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2-(-2-Benzofuranyl)-2-imidazoline reciprocally regulates Th17/Treg balance induced by ischemic stroke in rats

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Our group previously showed that 2-(-2-benzofuranyl)-2-imidazoline (2-BFI) is a potent neuroprotective agent in the treatment of ischemic stroke in rats. As its mode of action was not well defined, we determined if its therapeutic effect includes altering an immune response to experimental ischemic stroke in rats. In the current study, 2-BFI significantly reduced stroke-induced brain infarct volume and it also decreased neurological deficits. Its anti-immune effects were determined based on flow cytometry measurements of both the 2-BFI-induced changes in the Th17/ Treg cell balance ratio and ELISA measurements of proinflammatory IL-17A and anti-inflammatory IL-10 cytokine expression levels in the brain and peripheral blood following ischemic strokes. 2-BFI blunted the stroke-induced increases in this ratio, which resulted from suppression of the rises in the Th17 cell number whereas the proportion of Treg cells increased. Stroke also induced increases in IL-17A expression levels whereas the IL-10 expression levels declined. 2-BFI treatment inhibited the rises in IL-17A expression levels whereas the corresponding declines in IL-10 were suppressed by this agent. Therefore, one of the neuroprotective effects of 2-BFI in the treatment of cerebral strokes stems from its suppression of rises in the Th17/ Treg balance along with corresponding changes in related cytokines modulating development of this condition.

1. Introduction

Acute ischemic stroke is one of the main causes of death and it is a risk factor that can result in chronic physical and cognitive disability. (Wu et al. 2019). Ischemic stroke resulting from obstruction of cerebral blood flow accounts for most of the cases of strokes. Even though it is known that neuro-inflammation is a contributing factor to its pathology, there is no effective treatment that selectively targets the acute immune response that develops in the brain during cerebral ischemia (Rayasam et al. 2018). Therefore, there is a pressing need to identify novel targets for reversing immune system activation induced by ischemic stroke. Such an undertaking benefits from the results of studies that identify agents which reduce ischemic stroke neurological deficits through suppressing neuro-inflammation. These studies provide the needed insight to develop effective and selective new drugs for treatment of ischemic stroke.

It is well-known that the immune system is active in response to ischemic stroke. The innate system and the adaptive system play key roles following ischemia. The innate system can be activated by damage-associated molecular patterns (DAMPs), which are released from the ischemic region. Damaged cells also release mediators that can increase inflammation and damage the integrity of the blood-brain barrier (BBB). If this occurs, inflammatory factors enter the peripheral circulation by permeating through the disrupted BBB and activate the adaptive immune system. During this phase, T cell and B cell mediate the inflammatory response through the release of various interleukins and proinflammatory cytokines. Accordingly, T cell infiltration across the BBB into the brain may contribute to the neuronal damage and activation of the repair process (Qin et al. 2020; Tsygan et al. 2019; Xie et al. 2019). $CD4^+$ T cells are divided into conventional helper T cells and regulatory T cells (Qin et al. 2020). Regulatory T cells (Tregs) express the forkhead/winged helix transcription factor (Foxp3), which makes up approximately 10% of the $CD4^+$ T cells. Treg cells have important effects on the control of these autoreactive T cells and the maintenance of immune homeostasis through either contact-dependent suppression or the release of anti-inflammatory cytokines. Tregs also have key roles in the immune responses to infectious diseases, cancer, and transplantation tolerance (Göschl et al. 2019). Th17 cells express the master transcription factor retinoic acid-related orphan receptor γt (RORγt) and secrete interleukin-17 (IL-17), tumor necrosis factor (TNF)-α and IL-6. These mediators play critical roles in the development of many autoimmune diseases and inflammatory conditions (Bellone et al. 2020). In previous studies, the Th17/Treg cell imbalance was identified as an important mechanism for initiating immune responses induced by the onset and progression of acute ischemic stroke (Dolati et al. 2018; Hu et al. 2014; Zhang et al. 2014).

2-(2-Benzofu-ranyl)-2-imidazoline (2-BFI), a ligand of the type 2 imidazoline receptor (I_2R) was identified as a potently neuroprotective agent against ischemic damage in our previous studies (Han et al.2009, 2010, 2012, 2013). However, it was unknown whether or not 2-BFI reduces changes in the Th17/Treg imbalance induced by an acute ischemic stroke. To address this question, 2-BFI was administered to determine if it suppresses this balance in the brain and the peripheral blood induced by middle cerebral artery occlusion (MCAO) in rats.

2. Investigations and results

2.1. 2-BFI protects the brain against MCAO

We performed 2,3,5-triphenyltetrazolium chloride (TTC) staining to measure the infarct volume 72 h after MCAO. As shown in Fig.

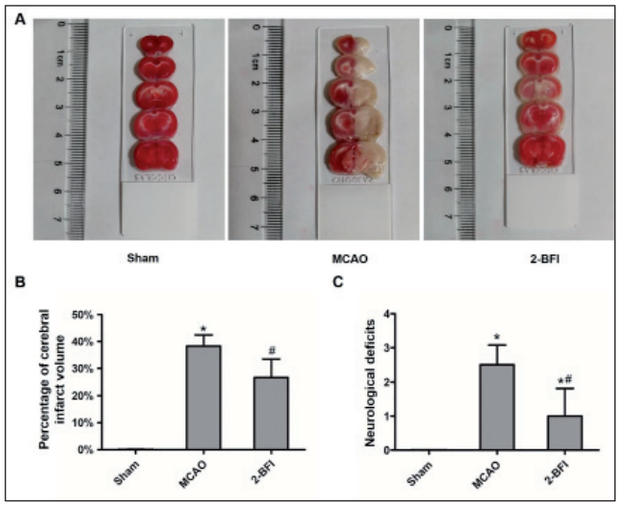

Fig.1: 2-BFI treatment suppresses middle cerebral artery occlusion-induced neuronal damage. Rats were subjected to 2-hour of MCAO followed by reperfusion.2-BFI (3 mg/kg) was injected by i.p.at 0h, 22h, 46h after initiating reperfusion. (A) TTC measurement of the infarct volume was determined after 72 hours of MCAO. (B) Graphical representation of changes in infarct volume. n=6. (C) Neurological deficits were assessed at 72 hours of MCAO. n=6. *, p<0.01, vs Sham; #, p<0.05, vs MCAO. Sham: vehicle-treated rats; MCAO: middle cerebral artery occlusion; 2-BFI: 2-(2-benzofuranyl)-2-imidazoline.

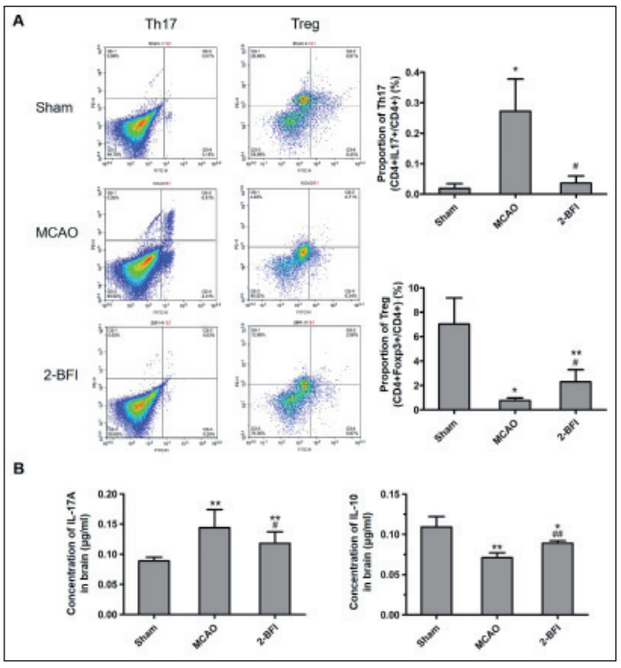

Fig.2: Effects of 2-BFI treatment on stroke-induced changes in Th17/Treg cell balance and IL-17A and IL-10 expression levels in the brain(A) Flow cytometry was performed to assess the proportion of Th17 cells and Treg cells in the brain. (B) ELISA determination of IL-17A and IL-10 expression levels in the brain. *, p<0.05 vs Sham; **, p<0.01 vs Sham; #, p<0.05 vs MCAO; ##, p<0.01 vs MCAO. Sham: vehicle-treated rats; MCAO: middle cerebral artery occlusion; 2-BFI: 2-(2-benzofuranyl)-2-imidazoline.

1A, no infarct area developed in the Sham group whereas infarcts appeared on the ipsilateral side in the MCAO group (p<0.01). The brain infarct volume expressed as a percentage of the whole brain volume was smaller in the 2-BFI group than in the MCAO group (p=0.028<0.05).

Neurological deficit scores were obtained to determine whether 2-BFI treatment reduced the changes in the MCAO model. Neurological deficits were obvious and readily seen at 72 h after MCAO whereas they were absent in the Sham group (p<0.01). Compared with the MCAO group, 2-BFI significantly reduced neurological deficit scores (p=0.016<0.05) (Fig. 1B).

2.2. 2-BFI treatment reduces MCAO-induced changes in the Th17/Treg balance in the brain

Flow cytometry was performed to determine the effects of 2-BFI on the changes in the proportions of Th17 cells and Treg cells in the brain induced by MCAO. The results show that the proportion of Th17 cells significantly increased (p=0.049) while Treg cells significantly decreased (p=0.014), in the MCAO group compared with the Sham group. Compared with the MCAO group, the proportion of Th17 cells significantly decreased in the 2-BFI group (p=0.045), whereas the proportion of Treg cells significantly increased (p=0.0036) (Fig. 2A).

The results of ELISA showed that the IL-17A expression level increased (p=0.003) in the MCAO group, compared with the Sham group in the brain tissue. In contrast, the level of IL-10 significantly decreased (p=0.0044). However, the level of IL-17A in the 2-BFI group significantly decreased (p=0.032) and IL-10 significantly increased (p=0.0026), compared with the MCAO group in the brain tissue. (Fig. 2B).

2.3. 2-BFI treatment suppresses MCAO-induced changes in Th17/Treg balance in the peripheral blood

To determine if the changes in the Th17/Treg balance induced by 2-BFI following MCAO in the brain correspond to those in the peripheral blood circulation, flow cytometry was performed. As in the brain, the proportion of Th17 cells in peripheral blood of the MCAO group significantly increased (p = 0.0002), whereas Treg cells significantly decreased (p = 0.0038), compared with the Sham group. Similar to the effects of 2-BFI in the brain, the proportion of Th17 cells in peripheral blood of the 2-BFI group significantly decreased (p=0.0001), Treg cells significantly increased (p=0.026), compared with the MCAO group (Fig. 3A).

The results of ELISA showed that compared with the Sham group, the levels of IL-17A and IL-10 of the MCAO group significantly increased (p=0.0023) and significantly decreased (p=0.0001) in the peripheral blood. On the other hand, the levels of IL-17A of 2-BFI group decreased significantly (p=0.0022) whereas IL-10 in the 2-BFI group significantly increased in the peripheral blood (p=0.044), compared with the MCAO group (Fig. 3B).

3. Discussion

Characterization of changes in the Th17/Treg balance provides important insight into mechanisms modulating immune processes in ischemic stroke. Dolati et al. (2018) demonstrated that changes in the balance of the Th17/ Treg ratio affect immune responses underlying the pathogenesis of ischemic stroke. The increases in this ratio that occur in ischemic stroke patients are a consequence of increases in Th17 cells along with decreases in Treg cell. Li et al. (2013) described the changes of the Th17/Treg cells in the peripheral blood of atherosclerotic cerebral infarction patients and the impact of oxidized low-density lipoprotein (ox-LDL) on this ratio. This rise suggests this condition creates an imbalance of the Th17/Treg cells ratio and ox-LDL may contribute to this imbalance (Li et al. 2013). In the present study, we compared the changes in the proportions of Th17 and Tregs cells, and their related inflammatory mediator levels in both the brain and the peripheral blood of MCAO rats with those in the sham-operated control rats. The results indicate that the Th17 cell content increased whereas Treg cell content decreased in both the brain and peripheral blood of MCAO rats compared with the ratio in the sham-operated rats. This correspondence between their brain and blood levels suggests that stroke-induced disruption of the blood brain barrier integrity may enable activated immune cells in the brain to interchange with those in the peripheral circulation.

In our previous study, 2-BFI reduced the neurological deficit scores, promoted the recovery of limb function and inhibited apop-

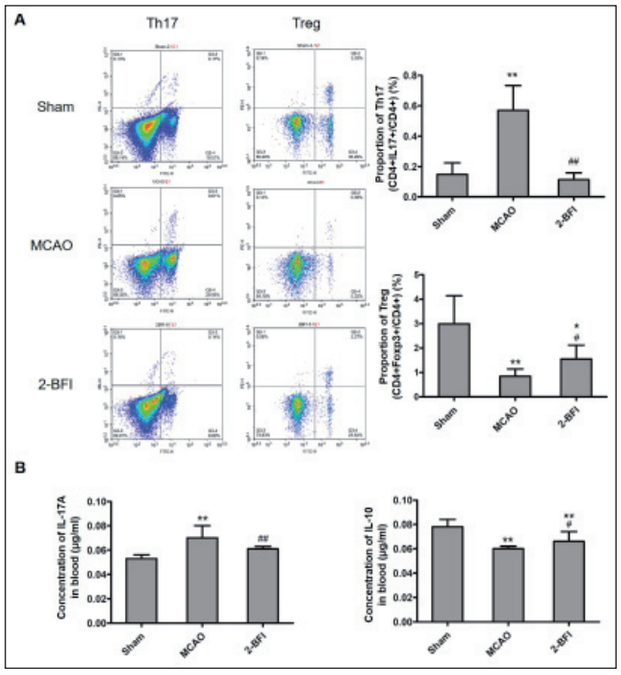

Fig. 3: Effects of 2-BFI treatment on stroke-induced changes in Th17/Treg cell balance and IL-17A and IL-10 expression levels in the peripheral blood. (A) Flow cytometry was performed to assess the proportion of Th17 cells and Treg cells in the peripheral blood. (B) ELISA determined the IL-17A and IL-10 expression levels in the peripheral blood. *, p<0.05 vs Sham; **, p<0.01 vs Sham; #, p<0.05 vs MCAO; ##, p<0.01 vs MCAO. Sham: vehicle-treated rats; MCAO: middle cerebral artery occlusion; 2-BFI: 2-(2-benzofuranyl)-2-imidazoline.

tosis. Suppression of apoptosis was attributed to a decline in the expression of caspase-3 in ischemic brain tissue (Han et al. 2009). Furthermore, 2-BFI contributed to increased cell survival based on an increase in Bcl-2 gene expression following MCAO induced cerebral ischemia (Han et al. 2010). Additional support consistent with these beneficial effects is that 2-BFI reduced the infarct volume, and promoted angiogenesis (Han et al. 2012). 2-BFI also displayed a neuroprotective role by blocking NMDA receptor mediated intracellular calcium influx and calcium overload along with inhibiting glutamate excitotoxicity (Han et al. 2013). In this study, we found that 2-BFI reduced the infarct volume and reduced neurobehavioral deficits in MCAO rats. This study also showed that the number of Th17 cells in both peripheral blood and brain significantly declined whereas the number of Treg cells significantly increased after the administration of 2-BFI in MCAO rats. These results suggest that the improved functional outcome after MCAO is mainly due to inhibiting the rises in the Th17/Treg ratio. The appropriate expression of proinflammatory cytokines plays critical roles in maintaining the Th17/Treg balance. The increase in this balance is consistent with increases in IL-17A secretion by Th17 cells and a decline in IL-10 secretion by Treg that were identified in ischemic stroke patients and stroke animal models (Dolati et al. 2018; Hu et al. 2014; Zhang et al. 2014). IL-17A is mainly secreted by Th17 cells. It is one of key cytokines in inflammation-associated diseases (Bunte et al. 2019). In an ischemic/reperfusion model of cerebral infarction, the expressions of IL-17 mRNA and protein were significantly increased on the first day after cerebral infarction, and the peak increase of IL-17 mRNA expression was reached on the third day after cerebral infarction, while the high level of its protein product was maintained until the sixth day (Zhang et al. 2014). The elevated IL-17 expression levels were significant post 1, 5, and 10 days in ischemic stroke patients compared with the control group (Dolati et al. 2018). Anti-inflammatory cytokine IL-10 secreted by Treg cells could provide neuroprotective effects (i.e. reduced infarct size) after ischemic stroke by inhibiting inflammatory responses (Zhang et al. 2016). However, the IL-10 expression levels were instead lower in ischemic stroke patients compared patients with no ischemic stroke (Hu et al. 2014). Furthermore, we found in the current study that the expression level of IL-17A in both the peripheral blood and the brain tissue markedly increased in the MCAO rats, but significantly fell in the 2-BFI treatment group. In addition, the expression of IL-10 in both peripheral blood and brain tissue decreased by a large amount in MCAO rats, whereas 2-BFI treatment markedly attenuated this decline. These effects are in agreement with ELISA measurements showing that 2-BFI blunted the IL-17A expression levels and lessened the declines in IL-10 expression levels which contribute to the overall neuroprotective role of 2-BFI in the brain and peripheral blood of ischemic stroke rats.

The current study has some limitations. The dose of 2-BFI was only 3 mg/kg which is lower than the highest dose used in our previous study. In that case, a dose response study was performed. The 2-BFI dose ranged between 1.5 mg/kg and 6 mg/kg. With 3 mg/kg, the number of TUNEL positive cells in the infarct area decreased. This result suggested that 3 mg/kg of 2-BFI had an adequate neuroprotective effect (Han et al. 2010). Secondly, the time of 2-BFI administration was only at 0 h after reperfusion (2 h after MCAO), which is difficult to realize in the clinic. On the other hand, in our previous study, 2-BFI (3 mg/kg) was given at 0, 1, 3, 5, 7, and 9 h after reperfusion. The results showed 2-BFI consistently at all of these time points decreased the infarct volume and the time of the largest decline infarct volume occurred at 0 h after reperfusion (Zhang et al. 2018). Thirdly, glial cells have been implicated in stroke immune regulation and exert both beneficial and detrimental effects during ischemic stroke (Xu et al. 2020). However, their involvement was not dealt with in this study.

Taken together, 2-BFI has a neuroprotective effect on acute ischemic stroke since it suppressed increases in the Th17/Treg balance. Accompanying these changes, the expression level changes in IL-17A and IL-10 corresponded with the effects of 2-BFI on Th17 and Treg cell numbers. These results suggest that 2-BFI has the potential to be used to treat ischemic stroke in a clinical setting if its effects are first replicated in future studies using other experimental models.

4. Experimental

4.1. Experimental animals and reagents

Sprague-Dawley rats (male, 3-4 months old, 250-300 g body weight) were obtained from Gempharmatech Bioscience Co., (Nanjing China). All animals were fed with food and water in the light dark cycle for 12 h a day. All experimental procedures followed the Guide for the Care and Use of Laboratory Animals of the China National Institutes of Health. All procedures were approved by the Animal Care and Use Committee of Wenzhou Medical University. Rats were randomly allocated to three different experimental groups: sham-operated group (Sham, n =18), MCAO only group (MCAO, n = 18), MCAO with 2-BFI group (2-BFI, n = 18). 2-BFI was purchased from Tocris (UK) and TTC staining solution (2,3,5-triphenyltetrazolium chloride) was obtained from Sigma Chemical Co., USA. IL-10 ELISA kit (Cat. No. 70-EK3172/2) and IL-17A ELISA kit (Cat. No. 70-EK3102/2) was purchased from MultiSciences Biotech, Co., China. A primary antibody including FOXP3 Antibody, CD4 Antibody, IL-17A Antibody and IL-10 Antibody were purchased from ebioscience (USA).

4.2. Middle cerebral artery occlusion (MCAO) model

Rats were anesthetized with 10% chloral hydrate. Middle cerebral artery occlusion (MCAO) model was produced following the animal procedures for transient occlusion of the middle cerebral artery as previously described (Han et al. 2012). Briefly, the skin was cut about 2-3 cm in the middle of the neck, and the right common carotid artery, the external carotid artery, and internal carotid artery were blunt separated. A monofilament silicon-coated nylon suture was inserted from the right common carotid artery to the right internal carotid artery (The insertion depth of suture was from the 18±0.5 mm at common carotid bifurcation) to occlude the right middle cerebral artery. After 120 min of occlusion, the thread was removed to allow for reperfusion. Sham-operated rats underwent an identical surgery except that the suture was not inserted. Immediately following the thread being removed to allow for reperfusion, rats were administered with 2-BFI at dose of 3 mg/kg at 0 h, 22 h, 46 h after reperfusion (2-BFI group) or injected with saline solution through i.p. (MCAO group).

4.3. Assessment of the neurological deficit

The neurologic examination of the first time was performed when rats woke up after anesthesia. Animals with a neurological symptom score of 0 and 4 after MCAO were excluded. The last neurologic examination of the was performed at 24 h after

terminating 2-BFI injection (after 72 hours of MCAO). The neurologic deficit scores of rats were evaluated using the Zealonga five-point scale: 0 score: no symptoms of nerve damage; 1 score: unable to fully extend left front paw; 2 scores: circling to the left side; 3 scores-incline to the left side; 4 scores: unable to walk spontaneously with loss of consciousness.

4.4. 2,3,5-Triphenyltetrazolium chloride (TTC) staining

To evaluate the infarct volume in rat brain, 2,3,5-triphenyltetrazolium chloride (TTC) staining was performed. First, rats were anesthetized and euthanized three days after MCAO. Then the brains were removed, sectioned into five 2-mm-thick coronal slices, immersed into a 2% TTC solution and incubated for 30 min at 37 °C. Infarcted areas lacked typical brick-red staining in the normal brain tissue. These areas were quantified with ImagePro Plus software (V6.0) (Media Cybernetics, Inc., USA). Infarct volume was calculated by multiplying the corrected area by the slice thickness and summing the volume.

4.5. Flow cytometry

Flow cytometry was used for the measurement of the proportion of Th17 and Treg cells in brain or peripheral blood of rats. After rats were anesthetized and euthanized, brain tissue was acquired by removing the cerebellum and the brainstem from the ischemic brain. The brain tissue was washed with normal saline, digested for 10 min with 0.25% trypsinase, centrifuged for 5 min at 1000 rpm; and diluted with PBS. A cell suspension (5×10^6/ml) was prepared for flow cytometry analysis. Blood samples were collected from the abdominal aorta. Peripheral blood mononuclear cells (PBMCs) were isolated and collected from blood samples (Dolati et al. 2018). For the analysis of flow cytometry, PBMCs were suspended at a density of 2.0×10^6 cells/ml in Roswell Park Memorial Institute (RPMI) 1640 Medium (Sigma, USA).

Before flow cytometry, 100 µl of the cell suspension was fixed with 4% paraformaldehyde for 30 min and treated with 0.1% Triton X-100 for 15 min at room temperature after centrifugation for 5 min at 1000 rpm. The cell suspension was resuspended in 100 µl 10% BSA-PBS and stained with phycoerythrin (PE)-conjugated anti-CD4 (Cat. No. 17-0040-80, ebioscience, San Diego, CA, USA), fluorescein isothiocyanate (FITC)-conjugated anti- FOXP3 (Cat. No. 53-5773-80, ebioscience, San Diego, CA, USA), fluorescein isothiocyanate (FITC)-conjugated anti- IL-17A (Cat. No. 17-4724-42, ebioscience, San Diego, CA, USA), respectively. Stained cells were assessed by using NovoCyte Flow cytometer (ACEA Biosciences Inc., USA) with NovoExpress Software 1.2.5 (ACEA Biosciences Inc., USA) according to the manufacturer's instructions.

4.6. ELISA

Three days after MCAO in each of these three groups, abdominal anesthesia was performed. The brain was extirpated and the cerebellum and the brain stem were removed. A single cell suspension of dispersed brain tissue was prepared (Campanella et al. 2002). Blood samples were collected from the abdominal aorta. ELISA was used to detect IL-10 and IL-17A expression levels. ELISA detection step was followed exactly as the manufacturer recommended. Briefly, 50 µl of samples were added to the ELISA plate and incubated for 2 h at room temperature. After washing, 50 µl of antibody conjugate was added to each well and incubated for 2 h, followed by the addition of 100 µl of substrate solution to each well. After 30 min of incubation at room temperature protected from light, 100 µl of stop solution was added to each well resulting in a color change from blue to yellow. Dual-wavelength detection was performed using a microplate reader to determine the maximum absorption wavelength at 450 nm and the OD value at either a reference wavelength of 570 nm or 630 nm.

4.7. Statistical analysis

All data were analyzed by SPSS 20.0 (SPSS Inc., Chicago, Illinois, USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test for making a comparison among multiple groups. Data is displayed as mean ± standard deviations (SD). $p<0.05$ represented statistically significant.

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Conflicts of interests: Non declared.

References

Bellone M, Brevi A, Huber S (2020) Microbiota-propelled T helper 17 cells in inflammatory diseases and cancer. Microbiol Mol Biol Rev 84: e00064-19.

Bunte K, Beikler T (2019) Th17 cells and the IL-23/IL-17 axis in the pathogenesis of periodontitis and immune-mediated inflammatory diseases. Int J Mol Sci 20: 3394.

Campanella M, Sciorati C, Tarozzo G, Beltramo M (2002) Flow cytometric analysis of inflammatory cells in ischemic rat brain. Stroke 33: 586–592.

Dolati S, Ahmadi M, Khalili M, Taheraghdam AA, Siahmansouri H, Babaloo Z, Aghebati-Maleki L, Jadidi-Niaragh F, Younesi V, Yousefi M (2018) Peripheral Th17/Treg imbalance in elderly patients with ischemic stroke. Neurol Sci 39: 647–654.

Göschl L, Scheinecker C, Bonelli M (2019) Treg cells in autoimmunity: from identification to Treg-based therapies. Semin Immunopathol 41: 301–314.

Han Z, Cheng ZH, Liu S, Yang JL, Xiao MJ, Zheng RY, Hou ST (2012) Neurovascular protection conferred by 2-BFI treatment during rat cerebral ischemia. Biochem Biophys Res Commun 424: 544–548.

Han Z, Xiao MJ, Shao B, Zheng RY, Yang GY, Jin K (2009) Attenuation of ischemia-induced rat brain injury by 2-(-2-benzofuranyl)-2-imidazoline, a high selectivity ligand for imidazoline I (2) receptors. Neurol Res 31: 390–395.

Han Z, Yang JL, Jiang SX, Hou ST, Zheng RY (2013) Fast, non-competitive and reversible inhibition of NMDA-activated currents by 2-BFI confers neuroprotection. PLoS One 8: e64894.

Han Z, Zhang HX, Tian JS, Zheng RY, Hou ST (2010) 2-(-2-Benzofuranyl)-2-imidazoline induces Bcl-2 expression and provides neuroprotection against transient cerebral ischemia in rats. Brain Res 1361: 86–92.

Hu Y, Zheng Y, Wu Y, Ni B, Shi S (2014) Imbalance between IL-17A-producing cells and regulatory T cells during ischemic stroke. Mediators Inflamm 2014: 813045.

Li Q, Wang Y, Yu F, Wang YM, Zhang C, Hu C, Wu Z, Xu X, Hu S (2013) Peripheral Th17/Treg imbalance in patients with atherosclerotic cerebral infarction. Int J Clin Exp Pathol 6: 1015–1027.

Longa EZ, Weinstein PR, Carlson S, Cummins R (1989) Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20: 84–91.

Qin X, Akter F, Qin L, Cheng J, Guo M, Yao S, Jian Z, Liu R, Wu S (2020) Adaptive immunity regulation and cerebral ischemia. Front Immunol 11: 689.

Rayasam A, Hsu M, Kijak JA, Kissel L, Hernandez G, Sandor M, Fabry Z (2018) Immune responses in stroke: how the immune system contributes to damage and healing after stroke and how this knowledge could be translated to better cures? Immunology 154: 363–376.

Tsygan NV, Trashkov AP, Litvinenko IV, Yakovleva VA, Ryabtsev AV, Vasiliev AG, Churilov LP (2019) Autoimmunity in acute ischemic stroke and the role of blood-brain barrier: the dark side or the light one? Front Med 13: 420–426.

Wu S, Wu B, Liu M, Chen Z, Wang W, Anderson CS, Sandercock P, Wang Y, Huang Y, Cui L, Pu C, Jia J, Zhang T, Liu X, Zhang S, Xie P, Fan D, Ji X, Wong KL, Wang L; China Stroke Study Collaboration (2019) Stroke in China: advances and challenges in epidemiology, prevention, and management. Lancet Neurol 18: 394–405.

Xie L, Li W, Hersh J, Liu R, Yang SH (2019) Experimental ischemic stroke induces long-term T cell activation in the brain. J Cereb Blood Flow Metab 39: 2268–2276.

Xu S, Lu J, Shao A, Zhang JH, Zhang J (2020) Glial cells: role of the immune response in ischemic stroke. Front Immunol 11: 294.

Zhang L, Huang Y, Lin Y, Shan Y, Tan S, Cai W, Li H, Zhang B, Men X, Lu Z (2016). Anti-inflammatory effect of cholera toxin B subunit in experimental stroke. J Neuroinflammation13: 147.

Zhang J, Mao X, Zhou T, Cheng X, Lin Y (2014) IL-17A contributes to brain ischemia reperfusion injury through calpain-TRPC6 pathway in mice. Neuroscience. 274: 419–428.

Zhang Z, Yang JL, Zhang LL, Chen ZZ, Chen JO, Cao YG, Qu M, Lin XD, Ji XM, Han Z (2018) 2-(2-Benzofuranyl)-2-imidazoline treatment within 5 hours after cerebral ischemia/reperfusion protects the brain, Neural Regen Res 13: 2111–2118.