

## Therapeutic Effects of Dimethyl Fumarate on the Rat Model of Brain Ischemia

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Blood-brain barrier (BBB) disruption, inflammation, and cell death are major pathogenic mechanisms in ischemic stroke. Dimethyl fumarate (DMF) has anti-inflammatory and immunomodulatory effects. So, this study aimed to elucidate the effects of DMF on brain ischemia in the middle cerebral artery occlusion (MCAO) model. 69 Sprague-Dawley male rats were allocated into a sham group that was just subjected to surgery stress; vehicle and DMF groups, after MCAO, received vehicle or 30 mg/kg DMF for three days. Neurological scores were evaluated every day. BBB disruption was evaluated by the extravasation of Evans blue. In addition to the measurement of brain water content, the total and infarct volume, numerical density, and the total number of neurons, non-neurons, and dead neurons in the right cortex were estimated by stereological methods. RT-PCR was done to analyze the expression levels of NF- $\kappa$ B and Nrf2. Although brain ischemia treatment with DMF did not have a significant effect on the infarction size, it improved neurobehavioral function, BBB disruption, cerebral edema, increased number of neurons, and expression of Nrf2. It also decreased the number of dead neurons and the expression of NF- $\kappa$ B. DMF beneficial effects on stroke may be mediated through both increase of the Nrf2 and decrease of NF- $\kappa$ B expression.

**Keywords:** Dimethyl fumarate. Brain ischemia. Brain edema. cell death. Nrf2. NF- $\kappa$ B.

### INTRODUCTION

Ischemic stroke, one of the most common neurological disorders, is caused by a sudden occlusion in an artery that supplies blood to the brain by a thrombus or embolism. It is leading to depletion of ATP and oxygen-glucose deprivation in the cerebral tissue (Petty, Wettstein, 2001). Cerebral ischemia and post-ischemic reperfusion cause multiple events at cellular and molecular levels. It leads to neuronal death including local reduction of oxygen or glucose, excitotoxicity induced by glutamate and ionic imbalance, oxidative/nitrative stress, loss of blood-brain

barrier (BBB) integrity in partitioning ions, inflammation, cerebral edema, induction of apoptosis, alteration of gene expression, protein, and reaction products and ultimately DNA damage (Dirnagl, Iadecola, Moskowitz, 1999).

Today, there is only one Food and Drug Administration (FDA)-approved medication for ischemic stroke; i.e., tissue plasminogen activator (tPA). However, it should be administered within three hours of symptom onset and has many other limitations such as high cost, low availability, increasing intracranial bleedings, and other serious side effects (Gravanis, Tsirka, 2008). There is an urgent necessity to find a new, high-throughput, and inexpensive drug to minimize these limitations. Dimethyl fumarate (DMF), a novel (FDA-approved) orally administered effective treatment in neurological diseases such as multiple sclerosis. It has been shown that DMF has a variety of protective effects through its potent immunomodulatory and antioxidant properties in

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neurons (Linker, *et al.*, 2011). Furthermore, this drug can reduce brain edema and improve neurological outcomes and BBB disruption in a mouse model with ischemic stroke (Kunze, *et al.*, 2015).

Nuclear factor erythroid 2-related factor 2 (Nrf2) signaling regulates antioxidant response element (ARE)-mediated expression of phase II detoxifying and antioxidant enzymes (Kensler, Wakabayashi, Biswal, 2007). Nuclear factor  $\kappa$ B (NF- $\kappa$ B) contributes to neuronal cell death and inflammatory processes in cerebral ischemia (Ridder, Schwaninger, 2009).

In the present study, as DMF is a relatively safe, inexpensive, and novel neuroprotective medicine in the neurological disorder, we evaluate its potential therapeutic effects on neurobehavioral function, BBB integrity, brain water content, and histologic changes in the sub-acute stage of brain ischemia in rats. Because of the therapeutic effects of this drug may be mediated via Nrf2 and NF- $\kappa$ B expression, their levels have been evaluated.

## MATERIAL AND METHODS

### Experimental Animals

All experiments were approved by the local Ethics Committees of Shahid Beheshti University, Tehran, Iran. Adult male Sprague-Dawley rats (250–300 g) were purchased from the Comparative and Experimental Medical Center of Shiraz University of Medical Sciences (SUMS, Shiraz, Iran). The rats had free access to food and water in a room with temperature 21–23°C and a 12 h light/12 h dark cycle (lights on at 08:00). After the adaptation period, 69 rats were randomly divided into three homogeneous groups (n=23/group). The sham group suffered from surgery without middle cerebral artery occlusion (MCAO) procedure or drug administration. The vehicle and DMF groups, after MCAO, treated with vehicle (0.08 % methocel/H<sub>2</sub>O, methylcellulose, Sigma-Aldrich, St. Louis, MO, USA) or DMF (30 mg/kg body weight, Merck, Darmstadt, Germany) in 0.08 % methocel/H<sub>2</sub>O, with oral gavage post-operative days 0, 1, 2 and 3. Two, 24, 48, and 72 h after brain ischemia induction, neurobehavioral functions were evaluated. At the end of third day after brain ischemia, rats in each

group were deeply anesthetized and then euthanized for assessment of BBB integrity (n=5), brain water content (n=5), reverse transcription-quantitative real-time PCR (RT-qPCR) (n=6), and TTC staining evaluation (n=1). The remaining rat brains (n=6), after fixation with 4% buffered paraformaldehyde, were serially and coronally sectioned at the 30- $\mu$ m thickness, using a cryostat (Leica, Germany) and stained with cresyl violet for stereological studies. All evaluations have been done by an investigator that was blinded to groups.

### Middle Cerebral Artery Occlusion (MCAO) Procedure

The rats were subjected to MCAO by inserting monofilament suture into the right common carotid artery as described by Koizumi (1986). Briefly, the rats were anesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg, Merck, Darmstadt, Germany). A midline incision was made on the neck. The right common carotid artery (CCA) was carefully dissected and isolated from the vagus nerve and its sheath, external (ECA) and internal carotid artery (ICA). A heat-rounded and silicone-coated 3-0 monofilament nylon suture was advanced through the common carotid artery into the lumen of the ICA until it occluded the origin of the MCA to avoid backflow leakage through CCA. One hour after MCAO, reperfusion was done by the withdrawal of the suture. After suturing, the neck incision became sterile with a spray containing oxytetracycline. The body temperature was controlled and maintained about 37  $\pm$  0.8 °C throughout and after surgery with an electrical blanket. 2, 3, 5-triphenyltetrazolium chloride (TTC) was just used to ensure that brain ischemia was successfully induced and the rats were suffering from ischemic stroke (n=1 randomly in each group). The brain of each animal was obtained at the end of third day post-ischemia and immediately sliced into coronal sections (2 mm thick) from the rostral to the caudal frontal tip using the brain matrix for rat (Zivic Instruments, Pittsburgh, USA). The sections were stained with 2 % TTC (Sigma-Aldrich, Saint Louis, MI, USA), followed by immersion in normal saline at 37 °C for 15 min. The brain sections

were then photographed with a stereomicroscope (Nikon, SMZ745T, Japan).

## Body Weight and Neurological Scores

### Body Weight

Bodyweight was monitored before and on day three after surgery using a Digital Weighing Scale (Acculab ALC210.4, USA). Changes in the body weight of animals in different groups were calculated.

### Neurological Scores Examination

Each rat was subjected to neurobehavioral tests to evaluate its function at 0, 1, 2, and 3 days after MCAO (n=12 rats/group). These tests were expressed as the modified neurologic severity scores (mNSS). The mNSS is a composite of motor, sensory (visual, tactile, and proprioceptive), reflex, and balance tests. Neurological function was graded on a scale of 0–18 as previously described (Zhou, *et al.*, 2011). If the animal score was below three or died from brain ischemia, they excluded from the study, and another rat was substituted.

### Evaluation of Blood-Brain Barrier Permeability

BBB integrity was determined by quantifying the Evans blue (EB) leakage into brain tissue, according to Uyama *et al.* (1988). Briefly, the right femoral vein was cannulated 48 h after MCAO induction. EB prepared as 2% in saline, and 4 ml/kg was injected. Under deep anesthesia, the chest wall was opened 72 h after ischemia (24 h after EB injection). To eliminate the EB from cerebral circulation, 250 ml of 0.9% saline was transcardially perfused for 20 min. The whole brain was immediately removed after guillotine decapitation and brain hemispheres were separated. Its wet weight was immediately measured. The hemispheres were homogenized in 2.5 ml PBS, and the samples were then mixed with 2.5 ml of 60% trichloroacetic acid (Merck, Darmstadt, Germany) to precipitate protein, and centrifuged for 30 min at 3500 rpm. The supernatant (0.2 ml) was utilized to measure the extracted EB absorbance

at 610 nm with an Epoch microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The tissue content of EB was quantified from a standard curve derived from the known amounts of dye. Calculations were based on external standards in the same solvent and data were expressed as micrograms per gram ( $\mu\text{g/g}$ ) of brain tissue based on wet weight, and calculated according to a standard curve. The EB extravasation ratio was calculated by applying the following formula: EB extravasation ratio = [right hemisphere EB ( $\mu\text{g}$ ) / right hemisphere weight (g)] / [left hemisphere EB ( $\mu\text{g}$ ) / left hemisphere weight (g)] (Uyama, *et al.*, 1988).

### Brain water content measurement

Brain water content (BWC), as a marker of cerebral edema, was measured based on previous studies (Vakili, Hossienzadeh, Sadogh, 2007). Briefly, at 72 h after ischemia/reperfusion (I/R), five rats in each group were sacrificed by a guillotine, and brains immediately removed. Then, the cerebellum and olfactory bulb were cut, and the hemispheres were separated. Each cerebral hemisphere was weighed to obtain their wet weight. Then, the brains were dried in an oven at 120°C for 24 h to obtain dry weight. BWC percentage was determined using the following formula: [(wet weight – dry weight) / (wet weight)]  $\times$  100.

### Tissue processing

After the sub-acute treatment period, six rats in each group were anesthetized with chloral hydrate (400 mg/kg) and transcardially perfused with 0.09% saline, followed by 4% buffered paraformaldehyde. Brains were dissected, removed, and kept in the same fixative overnight. The brains were then transferred to 30% sucrose (Sigma-Aldrich Inc., St. Louis, USA) in phosphate-buffered saline for 72 hours. Finally, the dissected brains were swiftly frozen in isopentane (2-methylbutane) and stored at -80°C until further process. The brains were coronally and serially sectioned using a cryostat (Leica Biosystems, Wetzlar, Germany) at a thickness of 30  $\mu\text{m}$ , and immersed into 12-well plates containing cryoprotectant solution and maintained at -20°C until staining. Every 48 section

(1.44 mm interval) was selected by a systematic uniform random sampling method. The first section was 2-mm posterior to the frontal tip. An average of 8 ( $\pm 1$ ) sections per brain fixed on gelatin-coated slides and stained with cresyl violet.

### Stereological evaluations

In cresyl violet staining, the normal area appears in dark blue to purple, while the infarct area in the right (ischemic) hemisphere does not stain with this color and appears very pale blue to white. The total and infarct volume of the brain and hemispheres, and its hippocampus, striatum, and cortex subfields were separately estimated by a point-counting technique based on Cavalieri's principle (Namavar, *et al.*, 2012) using stereology software (StereoLite, SUMS, Shiraz, Iran). The volume of the hippocampus, striatum, cortex, and hemispheres ( $V_{ref}$ ) were determined by applying the following formula:  $V_{ref} = d \times a(p) \times \Sigma P$ , where,  $d=1.44$  mm; equals to the section interval or distance from one section to the next.,  $a(p)= 1.23076$  mm<sup>2</sup>; equal to the area associated with one point in the grid to the next, and  $\Sigma P$ =number of points separately hitting the rat brain section of the right and left total cortex, striatum, hippocampus and hemisphere and ischemic areas in them (Namavar, *et al.*, 2012).

Furthermore, the numerical density and the total number of neurons, non-neurons and dead neurons in right hemisphere cortex were quantified using the optical disector technique (Namavar, *et al.*, 2012), with a high-numerical-aperture (NA=1.30)×40 oil-immersion objective, connected to a video camera, which transmits the microscopic image to a monitor. To assess the movements in the Z-direction, an electronic microcator with digital readout (MT12, Heidnehain, Traunreut, Germany) was used. The neuronal, non-neuronal or dead neuronal numerical density is defined as follow:  $N_v = \Sigma Q / [\Sigma P \times a(f) \times h]$ , where  $\Sigma Q$  is the number of neurons, non-neurons or dead neurons counted within the sampling frame or disector,  $\Sigma P$  is the number of disector,  $a(f)= 0.00105365$  mm<sup>2</sup>, is the area of the sampling frame,

and  $h=0.015$  mm, is the height of the sampling frame in the Z-direction. We used the key features to identify neurons and non-neurons in Nissl stained (cresyl violet) sections according to Gabbott and Stewart (1987) and Ling *et al.* (1973) reports. The brain cells were divided into two types: neurons and "others", which were termed non-neurons. The basic morphology of a normal neuron in cresyl violet staining consists of large cell body or perikaryon with neurites (dendrites and axon) emerging from the cell body, Nissl substance in perikaryon (and dendrites), an identifiable nucleus that is invariably pale or euchromatic with discrete nucleolus (Abusaad, *et al.*, 1999). Dead or dying neurons (i.e., apoptotic or necrotic cells) display accumulation of dense globular material in the cytoplasm with evidence of nuclear fragmentation, shrunken perikarya, and darkly stained nuclei of reduced size (Csordas, Mazlo, Gallyas, 2003).

### RNA isolation, cDNA synthesis and Real-time PCR analysis

At the end of third day after surgery, rats from all groups were sacrificed and brains immediately removed. RNA was directly isolated from the right (ipsilateral to MCAO) hemisphere cerebral cortex with QIA Shredder and RNeasy Mini kits (Qiagen, Iran) according to the manufacturer's instruction. The RNA concentration was quantified by ultraviolet spectrophotometry at 260/280 nm. Complementary DNA (cDNA) was synthesized, using SuPrime Script RT Premix (2X) cDNA Synthesis Kit (GeNet BIO Inc.; Daejeon, South Korea) for RT-PCR, according to the protocol suggested by the company. Quantitative RT-PCR was performed using the cDNA for RNA quantitation with Power SYBR Green PCR Master Mix and a StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Beta-2 -microglobulin (B2M) was used as an internal control in expression studies. Relative quantification was measured using the comparative Ct ( $2^{-\Delta\Delta Ct}$ ) method (Livak, Schmittgen, 2001). Primers for real-time PCR were designed using the Primer-BLAST, and are listed in Table I.



**TABLE I** - Primer sequences

Gene	Sequence	Primer efficiency (%)
Nrf2	forward 5'- CACATCCAGACAGACACCAGT-3' reverse 5'- CTACAAATGGGAATGTCTCTGC-3'	98.7
NF-κB	forward 5'- AGGCCATTGAAGTGATCCAG -3' reverse 5'- GAGCTCATCTATGTGCTGTCTT -3'	92.0
B2M	forward 5'- CGTGCTTGCCATTCAGAAA -3' reverse 5'- ATATACATCGGTCTCGGTGG -3'	92.2

### Statistical Analysis

All data were analyzed by SPSS 18.0 software and expressed as mean  $\pm$  SEM. Statistical differences were measured by one-way analysis of variance (ANOVA) combined with Tukey post hoc test for multiple group comparisons. Values of  $P < 0.05$  were considered significant.

## RESULTS

### Body weight changes

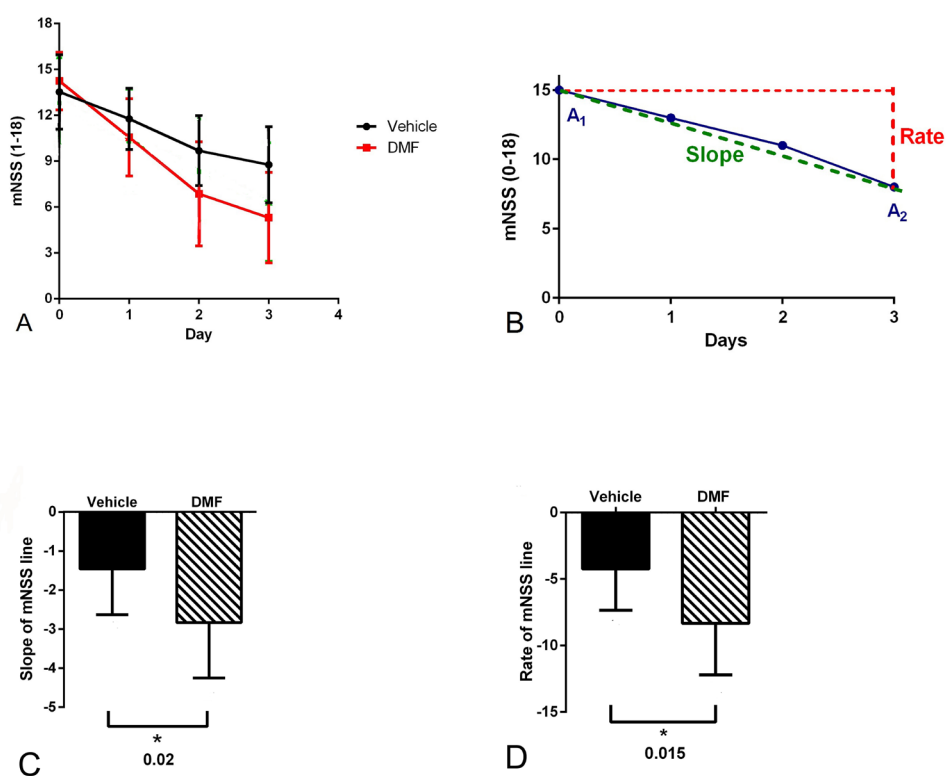
At the end of third day after cerebral ischemia, rats in the vehicle group showed a significant decrease (21.8%) in body weight as compared to rats in the sham group ( $P < 0.01$ ). However, DMF-treated rats showed no significant difference in body weight change in comparison with the vehicle-treated and sham group (data not shown,  $P > 0.05$ ).

### Neurobehavioral Deficits Evaluation

MCAO causes neurobehavioral deficits that include motor and sensory ones that constitute an adequate model for ischemic stroke. To examine whether DMF treatment affects neurobehavioral deficits induced by MCAO, we evaluated an 18-point modified neurological

severity score (mNSS) comprising tasks for motor ability, alertness, sensory, balance, and general behavior (n=12 rats/group) (Zhou, *et al.*, 2011). Because the sham group did not have neurobehavioral deficits, we did not consider this group for evaluating mNSS.

On 2–3 h after MCAO (day 0), all animals had an average mNSS score of 12-15 that shows MCAO was successful. There was no statistically significant difference in mNSS on day 0 between groups. As shown in figure 1A, the downward line of reduction of mNSS (improvement of neurobehavioral function) was observed in all group rats throughout the treatment period. However, an analysis of variance with repeated measures showed no significant difference between groups. Therefore, we evaluated two more indices include slope and rate of these behavioral changes (Figure 1.B) (Owjfard, Bahaodini, Tamadon, 2017). The slope is the trend of the descending curves of improvement of neurobehavioral function during three days after MCAO, using trend line formula in Excel software. Rate is the subtraction of the mNSS on day three after MCAO (A2) from day 0 (A1). If slope and rate were changed, the difference between control and treated groups would increase by time. According to the slope and rate of mNSS, three days after ischemia-reperfusion injury, rats that received DMF showed a significant decrease in mNSS compared with the vehicle group (Figure 1.C and D).

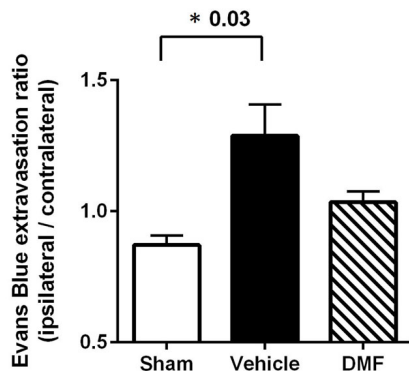


**FIGURE 1** - A, The downward line of mNSS in DMF -treated rats with transient brain ischemia are comparable to those of vehicle-treated rats on days 0, 1, 2 and 3 post-ischemia-reperfusion injury; B, the improvement of neurobehavioral function was evaluated using two indices: slope and rate.; C, D significant neurobehavioral improvement in mNSS scores was evident in DMF treatment as compared to vehicle after three days post-ischemia–reperfusion injury ( $*P<0.05$ ). DMF; dimethyl fumarate, mNSS, modified neurological severity score.

### Assessment of BBB Permeability

BBB protection has been proposed as a promising target for treating ischemic stroke. To evaluate whether the beneficial effect of DMF was mediated by reducing BBB leakage, we determined EB dye extravasation in the ischemic and control hemispheres 24 h after EB injection. EB dye extravasation in the ischemic (right) hemisphere was significantly increased in all MCAO groups when compared with the control (left) hemisphere at 72 h after MCAO (data have not shown). EB extravasation

ratio [right hemisphere EB ( $\mu\text{g/g}$ ) / left hemisphere EB ( $\mu\text{g/g}$ )] in the ipsilateral hemisphere to brain ischemia in the vehicle group ( $1.28 \pm 0.11$ ) increased statistically in comparison with the sham group ( $0.87 \pm 0.03$ ) ( $P<0.05$ ). Besides, rats with MCAO that treated with DMF during the sub-acute phase of cerebral ischemia decreased EB extravasation ratio ( $1.03 \pm 0.04$ ) in comparison with the vehicle group ( $1.28 \pm 0.11$ ), however, this decrease was not statistically significant ( $P>0.05$ , Figure 2). This graph shows that this drug retained BBB integrity in the level of sham group or prevented from its disruption.

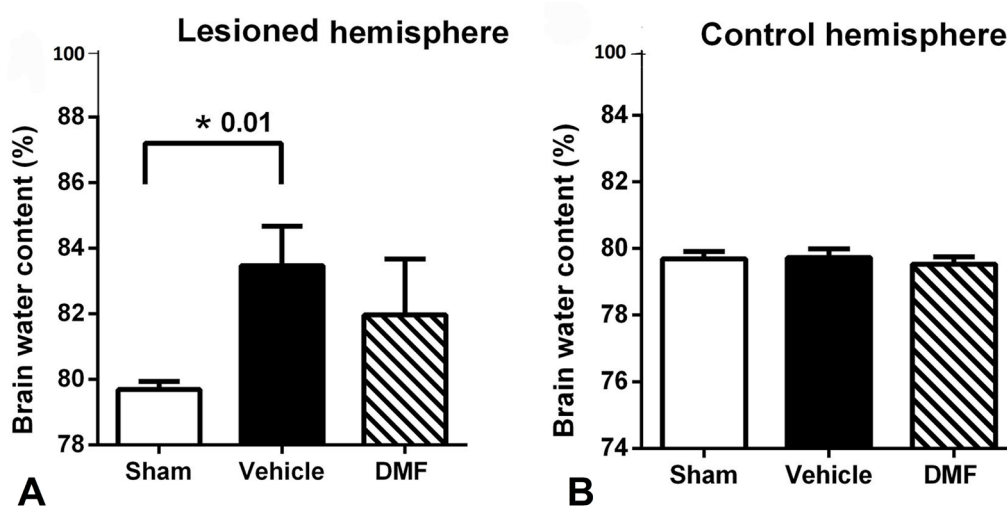


**FIGURE 2** - Alterations in the permeability of the blood-brain barrier in rats three days after middle cerebral artery occlusion (MCAO). Quantitative analysis of Evans blue extravasation ratio (ipsilateral/ contralateral) from rat brain extracts at 72 hours after MCAO (n=5 rats/group). \* $P < 0.05$ .

### Brain water content (BWC)

The evaluation of BWC in the left hemisphere (contralateral to ischemia) showed no significant difference in this parameter between the MCAO-tolerated and sham groups ( $P > 0.05$ , Figure 3B). However, in the right hemisphere, the vehicle group, showed a significant increase in the BWC percentage when compared with

its contralateral hemisphere (3.73%, Figure 3A and B, comparison have not shown) and the sham group (3.78%) ( $P < 0.05$ ). BWC in the right (ischemic) brain hemisphere of the rats with oral administration of DMF ( $81.65 \pm 1.49$  %) reduced as compared with vehicle ( $83.46 \pm 1.2$  %); however, this reduction was not statistically significant ( $P > 0.05$ , Figure 3A).

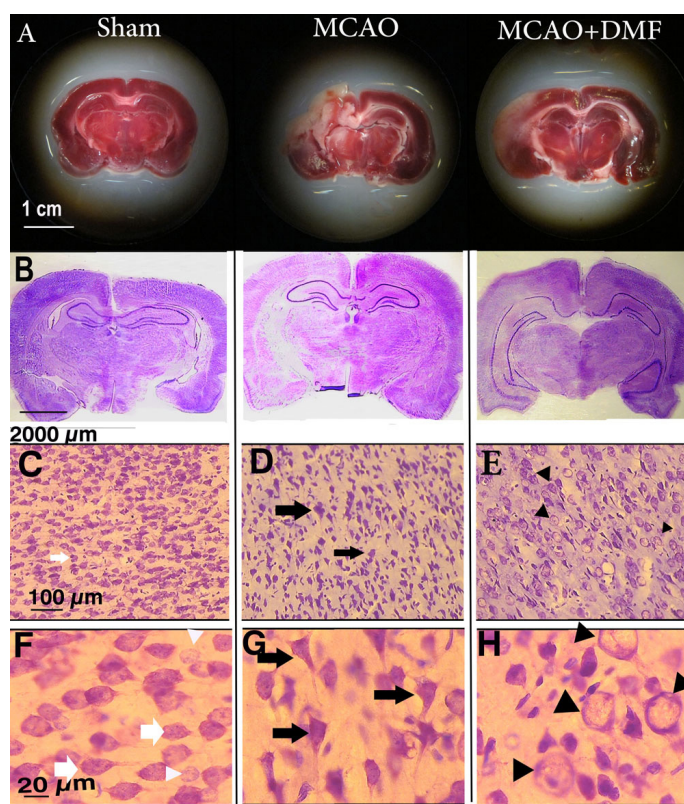


**FIGURE 3** - Percentage of brain water content (BWC) was measured 72 h after MCAO. A, in the right (ischemic) hemisphere and B, left (control) hemisphere by the wet/dry weight method; Data are expressed in percentage as mean  $\pm$  SD (n = 5 per group). \* $P < 0.05$ .

## Histological and Stereological findings

TTC was just used to ensure that brain ischemia was successfully induced and the rats were suffering from ischemic stroke (n=1 randomly in each group). With this staining method, viable tissues stain deep red based on intact mitochondrial function, while infarcts remain white (Figure 4A). The stained brain sections with cresyl violet showed normal area (dark blue to purple staining) in both hemisphere of sham group and the left hemisphere of other groups, but very pale blue or white (ischemic or infarct) area in the right (ischemic) hemisphere including cortex, striatum, and hippocampus in the brain ischemia-induced groups (control and DMF-treated rats). In addition, rats with MCAO that treated with DMF reduced ischemic

area (Figure 4B). Both TTC and cresyl violet stained sections proved brain ischemia in the MCAO groups. In cresyl violet staining of the right cerebral cortex, the sham group showed that neuronal cells are normal looking with obvious nucleolus and euchromatin nucleus and Nissl stained cytoplasm, and dead cells were not observed (Figure 4C and F). Numerous dark neurons with shrunken nuclei and perikaryon that are classified as dying or dead neurons were observed in the cortex of rats, which were subjected to MCAO and treated with vehicle (Figure 4D and G). Treatment with DMF decreased the dead neuron; however, in this group, the neuronal cells that appeared slightly swollen (hypertrophied) were observed and showed that these neurons are in ischemic condition (Figure 4E and H).



**FIGURE 4** - A, a representative TTC-stained cerebral tissue section in normal uninjured (sham), vehicle, and DMF-treated MCAO rats, the normal uninjured area appears in red, while infarct area in the right (ischemic) hemisphere does not stain with this color and appears white. B, In cresyl violet staining, the normal area in both hemispheres appears in dark blue to purple, while the infarct area in the right (ischemic) hemisphere does not stain with this color and appears very pale blue to white. C and F, Representative cresyl violet-stained photograph of the right cerebral cortex in sham surgery group with normal neurons (white arrow) and non-neuron cells (white arrowhead); D and G, Rats that were subjected to MCAO and treated with vehicle showed frequent shrinkage and pyknotic (dead) cells (Black arrow); E and H, DMF treatment group after MCAO show less dead neuron (black arrow); however, hypertrophied neurons appeared (black arrowhead). DMF, Dimethyl fumarate; MCAO, middle cerebral artery occlusion; TTC, 2, 3, 5-triphenyltetrazolium chloride.



The total volume of right and left hemispheres and total brain volume are shown in Table II. Our data indicate that there was no significant difference in the total volume of right and left hemisphere and total brain volume in the sham group compared with vehicle and DMF-treated groups after MCAO (Table II,  $P>0.05$ ). Since there was no sign of infarction in the sham group,

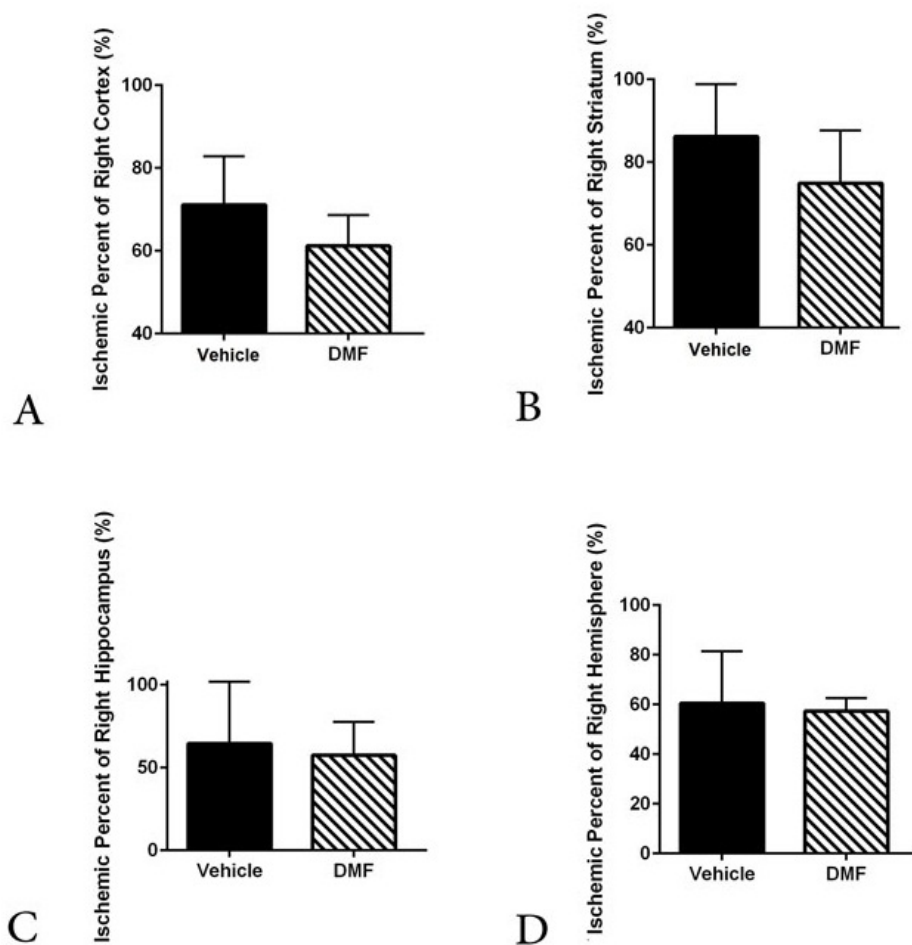
we did not compare this group with the other groups for infarct volume. Table II also shows the infarct volume of the right cortex, striatum, hippocampus, and total hemisphere. Our results show that there was no significant difference in the infarct volume of the right cortex, striatum, hippocampus, and total hemisphere between treated groups after MCAO (Table II,  $P> 0.05$ ).

**TABLE II** - The total and infarct volume (mean  $\pm$  SD) of the different parts of the brain ( $\text{mm}^3$ )

Stereological parameters	Groups		
	Sham	Vehicle	DMF
Right Hemisphere Total Volume	534.41 $\pm$ 51.3	468.03 $\pm$ 50.34	564.4 $\pm$ 44.99
Left Hemisphere Total Volume	527.14 $\pm$ 42.35	505.74 $\pm$ 56.32	537.13 $\pm$ 52.84
Total Brain Volume	1061.55 $\pm$ 93.25	973.78 $\pm$ 94.43	1101.54 $\pm$ 95.37
Right Cortex Infarct Volume	-	164.53 $\pm$ 37.86	171.77 $\pm$ 31.57
Right Striatum Infarct Volume	-	73.85 $\pm$ 25.72	67.61 $\pm$ 15.21
Right Hippocampus Infarct Volume	-	27.11 $\pm$ 22.31	27.26 $\pm$ 15.16
Ischemic Right Hemisphere Infarct Volume	-	285.74 $\pm$ 102.58	325.01 $\pm$ 51.37

The percentage of the ischemic volume was separately calculated for all parts of the right hemisphere, using the following formula:  $[(\text{Infarct Volume})/(\text{total volume})] \times 100$ . By comparing the percentage of ischemia in different regions of the brain, we observed that the highest ischemia percentage occurred in the striatum

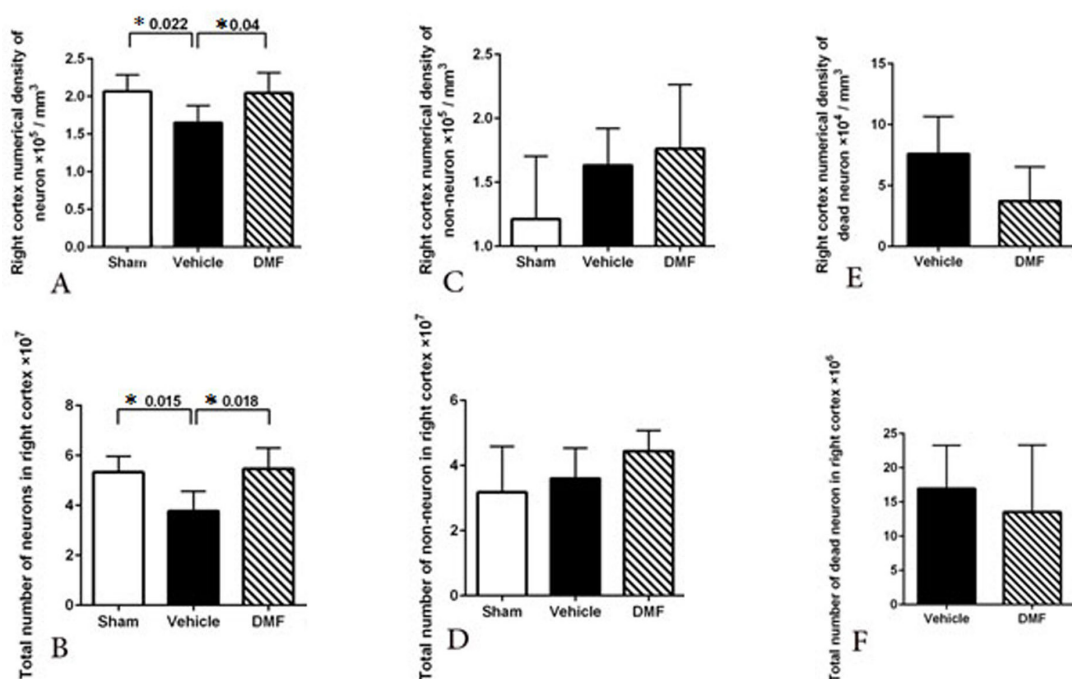
(86.24 $\pm$ 12.6%) while the cortex (71.07 $\pm$ 11.7%), and the hippocampus (64.53 $\pm$  37.4%), showed relatively less ischemia in this MCAO model. The present data also showed that although DMF reduced ischemic volume percentage in all parts of the right hemisphere, this decrease was not statistically significant (Figure 5).



**FIGURE 5** - Ischemic percent (mean  $\pm$  SD) in the right cortex, striatum, hippocampus, and hemisphere in the vehicle and dimethyl fumarate (DMF) - treated rats.

Numerical density and the total number of neurons in the brain right cortex of the vehicle group significantly decreased compared with the sham group (Figure 6A and B,  $P < 0.05$ ). Treatment with DMF significantly increased the numerical density and the total number of neurons in the right cortex in comparison with the vehicle-treated group, and this increase was almost the same as the sham group level ( $P < 0.05$ , Figure 6A and B). Also, present data showed that numerical density and the

total number of non-neurons in the right-cortical brain of all MCAO groups (vehicle and DMF) non-significantly increased compared with the sham group ( $P > 0.05$ , Figure 6C and D). Furthermore, MCAO significantly increased numerical density and the total number of dead neurons in the right-cortical brain (data not shown). Although treatment with DMF decreased these parameters, this decrease was not statistically significant ( $P > 0.05$ , Figure 6E and F).

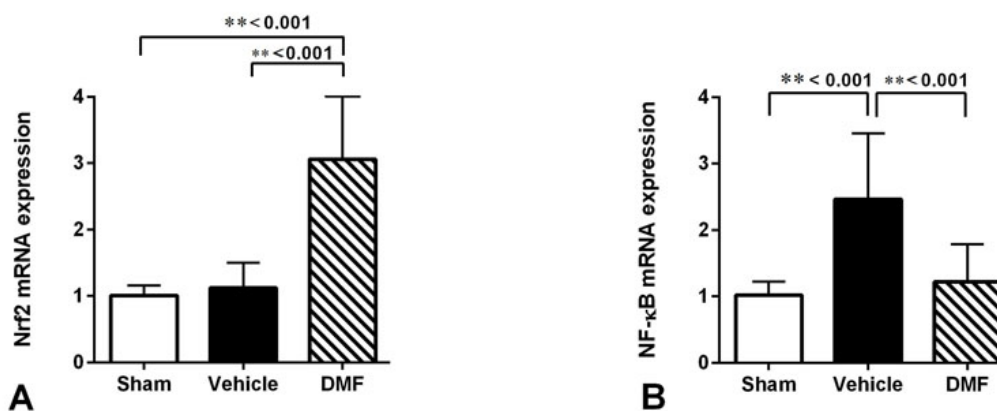


**FIGURE 6** - The numerical density and the total number of neurons, non-neurons, and dead neurons (mean  $\pm$  SD) in the sham, vehicle, and dimethyl fumarate (DMF) –treated groups.  $*P < 0.05$ .

### Expression of Nrf2

Our findings showed that MCAO did not significantly change the Nrf2 expression level in the right cortex.

However, the treatment of brain ischemia with DMF markedly increased Nrf2 levels at day three post-ischemia compared to vehicle-treated and sham groups ( $P < 0.001$ , Figure 7A).



**FIGURE 7** - The expression of Nrf2 (A) and NF- $\kappa$ B (B) in the brain cortex of the right hemisphere 72 h after one h brain ischemia in the sham, vehicle, dimethyl fumarate (DMF) -treated rats (n = 6 rats/group).  $** P < 0.001$ .

## Expression of NF- $\kappa$ B

The expression level of NF- $\kappa$ B in the MCAO vehicle group significantly increased when compared to the sham group. It means that brain ischemia significantly increased NF- $\kappa$ B expression. However, DMF treatment significantly reduced the expression level of this gene in comparison with the vehicle-treated group ( $P < 0.001$ , Figure 7B).

## DISCUSSION

Ischemic stroke causes neurological deficits including motor and sensory ones. Treating brain ischemia during the sub-acute phase with DMF significantly improved neurobehavioral function (Figure 1). These findings are in line with several recent reports that used DMF to treat brain ischemia in mice for 7 days (Yao, *et al.*, 2016), rats for 72 h (Lin, *et al.*, 2016), and also for traumatic brain injury (TBI) in C57Bl/6 mice (Kramer, *et al.*, 2017), and mice (Iniaghe, *et al.*, 2015), and intracerebral hemorrhage (ICH) in rats and C57BL/6 mice (Zhao, *et al.*, 2015).

Stroke can cause BBB disruption and increase its permeability. In the BBB breakdown, there is a massive infiltration of activated immune cells into the brain, causing secondary brain injury and loss of function. Our results indicated that MCAO increased EB extravasation that is in line with previous studies (Fernández-López, *et al.*, 2012). DMF decreased EB extravasation in comparison with the vehicle group, although this decrease was not statistically significant (Figure 2). Our results regarding DMF and BBB permeability is somewhat in line with Benardais *et al.* findings where DMF had no consistent modulatory effect on the expression of tight junction molecules, neither in a human brain microvascular endothelial cell line *in vitro* nor in an experimental autoimmune encephalomyelitis (EAE) model *in vivo* (Benardais, *et al.*, 2013). It is also in line with a recent article by Kramer *et al.* (2017) that showed DMF treatment did not affect TBI-induced BBB leakage. However, these are in contrast to some studies that indicated DMF stabilizes BBB by maintaining endothelial tight junctional barriers in cerebral microvessels, which limit the paracellular influx of blood-borne material

into the brain parenchyma (Chen, *et al.*, 2014; Iniaghe, *et al.*, 2015; Kunze, *et al.*, 2015; Linker, *et al.*, 2011). Other studies have also reported that DMF reduced amount of extravasated EB dye within brain tissue in the ipsilateral hemisphere in a mouse stroke model (Kunze, *et al.*, 2015), murine ICH (Iniaghe, *et al.*, 2015) and mouse models of EAE (Chen, *et al.*, 2014). There might be several explanations for these contrasts, including protocol differences, in particular, the severity of the ischemic injury, the timing or interval between MCAO and sacrificing the animals, dose of DMF, animal species and the timing or interval between EB administration and brain removing (24 h in this study). Although DMF did not significantly affect MCAO-induced BBB leakage, our results show that, at least, it retained BBB integrity in the level of the sham group (Figure 2). It means that this drug probably has partly prevented ischemia-induced BBB disruption.

Our results indicated that MCAO increased brain edema or BWC in the ischemic hemisphere (Figure 3A), which is in line with other studies (Pillai, *et al.*, 2009; Zhao, *et al.*, 2015). In this study, DMF reduced MCAO-induced brain edema in the ipsilateral ischemic hemisphere in comparison with the vehicle group; however, this decrease was not statistically significant (Figure 3A). As figure 3A shows, the treatment of brain ischemia with this drug showed a non-significant difference with the sham group. It means this drug suppressed brain edema resulted from brain ischemia and relatively retained it in almost at the level of the sham group. Our result regarding DMF and BWC is somewhat in line with Kramer *et al.* (2017) findings, which indicated that DMF treatment (80 mg/kg body weight) for three consecutive days did not affect BBB leakage, immune cell invasion and inflammatory marker expression in experimental traumatic brain injury (Kramer, *et al.*, 2017). Furthermore, as Pillai *et al.* previously have indicated, BBB permeability following I/R injury has biphasic nature. The first phase of differential permeability characteristics at the BBB lasting up to 24 hours led to progressive edema, whereas the second phase (after 24 h, similar to the present study) did not contribute to edema formation and might facilitate edema decrease (Pillai, *et al.*, 2009). However, some studies indicated



DMF reduces cerebral edema formation. For example, Kunze *et al.* (2015) used DMF as a pretreatment for brain ischemia in mouse and Zhao *et al.* for treatment of ICH (Zhao, *et al.*, 2015) reported its effectiveness in the inhibition of cerebral edema. Although their dose and duration of treatment were similar to ours, they used DMF as a pretreatment and in mice (Kunze, *et al.*, 2015) or in the ICH model of stroke (Zhao, *et al.*, 2015) that are different from our study. Besides, Lin *et al.* reported that DMF (25 or 50 mg/kg for 14 days) treatment significantly reduced brain inflammation by suppressing multiple pro-inflammatory cytokines *in vivo* stroke models (Lin, *et al.*, 2016). Iniaqhe *et al.* (2015) reported that the decrease in the BWC or edema depends on the DMF dosage. They concluded that treatment with a high dose of DMF (100 mg/kg) significantly reduced BWC in the ipsilateral basal ganglia and cortex compared to vehicle-treated groups while its low dose (10 mg/kg) did not produce a significant reduction in BWC. The present and other studies regarding the effectiveness of DMF on the brain edema depends on different factors, such as dose of DMF, type of disorder, duration of treatment, and animal species.

The present findings regarding the comparison of right and left hemisphere volume based on Cavalieri's principle approves our BWC data. MCAO increased BWC and the mean total volume in the ipsilateral ischemic hemisphere when compared with contralateral one. However, there was no significant difference between the total volume of each hemisphere or the whole brain in different groups (Table II,  $P > 0.05$ ).

Under the present computer-assisted stereology analysis, which is unbiased, we observed that the highest ischemia percentage occurred in the striatum (Figure 5). These observations might support findings that cerebral blood flow (CBF) in cortical branches returns to baseline values within 120 minutes of reperfusion, whereas CBF values in the striatum remain depressed (Takagi, *et al.*, 1995). DMF treatment of MCAO reduced infarct volume in the right hippocampus, cortex, striatum, and hemisphere compared with vehicle treatment; however, this decrease was not statistically significant (Figure 5).

Regarding DMF effect on the size of brain ischemia, our findings are in agreement with Kunze *et al.* (2015)

results that showed oral administration of DMF for three consecutive days did not affect the infarct size after cerebral ischemia in male C57BL/6 mice. Yao *et al.* also estimated infarct volume with TTC staining and MRI, and reported that one-day treatment with DMF did not show a significant decrease in the infarct volume. However, the treatment of more than three days significantly decreased this parameter in mice. They also observed that a higher dose of DMF has a greater effect on the infarct volume reduction (Yao, *et al.*, 2016). In contrast to our data, Safari *et al.* (2017) used DMF for 14 days and Yao *et al.* (2016) and found that this drug significantly reduced the size of infarction. Furthermore, it was also reported that DMF treatment significantly reduced TBI-induced lesion volume in the ipsilateral ischemic hemisphere (Kramer, *et al.*, 2017). It seems that treatment with DMF is mostly dependent not only on the dose but also on the duration of treatment. Most studies that used more than three days DMF treatment, have shown reduced ischemic volume (Safari, *et al.*, 2017; Yao, *et al.*, 2016); whereas less than three days was not effective in reducing the infarct volume (Kunze, *et al.*, 2015; Yao, *et al.*, 2016).

Ischemic-induced neuronal death is an important factor to be considered as neurological deficiencies. Recent animal experiments have shown the involvement of apoptosis in neurodegeneration after ischemic brain injury. Necrosis is thought to occur in the ischemic core, whereas apoptotic like cell death is unmasked in the surrounding penumbra (Lipton, 1999). On the other hand, some evidence for functional neuronal replacement was reported in cerebral ischemia. Endogenous neurogenesis can occur in response to ischemic lesions of the brain, which might help to ameliorate neurological deficits (Shin, *et al.*, 2008). In this study, we showed that the brain ischemia significantly reduced both the numerical density and the total number of neurons in the ischemic hemisphere cortex in comparison with the sham group. It means that this disorder causes neuron loss in the cortex. However, treatment with DMF significantly increased the numerical density, and the total number of neurons in the right cortex (Figure 6A and B). Estimating the total number of dead neurons in the right cortex proved cell loss in the MCAO-induced brain ischemia (Figure 6E

and F). Moreover, although DMF decreased cell death, this decrease was not significant when compared with the control group (Figure 6E and F). In this study, necrosis or apoptosis was not separately assessed by specific methods such as TUNEL, and cell death were only estimated based on cresyl violet staining. To the best of our knowledge, there is no stereological study regarding the numerical density and the total number of neurons in this area of the brain in this model. The present study regarding cell death in the brain ischemia is in agreement with Kunze, *et al.* (2015) and Yao *et al.* results. They detected apoptotic cells in the ipsilateral ischemic hemisphere after 24 hours reperfusion by TUNEL-assay and reported that DMF decreased apoptotic neurons in the infarcted area of mice (Kunze, *et al.*, 2015; Yao, *et al.*, 2016). Increasing the numerical density and the total number of neurons in the cortex by DMF can be attributed to both decreasing cell death and increasing neurogenesis. Since the time for neurogenesis effect might not be enough in the present study (a three-day course) and also we did not directly evaluate neurogenesis, we propose that DMF has probably exerted its actions through preventing cell death. Nonetheless, neurogenesis studies regarding this drug is recommended in future studies.

In the present study, we evaluated the numerical density and the total number of non-neuronal cells by unbiased stereological methods for the first time. The non-neuron cells in the brain consist of both glial cells and endothelial cells. The endothelial cells are comprised of about 25% of non-neuronal cells (von Bartheld, Bahney, Herculano-Houzel, 2016). However, we did not use specific methods to detect glial cells, and we used non-neuronal terminology instead of glial cells. The present study indicated that ischemic stroke, despite the decreasing number of neurons, increased the number of non-neurons in the ischemic brain cortex (Figure 6C and D) which somewhat shows gliosis and inflammation after stroke. Our results are in line with Yao *et al.* who showed that brain tissue sections obtained from mice 72 h after transient MCAO increased astrocytosis and microgliosis (Yao, *et al.*, 2016). Other studies have also indicated that gliosis, is strongly stimulated in the infarct area of the ischemic brain, and studies imply that gliosis acts as inhibitors of axonal regeneration after cerebral

ischemia injury (negative effects) (McKeon, Hoke, Silver, 1995). Furthermore, Aldskogius and Kozlova (1998) proposed that increase in the total numbers of microglia/macrophages in these damaged regions is necessary to remove cellular debris of the dying cells after stroke (positive effects) (Aldskogius, Kozlova, 1998). DMF non-significantly increased non-neuronal numbers when compared with the control group. Based on our stereological study, it seems that this drug might have their effects mostly through the change in neuron and not glial cells, although proving this suggestion requires more investigation.

Nrf2 is a major regulator of the anti-oxidative defense responses, balancing between antioxidant defenses and ROS/RNS (Kensler, Wakabayashi, Biswal, 2007). Activation of the Nrf2-ARE pathway is a potential therapeutic approach in various neurodegenerative disorders (Iniaghe, *et al.*, 2015). Our findings, in contrast to Safari *et al.* (2019) and Shi *et al.* (2015) results showed that MCAO did not significantly change Nrf2 expression (Figure 7A). It appears that the effects of MCAO on Nrf2 activity might be dependent on brain injury severity, time course, and region of the brain that evaluates the Nrf2 levels following MCAO. However, our result regarding DMF is in line with previous studies which suggest that DMF can activate the Nrf2 transcriptional pathway, reducing oxidative stress, inflammation and neuronal demyelination in a mouse MCAO model (Kunze, *et al.*, 2015; Yao, *et al.*, 2016), ICH animal models (Iniaghe, *et al.*, 2015; Zhao, *et al.*, 2015), and rat MCAO model (Lin, *et al.*, 2016).

It is reported that NF- $\kappa$ B may have a contributing role in neurodegeneration in most experiments of cerebral ischemia (Ridder, Schwaninger, 2009). In line with previous studies that showed NF- $\kappa$ B was over-expressed following cerebral ischemia (Raza, *et al.*, 2013), we indicated that MCAO significantly increased NF- $\kappa$ B levels. However, DMF treatment significantly decreased the expression of this gene (Figure 7B). Our result is in agreement with previous investigations that reported DMF inhibits NF- $\kappa$ B signaling (Gillard, *et al.*, 2015). In conclusion, brain ischemia treatment in the sub-acute phase with DMF, although did not have a significant effect on brain ischemia size, but it retained BBB integrity

and cerebral edema in almost its normal condition. Furthermore, it significantly improved neurobehavioral function, increased the number of neurons, and decreased the number of dead neurons in the brain cortex of the ischemic hemisphere. These effects could be most likely attributed to the activation of the Nrf2 pathway and down-regulation of NF- $\kappa$ B pathway of this drug.

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