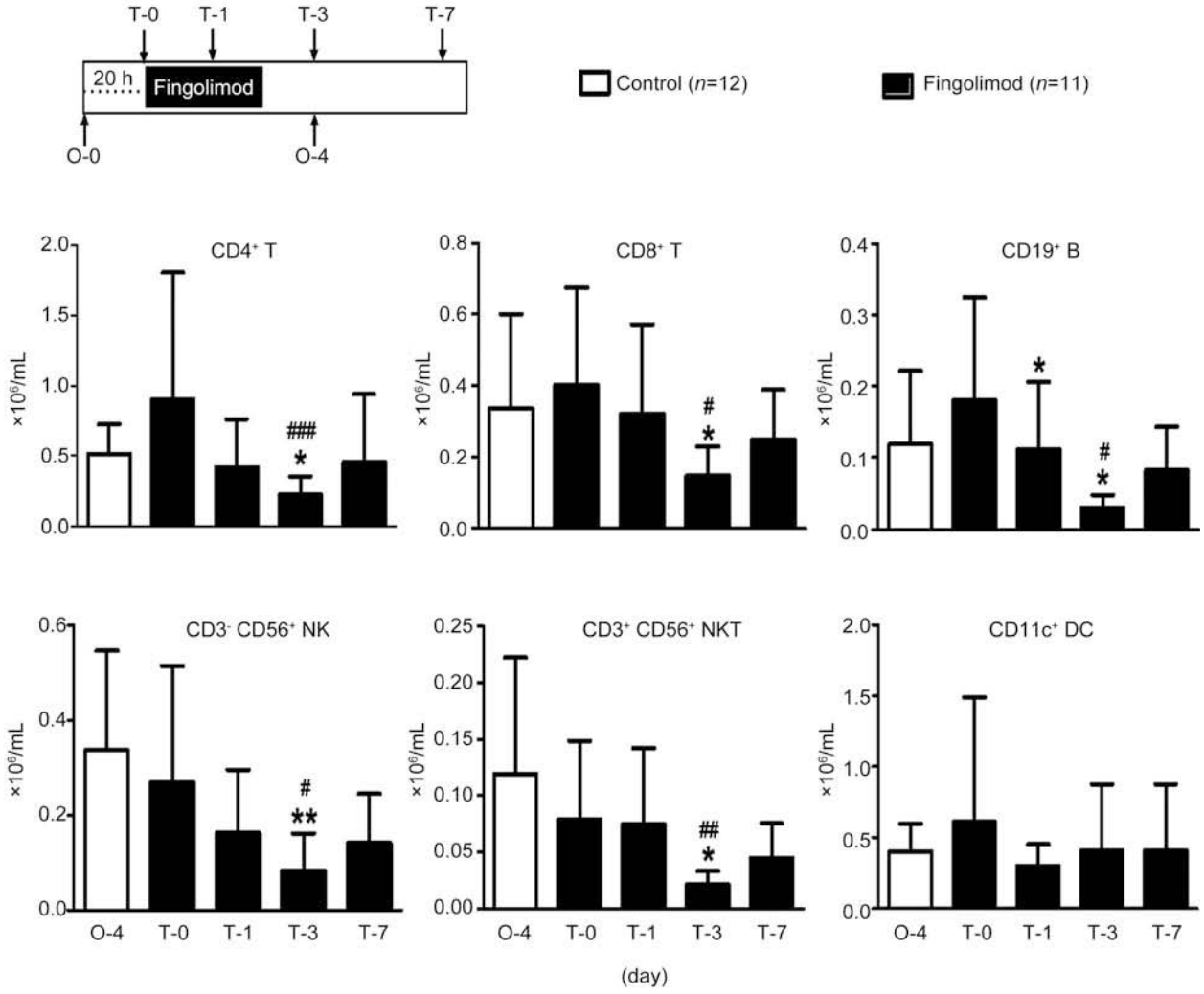

Fig. 1. Dynamic changes in lymphocyte subsets in ICH patients treated with fingolimod. Blood from fingolimod-treated patients was collected at baseline (19.5 ± 6.1 h) as well as on days 1, 3, and 7 after the first dose (T-0, T-1, T-3, T-7), and that from control patients on day 4 after the onset of symptoms (O-4). Mononuclear cells were purified and counted, the absolute numbers of $CD4^+$ T, $CD8^+$ T, $CD19^+$ B, NK, NKT, and dendritic cells (DCs) were calculated by total mononuclear cells/mL multiplying the percentage of these cells determined by flow cytometry. $^*P$ <0.05, $^{**}P$ <0.01 *versus* baseline of fingolimod-treated patients; $^{\#}P$ <0.05, $^{\#\#}P$ <0.01, $^{\#\#\#}P$ < 0.001 *versus* control; mean ± SD.

T cells[5, 7, 11, 12], and release of inflammatory cytokines[13, 14]. Major orchestrators of this inflammatory process are blood-derived immune cells, trafficking from the periphery to the brain parenchyma[15, 16]. In patients, peripheral immune cells start to infiltrate the brain between 6 and 12 h after the occurrence of symptoms, and the number of brain-infiltrating immune cells is positively correlated with neuronal apoptosis[17].

Having determined that fingolimod attenuates neurological deficits, promotes recovery, and has therapeutic potential for preventing secondary brain injury in ICH[8], we sought to understand how peripheral lymphocytes and cytokines are dynamically affected in these patients. Previously, we demonstrated that oral fingolimod reduces the counts of $CD4^+$ T, $CD8^+$ T, and $CD19^+$ B cells by 75%, 63%, and 83% respectively[8]. In this study, we showed that fingolimod treatment not only restricted the numbers of circulating T-lymphocytes, but also NK, NKT, B cells, and

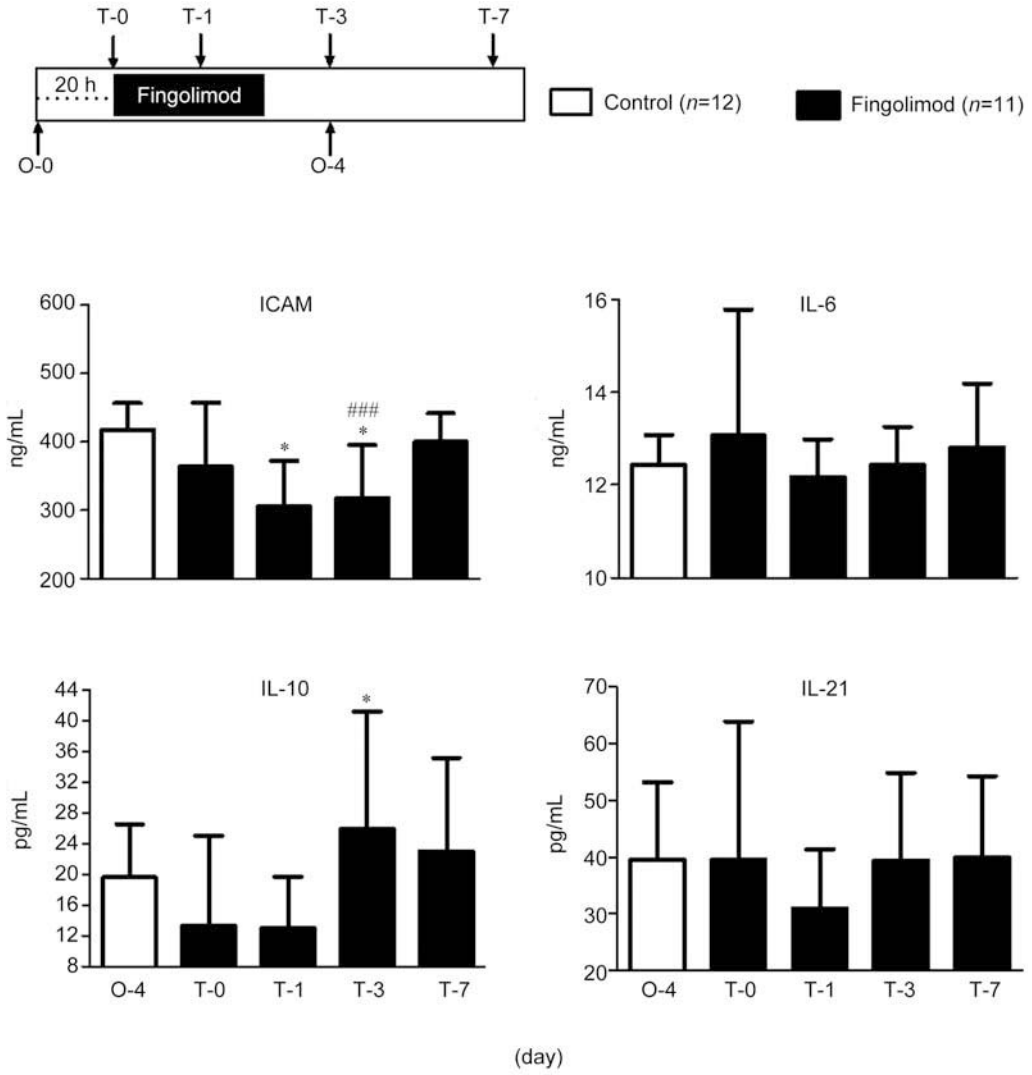

Fig. 2. ELISA analysis of dynamic changes of serum cytokines in ICH patients treated with fingolimod. Serum from fingolimod-treated patients was collected at baseline (19.5 ± 6.1 h) and on days 1, 3, and 7 after the first dose (T-0, T-1, T-3, T-7), and that from control patients on day 4 after the onset of symptoms (O-4). $^{*}P$ <0.05 *versus* T-0 (baseline); $^{###}P$ <0.001 *versus* control; mean ± SD.

neutrophils in the acute stage. Although stroke-induced immunosuppression syndrome has been identified in many studies[18], the fingolimod treatment group had fewer immune cells than the control group, suggesting a direct preventive effect of fingolimod on lymphocyte entry into the circulating pool. And all the subsets of lymphocytes quickly returned to the baseline level after cessation of fingolimod administration. These results suggested a rapid and short-term effect of fingolimod on the circulating lymphocyte pool that is beneficial for disease recovery, as inflammation in the early stage may cause brain injury, while the inflammatory milieu could promote neuroregeneration in the recovery stage[19]. Thus, this short period of fingolimod administration may have a less adverse effect of immune suppression[18]. Also, this rapid recovery of immune cells may benefit the neuroregeneration in the later stages of ICH. While circulating immune cells decreased, neutrophils in the fingolimod-treated group were normal, but lower than the control level. Studies on the natural course of leukocytes in ICH have reported that they increase after ICH, peak at 72 h, and almost disappear at 3 to 7 days, and these are associated with early neurological deterioration.

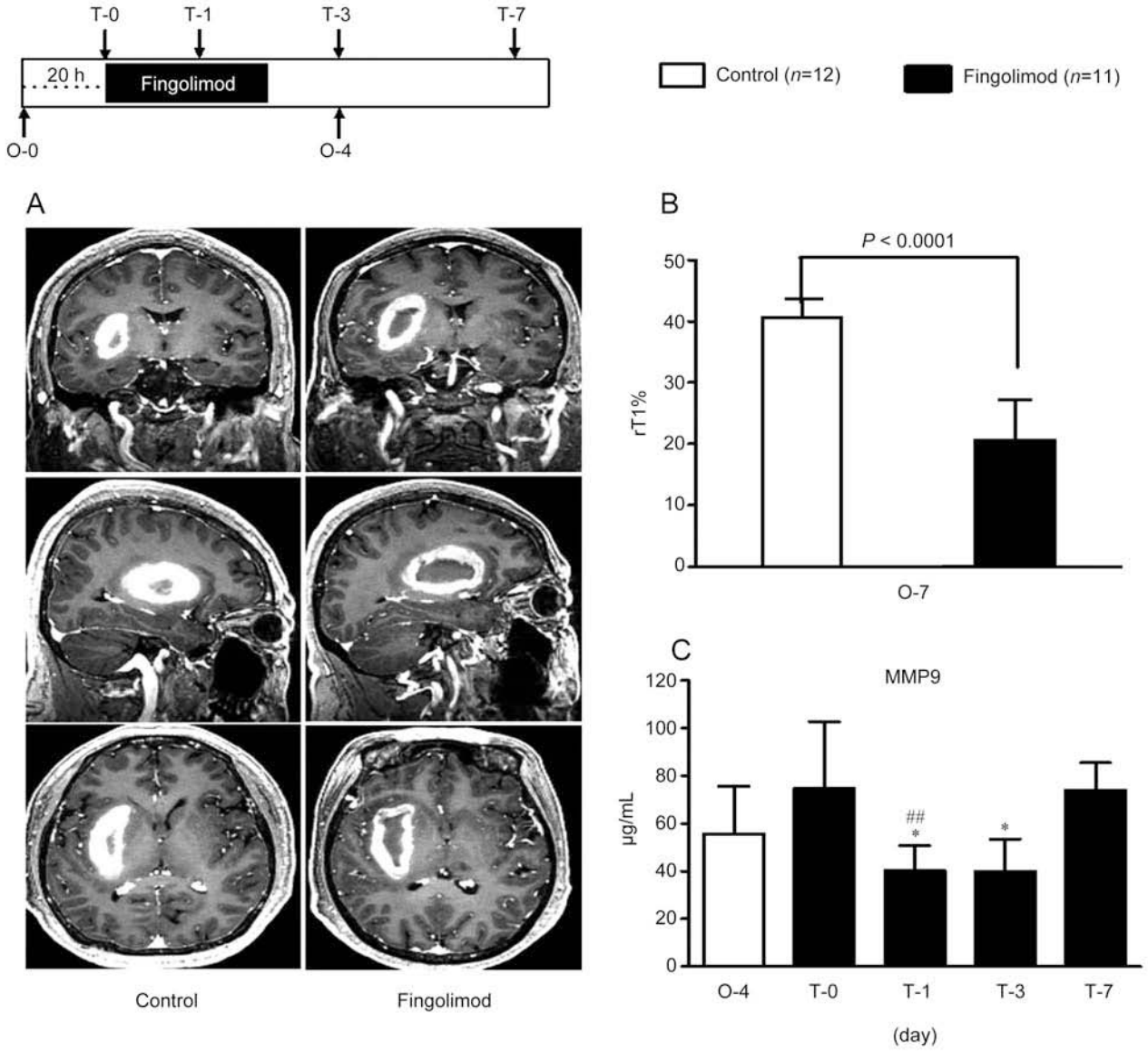

Fig. 3. Impact of fingolimod on vascular permeability. (A) Representative contrast-enhanced T1-weighted imaging (CET1-WI) scans show an acute cerebral hemorrhage in the right hemisphere in a control (left panels) and a fingolimod-treated patient (right panels). Parenchymal enhancement of the acute hemorrhage lesion in the control was much higher than in the fingolimod-treated patient. (B) Fingolimod significantly decreased the microvascular permeability of the infarct lesions, as indicated by rT1%, on day 7 after the onset of symptoms (O-7). (C) Serum MMP9 level changes in controls and patients treated with fingolimod. Serum was collected at baseline and on days 1, 3, and 7 after the first dose (T-0, T-1, T-3, T-7) from fingolimod-treated patients, and on day 4 from control patients after the onset of symptoms (O-4). Serum MMP9 levels were determined for each patient by ELISA. *P <0.05, *versus* baseline; $^{\#\#}P$ <0.01 *versus* control; mean ± SD.

Both human studies[20] and animal experiments have reported increased numbers of leukocytes at the first 72 h after onset[21]; however, here we found that the leukocyte count in the fingolimod-treated group was significantly lower on day 3 than the control, according with better clinical improvement.

Post-cerebral hemorrhage systemic inflammation, characterized by elevated plasma levels of inflammatory

mediators, is correlated with both ICH severity and poor clinical outcome[5, 6, 10]. To investigate the effect of fingolimod on inflammatory mediators, we focused on ICAM-1, IL-21, IL-6, and IL-10. ICAM-1 is an important adhesion molecule that promotes leukocyte infiltration[20-22]. In this trial, our data suggested that fingolimod inhibited secretion and decreased the plasma level of ICAM-1, which may prohibit leukocyte infiltration into the CNS during treatment. The plasma levels of IL-21 and IL-6 remained unchanged during fingolimod treatment. Increased serum concentrations of IL-6 as well as other cytokines have been found 24 h after ICH, and the IL-6 level has been correlated with blood volume and the mass effect of the hemorrhage[23]. In this study, the unchanged levels of the proinflammatory cytokines IL-6 and IL-21 may contribute to the effectiveness of fingolimod and this phenomenon suggests a therapeutic effect. In addition, the plasma level of IL-10, an anti-inflammatory cytokine, significantly increased 3 days after the first dose of fingolimod, and returned to baseline when PHE was relieved.

We also observed a reduction in the volume of PHE in our clinical trial[8]. However, the mechanisms need further study. Disruption of the BBB was the first issue to be further evaluated. CET1-WI has been used routinely in cerebral stroke studies[24]; the presence of parenchymal enhancement on CET1-WI is generally accepted as an indicator of contrast medium leakage across the disrupted BBB. The timeline for BBB disruption has been shown to include initiation on day 1, a peak on days 5–7, and can last 14 days or longer[25, 26]. Therefore, here we evaluated the microvascular permeability on day 7. Hematoma within the brain parenchyma triggers a series of events leading to secondary injury and severe neurological deficits. Although inflammation is triggered to remove blood and other residual debris, the by-products of this response are cytotoxic and lead to further tissue damage, BBB disruption, and edema. MMP9 has been widely implicated as a proinflammatory mediator of the development of BBB damage in cerebral hemorrhage. The significant reduction of rT1% in this study indicated a strong protective effect of fingolimod on the BBB. MMP9 is a plasma indicator of BBB disruption both in animal models[27, 28] and in human patients[25, 26]. It has also been reported that, among patients with deep ICH, an increase of MMPs is associated with PHE and the worsening of neurological deficits in the acute stage[29]. The mechanism of this effect may be summarized as follows: direct damage of brain cells, cell injury by processing death molecules, disruption of myelin, and the conversion of pre-forms of inflammatory molecules into mature forms that perpetuate inflammation. In this study, we further analyzed the plasma level of MMP9. Interestingly, the MMP9 levels significantly decreased starting from day 1 after the first dose of fingolimod and persisted to day 3, but returned to baseline on day 7. This result strongly indicated that fingolimod protected against BBB damage through the reduced MMP9. Since the role of MMP in ICH recovery is still controversial, given its desirable effect on neurogenesis, myelin formation, and axonal growth[30,31], the recovery of MMP9 levels on day 7 may be promising for potential benefits on later neurogenesis.

In conclusion, in this trial of oral fingolimod on ICH, a short period of fingolimod treatment in ICH patients effectively prevented the entry of immune cells into circulating pools, affected the plasma concentration of mediators of inflammation, and protected the vascular permeability as indicated by reduced plasma levels of MMP9 and decreased rT1%. These results suggest that fingolimod may serve as a potential therapeutic agent for ICH treatment through inhibiting the infiltration of immune mediators.

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