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FTY720 is Neuroprotective and Improves Functional Outcomes After Intracerebral Hemorrhage in Mice

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Abstract

Intracerebral hemorrhage (ICH) accounts for 20% of all strokes and is the most devastating form
across all stroke types. Lymphocytes have been shown to potentiate cerebral inflammation and
brain injury after stroke. FTY720 (Fingolimod) is an immune-modulating drug that prevents the
egress of peripheral lymphocytes from peripheral stores. We hypothesized that FTY720 would
reduce peripheral circulating lymphocytes, resulting in reduced brain injury and improved
functional outcomes. CD-1 mice were anesthetized and then injected with collagenase into the
right basal ganglia. Animals were divided into three groups: sham, ICH + Vehicle, and ICH +
FTY720, by the intra-peritoneal route at 1 h after ICH induction. Brain water content was
measured at 24 and 72 h. Neurobehavioral tests included corner test, forelimb use asymmetry, paw
placement, wire-hang test, beam balance test, and a Neuroscore. FTY720 significantly reduced
brain edema and improved neurological function at all time points tested. Lymphocyte modulation
with FTY720 is an effective neuroprotective strategy to reduce brain injury and promote
functional recovery after ICH.

Keywords

Intracerebral hemorrhage; FTY720; Fingolimod; Lymphocytes; Neuroprotection; Brain edema; Neurological function

Introduction

Intracerebral hemorrhage (ICH) is a spontaneous bleeding event in the brain for which there are no effective therapies. ICH accounts for about 20% of all strokes and affects 1 in 6,000 people each year [1]. ICH results in a higher mortality rate than ischemic stroke, and 40–50% of patients in the United States die within the first 30 days [2].

The inflammatory response after ICH is a highly complex process that includes acute and delayed events requiring local and peripheral cellular cross-talk. Significant orchestrators of this inflammatory process are blood-derived immune cells, trafficking from the periphery into the brain parenchyma [3]. Monocyte and neutrophil infiltration, along with microglia and macrophage activation, occur in and around the hematoma within hours after ictus and peak within a few days [3, 4], leading to the production of proinflammatory mediators, such as TNF-α, IL-1β, and proteolytic enzymes, such as MMP-9. While the myeloid branch of the immune system includes the above cell types involved in inflammation, lymphoid cells also interact with resident and infiltrated cells in the CNS, potentiating the inflammatory response. T-cells have been shown to play a major role in the numerous neurological diseases, such as Parkinson's disease, Alzheimer's disease, and multiple sclerosis [5–7], as well as the deleterious events following stroke [8]. It has been demonstrated that T-cells are significantly increased in the brain as early as 24 h after stroke [9]. These lymphocytes are responsible for further driving the local inflammatory reaction, leading to increased blood-brain barrier (BBB) permeability, edema formation, cell death, and neurological deficits following stroke.

FTY720 (Fingolimod), a sphingosine-1-phosphate(S1P) analog that is an agonist for S1P receptors (S1P1, 3, 4, and 5), has a half life of 20 h [10, 11]. FTY720's immunosuppressive activity results from inhibition of S1P1 receptor-dependent lymphocyte egress mediated by downregulating S1P1 receptor on T-cells [12]. Therefore, a single administration of FTY720 causes peripheral lymphopenia. It has been shown to be effective in several phase II clinical trials at reducing the incidence of relapsing/remitting MS [13, 14] and for renal transplantation [15]. In a recent study in our laboratory, FTY720 administration was shown to be neuroprotective following 2 h middle cerebral artery occlusion in rats [16].

In the present study, we hypothesized that FTY720 administration following ICH would reduce brain edema and improve neurological function in mice. To test this hypothesis, we administered FTY720 to reduce circulating peripheral lymphocytes, and then evaluated brain edema and neurological deficits 24 and 72 h following collagenase-induced intracerebral hemorrhage.

Materials and Methods

Experimental Animal Preparation

All procedures for this study were approved by the Animal Care and Use Committee at Loma Linda University, and were in compliance with the NIH *Guide for the Care and Use of Laboratory Animals.*

Eight-week-old CD-1 mice were used in our study and were housed on a 12:12 light/dark cycle in a pathogen-free facility with controlled temperature and humidity, and were given food and water ad libitum.

Experimental Protocol

In total, 30 mice were used in this study. Mice were divided into three groups: sham (needle insertion), ICH + vehicle (ICH), and ICH + FTY720 treatment (1 mg/kg, i.p). FTY720 was dissolved in 2% DMSO and given 1 h after ICH induction. Both sham and vehicle animals received the same volume of i.p. 2% DMSO in saline. All animals were neurologically tested, and brains were harvested 24 and 72 h after ICH induction. Evaluation of neurological deficits was carried out by a blinded investigator. Brain samples were collected for measurement of brain edema.

Intracerebral Hemorrhage Model in Mice

ICH was induced by intrastriatal collagenase injection as described by Rosenberg et al. [17, 18]. Briefly, mice were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) (2:1 v/v), and positioned prone in a stereotactic head frame (Kopf Instruments, Tujunga, CA). A borehole (1 mm) was drilled near the right coronal suture 1.7 mm lateral to the midline. A 27-gauge needle was inserted stereotactically into the right basal ganglia according to the coordinates: 0.9 mm posterior to the bregma, 1.7 mm lateral to the midline, and 3.7 mm below the surface of the calvaria. Clostridal collagenase (VII-S, Sigma; 0.1 U in 0.5 μL of saline) was infused into the brain over 2 min at a rate of 0.25 μL/min with a microinfusion pump (Harvard Apparatus, Holliston, MA). Sham-operated mice were subjected to needle insertion only. The needle was left in place for an additional 10 min after injection to prevent possible leakage or backflow of the collagenase solution. After removing the needle, the bore hole was closed with bone wax, the incision closed with sutures, and mice were allowed to recover. To avoid postsurgical dehydration, 0.5 ml of normal saline was given subcutaneously to each mouse after surgery.

Testing Neurological Function

All neurological tests were performed during the light cycle. Neurological deficits were evaluated by using a modified Garcia test [19], beam balance and wire-hanging tests [20]. In the modified Garcia test, seven items, including spontaneous activity, side stroke, vibrissae touch, limb symmetry, lateral turning, forelimb walking, and climbing were tested (the total possible neurological score was 21 for a healthy mouse). For both the modified beam balance and wire-hanging test, the total possible score for healthy mice was five. The behavior testing was conducted at 24 and 72 h after ICH induction by a blinded investigator.

Measurement of Brain Edema

Brain water content was measured as previously described [18]. Briefly, mice were decapitated under deep anesthesia. Brains were immediately removed and cut into 4-mm sections. Each section was divided into four parts: ipsilateral and contralateral basal ganglia, ipsilateral and contralateral cortex. The cerebellum was used as an internal control. Tissue samples were weighed to obtain the wet weight (WW) and then dried at 100°C for 24 h to determine the dry weight (DW). Brain water content (%) was calculated as $[(WW - DW)/WW] \times 100$.

Statistical Analysis

All the data were expressed as Mean ± SEM. Statistical differences were analyzed by one way-ANOVA Tukey test or Dunn's test on rank. A P value of < 0.05 was considered statistically significant.

Results

Brain Water Content

Brain water content was tested at 24 and 72 h after ICH (Fig. 1). There was a very significant increase in brain water content for the ICH + Vehicle group at both 24 and 72 h, respectively, in the ipsilateral basal ganglia (ICH + Vehicle[24 h], 83.62 ± 0.48 & ICH + Vehicle[72 h], 82.79 ± 0.41 vs. sham, 78.27 ± 0.24, $P < 0.05$) and cortex (ICH + Vehicle[24 h], 80.46 ± 0.36 & ICH + Vehicle[72 h], 80.48 ± 0.34 vs. sham, 78.96 ± 0.22, $P < 0.05$) compared to the sham group. Brain edema was also significantly increased in the contralateral basal ganglia for ICH + Vehicle at 24 and 72 h (ICH + Vehicle[24 h], 81.12 ± 0.20 and ICH + Vehicle[72 h], 80.34 ± 0.13 vs. Sham, 78.25 ± 0.11, $P < 0.05$) compared to the sham group. FTY720 treatment (1 mg/kg) significantly reduced brain edema in the ipsilateral basal ganglia at 24 and 72 h (ICH + FTY720[24 h], 82.16 ± 0.28 vs. ICH + Vehicle [24 h], 83.62 ± 0.48 and ICH + FTY720[72 h], 81.28±.33 vs. ICH + Vehicle[72 h], 82.79 ± 0.41, $P < 0.05$) and in the contralateral basal ganglia at 72 h after ICH (ICH + FTY720 [72 h], 79.70±.15 vs. ICH + Vehicle[72 h], 80.34±.13). FTY720 treatment also significantly reduced brain edema in the ipsilateral cortex (ICH + FTY720[72 h], 79.59 ± 0.17 vs. ICH + Vehicle[72 h], 80.48 ± 0.34, $P < 0.05$) and contralateral cortex at 72 h after ICH (ICH + FTY720[72 h], 78.89 ± 0.09 vs. ICH + Vehicle[72 h], 79.± 0.11).

Neurobehavioral Testing and Neurological Function

Neurobehavioral evaluations were completed at 24 and 72 h after ICH induction by using the modified Garcia test, wire-hanging test, and beam balance test (Fig. 2a–c). At 24 and 72 h after ICH, the ICH + Vehicle group demonstrated significant deficits compared to the sham group for the modified Garcia test, wire-hanging test, and beam walking test ($P < 0.05$). After FTY720 treatment, the Garcia neurological score was significantly improved at both time points ($P < 0.05$). FTY720 treatment significantly improved performance on the beam balance and wire-hanging tests at 24 h after ICH ($P < 0.05$) and showed a trend to improve performance at 72 h after ICH; however, we did not find significant results.

Discussion

Hemorrhagic stroke is the most fatal stroke type, and currently there is no effective treatment. The extravasated blood and blood components trigger a complex series of events leading to the production of inflammatory mediators and the infiltration of a wide variety of immune cells, resulting in edema formation, cell death, and permanent neurological deficits. The present study used a clinically relevant immunosuppressant that is already in clinical trials and has been submitted for regulatory approval in the US and EU for oral administration to treat MS patients. We found that administration of FTY720 reduced brain edema, improved neurological function, and offered neuroprotection after ICH. This is the first study to demonstrate the neuroprotective effect of FTY720 in a mouse intracerebral hemorrhage model.

Although T-cells are not the largest population of immune cells in the brain after stroke, their presence is certainly important. Inhibition of T-cell activation in rats with FK-506 and knockout mice for CD18 (a lymphocyte-associated adhesion molecule) leads to decreased cell death and improved functional outcome in the days and weeks following ICH [21, 22]. FTY720 binds to four of the five known S1P receptors and has been repeatedly shown to induce peripheral lymphopenia when administered at doses around 0.1 mg/kg [10, 23]. Our dosage of 1 mg/kg can be safely assumed to be causing the same effect in mice, which will reduce the amount of T-cells available for trafficking into the brain after ICH. This reduction

in T-cells available for trafficking in effect reduces the driving force behind inflammation, leading to reduced brain edema and neurological deficits.

FTY720 has been shown to have an endothelial barrier enhancement capacity by inhibiting VEGF induced vascular permeability in vitro and in vivo [24]. Moreover, FTY720 has been shown to induce the translocation of vascular endothelial cadherin and β-catenin, via S1P1 or S1P3 activation, to the focal contacts between endothelial cells, promoting adherens junction assembly [25]. Lastly, it has been demonstrated that activation of S1P1 with FTY720 induces anti-apoptotic signaling, rescuing cells from the brink of death. In a recent study in our laboratory, FTY720 treatment was shown to increase the levels of phosphor-Akt, phospho-Erk, and Bcl-2 as well as reduced cleaved caspase-3 after middle cerebral artery occlusion in rats.

In summary, FTY720 post-treatment reduced the brain edema and improved neurological function after intracerebral hemorrhage in mice. This result may be explained by its anti-inflammatory effect.

Acknowledgments

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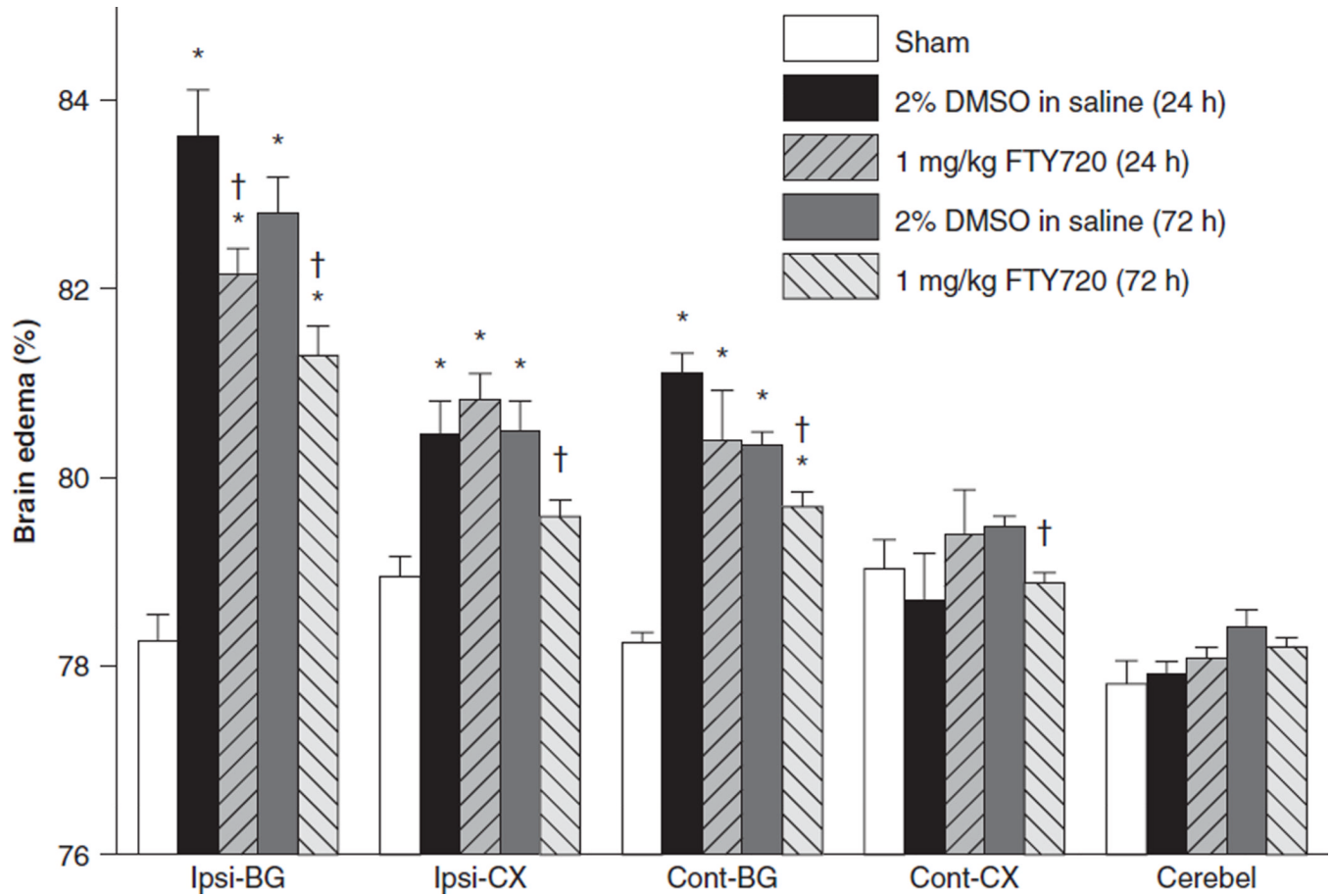
Fig. 1.
FTY720 treatment improved brain edema at 24 and 72 h after ICH. Cont-CX, contralateral cortex; Ipsi-CX, ipsilateral cortex; Cont-BG, contralateral basal ganglia; Ipsi-BG, ipsilateral basal ganglia; Cerebel, cerebellum. Values are expressed as mean ± SEM. *$P < 0.01$ vs. Sham; †$P < 0.05$ vs. Vehicle
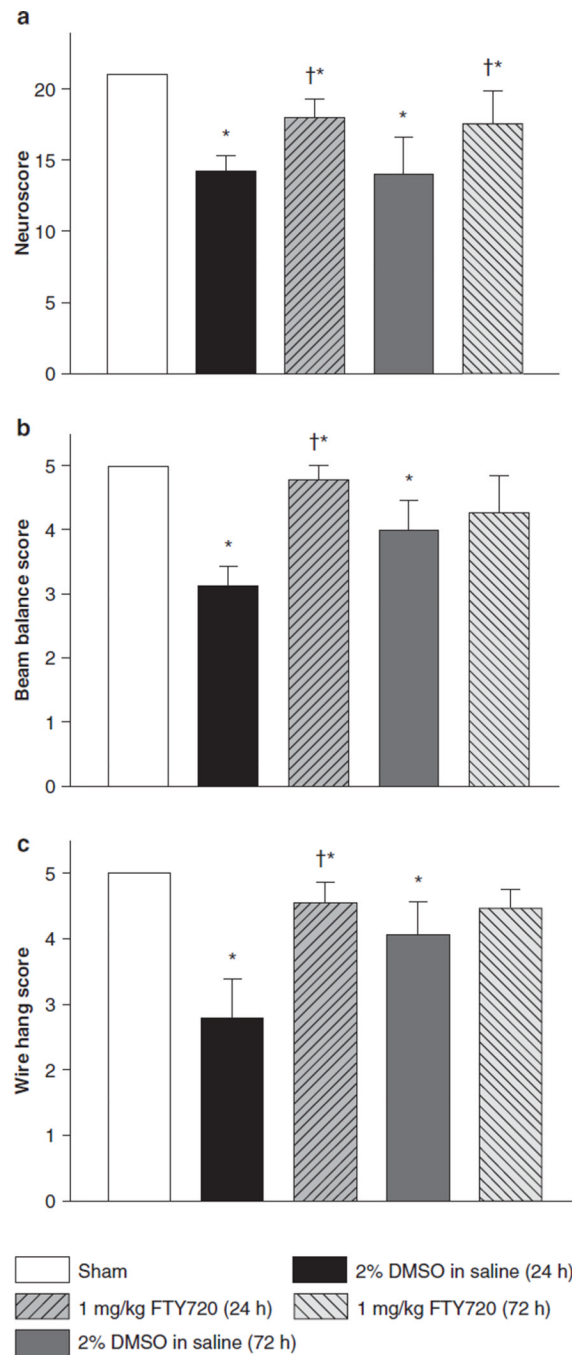
Fig. 2.
FTY720 treatment improved neurological function at 24 and 72 h after ICH. (**a**) Modified Garcia test; (**b**) wire-hanging test; (**c**) beam balance test. Values are expressed as mean ± SEM. *$P < 0.01$ vs. Sham; [†]$P < 0.05$ vs. Vehicle