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Neuro-protective effect of monomethyl fumarate on ischemia reperfusion injury in rats: Role of Nrf2/HO1 pathway in peri-infarct region

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ABSTRACT

Post stroke recanalization has been associated with increased risk of oxidative stress. Stimulating endogenous antioxidant pathway by activation of nuclear factor erythroid-2-related factor-2 (Nrf2) plays a key role in neuronal defense against inflammation and oxidative stress in penumbra. Here, we explored whether monomethyl fumarate (MMF) could produce neuro-protection after ischemia/reperfusion (I/R) injury via Nrf2/HO1 activation. In male SD rats, middle cerebral artery was occluded for 90 min and confirmed using Laser Doppler flowmeter. MMF (10, 20 and 40 mg/kg) was administered in two divided doses at 30 min post ischemia and 5–10 min after reperfusion. After 24 h, effect on neurobehavioral parameters, infarct damage by TTC staining and MRI, oxidative stress and inflammatory cytokines were assessed. Expression studies of nuclear Nrf2 and cytoplasmic HO1 were performed in peri-infarct cortex and striatum; followed by dual immunofluorescence study to check the specific cell type. I/R induced neurobehavioral deficits and infarct damage were significantly ($p < 0.05$) attenuated by MMF (20 and 40 mg/kg). MMF, 20 mg/kg, significantly normalized I/R induced altered redox status and increased levels of TNF-α, IL-1β in the ipsilateral cortex. MRI data showed significantly reduced infarct in cortex but not in striatum after MMF treatment. Expression of nuclear Nrf2 and cytoplasmic HO1 were significantly ($p < 0.05$) increased in peri-infarct cortex after treatment with MMF. Additionally, dual immunofluorescence showed increased Nrf2 expression in neurons and HO1 expression in neurons as well as astrocytes in peri-infarct cortex after MMF treatment. Our results show the neuro-protective potential of MMF probably by restricting the progression of damage from striatum to cortex through activation of Nrf2/HO1 pathway in peri-infarct cortex.

1. Introduction

In recent years, remarkable advances in the pathophysiology of stroke have been made. Yet, restoring cerebral blood flow by thrombolysis using tissue plasminogen activator (tPA) remains to be the only currently available treatment. Further, tPA benefits are restricted only to 2–5% of stroke patients because of narrow therapeutic window, risk of hemorrhage and late hospitalization (Deng et al. 2006; Miller et al., 2011) making stroke the third leading cause of death (Christopher and Murray, 2017) and the second leading cause of disability adjusted life years (DALY) lost, worldwide (Kassebaum et al., 2016). There is an evidence of decreased stroke related mortality in last two decades. However, incidence and DALY lost have been increasing with most of the burden in low to middle income countries (Krishnamurthi et al., 2013). Notably, reperfusion after thrombolysis using tPA or by mechanical means, can result in harmful consequences as a result of overproduction of reactive oxygen species (ROS) (Jung et al. 2010; Li et al., 2012; Peters et al., 1998). Therefore, finding an effective neuroprotective agent still remains to be a strongly sought after goal.

Cerebral stroke initiates a complex and multifactorial pathophysiological mechanism collectively known as ischemic cascade. Accumulating evidences suggest oxidative stress and neuro-inflammation as pivotal mediators of post stroke injury (Chan, 1994; Lakhan et al., 2009). Transient middle cerebral artery occlusion (MCAo) is a well-accepted model to study neuro-protection due to possibility of reperfusion as seen clinically after tPA or mechanical clot removal. Although, many exogenous antioxidants therapy have shown promising results in preclinical studies, but except edaravone (Lapchak, 2010), none have succeeded in clinical settings. The probable reasons for their failure may, in part, be their inability to cross blood brain barrier,

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shorter therapeutic time window, instability, dose and time of administration (Gilgun-Sherki et al., 2002). Hence, targeting oxidative stress and inflammation via induction of endogenous enzymatic antioxidants using alternate therapeutic approach such as activation of nuclear factor erythroid-2-related factor 2 (Nrf2) could play a vital role in cellular defense in ischemic penumbra.

The Nrf2 signaling pathway is a master regulator of endogenous antioxidant defense and acts as a potential therapeutic target for protection in ischemic event. Nrf2 activation leads to transcription of phase II genes involved in detoxification of xenobiotics, free radical scavenging and maintenance of redox potential. Many lines of evidence have demonstrated that activation of Nrf2 and heme oxygenase (HO1) pathway protects the brain against I/R injury (Alfieri et al., 2011; Shah et al., 2014; Yang et al., 2009; Zhang et al., 2014).

Monomethyl fumarate is a pharmacologically active metabolite of dimethyl fumarate (DMF), belonging to the family of fumaric acid esters (Litjens et al., 2004). Fumaric acid esters have been used for the treatment of psoriasis since a long time (Mrowietz et al., 1999). Recently, DMF has been approved by US-FDA for the treatment of relapsing remitting form of multiple sclerosis (Gold et al., 2012). Previous studies have shown DMF produced neuro-protection in multiple sclerosis (Linker et al., 2011) and STZ induced sporadic model of Alzheimer's disease (Majkutewicz et al., 2016). DMF has also been reported to reduce cerebral edema (Kunze et al., 2015) and neuro-inflammation (Wilms et al., 2010) by attenuating oxidative stress and inflammation. MMF has been reported to produce neuro-protection in retinal ischemia reperfusion injury (Cho et al., 2015) and post ischemic recovery in mice via reducing apoptosis and astrocytes activation (Yao et al., 2016). However, these studies did not reveal the effect of MMF intervention during acute phase of ischemic stroke injury and its effect on Nrf2/HO1 expression in specific cell type after I/R injury. Hence, the present study investigates the potential protective effect of MMF acute treatment in experimental model of ischemic stroke and the probable mechanism of neuro-protection.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats, weighing 270 ± 20 g, were procured from Central Animal Facility, All India Institute of Medical Sciences, New Delhi, India. Prior ethical approval was obtained from the Institutional Animal Ethics Committee (IAEC No. 819/IAEC/14). Rats were acclimatized, fed standard dry pellet diet; food and water were available *ad libitum*. Animals were randomly assigned into each experimental group. All experimental protocols were performed in compliance with the National Institute of Health (NIH) Guidelines for the Care and Use of the Laboratory Animals (NIH Publication no. 85 723, revised 1996). All efforts were made to minimize animal suffering and to reduce the number of animals used. Observers blinded to the experimental design obtained the data.

In the first study (dose selection study), rats were randomly divided into 5 groups: sham (all surgical procedures except insertion of filament), MCAo group (occlusion of 90 min followed by reperfusion) and 3 groups of MCAo + MMF treatment at three doses of 10 mg/kg, 20 mg/kg and 40 mg/kg administered at 30 min post ischemia and 5–10 min after reperfusion.

The second animal study elucidated the effect of MMF 20 mg/kg (the selected dose) on oxidative stress parameters and the levels of inflammatory cytokines using sham, MCAo and MCAo + MMF groups.

In the third animal study, magnetic resonance images (MRI) were acquired followed by effect of MMF on protein expression of Nrf2 and HO1 using western blot and immunofluorescence using sham, MCAo and MCAo + MMF groups.

2.2. Experimental stroke model

Focal cerebral ischemia was induced by occluding the middle cerebral artery using the intraluminal technique in the rats with some modifications (Chauhan et al., 2011; Longa et al., 1989). Briefly, post acclimatization, rats were anaesthetized by chloral hydrate (300 mg/kg i.p.). Regional cerebral blood flow (rCBF) was determined by Laser Doppler Flow meter (Biopac, LDF-100C, USA). CBF was measured before MCAo surgery as baseline, after MCAo to confirm the occlusion and post reperfusion to confirm recirculation. Briefly, in the scalp, a midline sagittal incision was made to expose bregma and 1 mm fiber optics probe was fixed over MCA territory (2 mm posterior and 5 mm lateral to bregma using stereotactic frame). Signals of laser Doppler were recorded as microvascular blood perfusion units (BPU) using AcqKnowledge 4.0 software. The rCBF data was expressed as percentage of the baseline value. After baseline blood flow estimation, animals were placed over operating table with thermostatically regulated heating pad for maintaining body temperature to 36–37 °C throughout the surgery and using blower till recovery. A midline incision was made on the ventral aspect of the neck and the left common carotid artery was exposed. Briefly, muscles of neck were separated further to expose internal carotid artery and external carotid artery. Through a small nick into external carotid artery (ECA), a 3 cm long 3–0 nylon monofilament suture (Ethicon, Johnsons & Johnsons Ltd., Mumbai, India) was introduced and gently advanced from ECA to the internal carotid artery lumen until resistance was felt once the filament went pass origin of middle cerebral artery. The filament was gently removed after 90 min of ischemia to allow reperfusion. Ischemia and reperfusion were confirmed by measuring cerebral blood flow. In sham-operated animals, all surgical procedures were carried out except the insertion of the filament.

2.3. MMF treatment

MMF (Sigma Aldrich, USA) was freshly dissolved in normal saline. Rats were administered MMF in the doses of 10, 20 and 40 mg/kg in two divided doses. First dose of MMF was administered after 30 min of ischemia followed by second dose at 5–10 min post reperfusion through intra peritoneal route (Schematic diagram Fig. 1). Sham and MCAo group rats were administered with same volume of the saline.

2.4. Assessment of neurobehavioral parameters

2.4.1. Neurological deficit score (NDS)

NDS was assessed by an examiner blinded to the experimental groups using the method described earlier (Longa et al., 1989). The neurological deficits were assessed after 24 h of MCAo and scored on 5 points scale - a score of 0 indicated no neurological deficits; a score of 1 if the rat failed to extend contralateral forepaw fully indicating mild focal neurological deficit; a score of 2 if the rat circled to the contralateral side indicating moderate neurological deficit; a score of 3 if the rat falls to the contralateral side indicating a severe focal deficit and finally, a score of 4 if the rats did not walk spontaneously indicating a depressed level of consciousness.

2.4.2. Assessment for motor coordination

Effect on motor coordination was assessed using rota rod (Ugo Basile, Italy) as described earlier (Rogers et al., 1997).

2.5. Assessment of brain infarct by 2,3,5- triphenyltetrazolium chloride (TTC)

After neurological examinations, deeply anesthetized rats were euthanized, perfused transcardially with normal saline and brains were isolated. 2 mm thick coronal sections from frozen brain were incubated with TTC (1% TTC in 10 mM PBS) at 37 °C for 15 min followed by

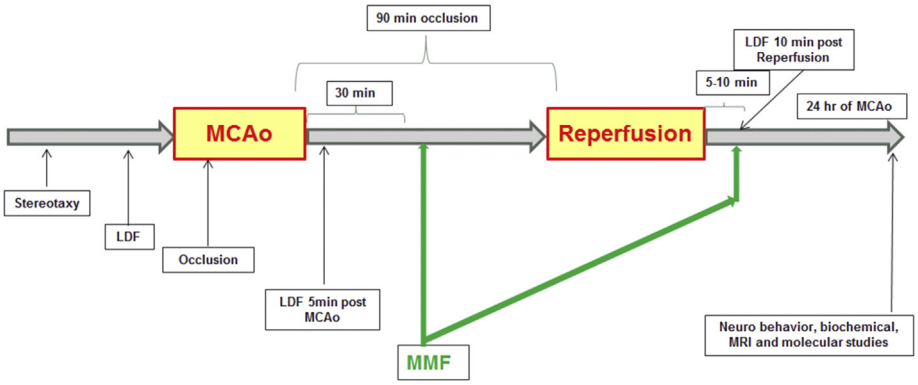

Fig. 1. Schematic plan of work showing experimental protocols including sequence of events, time of dosing, behavioral, biochemical and molecular studies.

fixation in 10% formaldehyde solution in PBS (Bederson et al., 1986). The unstained tissue representing infarct damage was calculated as % of ipsilateral hemisphere using computerized image-J analysis system.

2.6. Tissue preparation

After 24 h of MCAo, the animals were euthanized and the brains were quickly perfused with normal saline followed by ice cold PBS. Brains were quickly removed and kept over ice cold glass plate. Cerebral cortex and striatum were separated (Spijker, 2011) and stored at −80 °C. Cerebral cortex and striatum samples were thawed, homogenized in 10% (w/v) ice–cold 0.1 M phosphate buffer (pH 7.4). Supernatant was collected for estimation of markers of oxidative stress and inflammatory cytokines. Protein estimation was done using Bradford's assay. For western blot, peri-infarct region of cortex was selected based on TTC staining of the previous 2 mm section (Fig. 5A). Cytoplasmic and nuclear fractionation from peri-infarct cortex and whole striatum was done using NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, UK) as per the manufacturer's protocol. Cytoplasmic fraction was used to study HO1 expression whereas nuclear fraction was used to study Nrf2 expression.

2.7. Estimation of oxidative stress parameters

2.7.1. Estimation of brain glutathione levels

The reduced glutathione (GSH) levels were measured using method described by Ellman (1959).

2.7.2. Estimation of brain malondialdehyde levels

The levels of lipid peroxidation marker, malondialdehyde (MDA) was estimated as described previously (Ohkawa et al., 1979).

2.7.3. Estimation of brain superoxide dismutase levels

The levels of superoxide dismutase (SOD) was estimated using the method described by Marklund and Marklund (1974).

2.7.4. Estimation of brain nitric oxide levels

Nitric oxide levels were estimated using the method described earlier (Green et al., 1982).

2.8. Estimation of inflammatory cytokines in cortex and striatum

The levels of inflammatory cytokines like TNF-α, IL-10 and IL-1β were quantitatively determined in cortex and striatum using ELISA kit (Diaclone, France) according to manufacturer's protocol.

2.9. Assessment of brain damage by apparent diffusion coefficient (ADC) value, signal intensity and infarct damage using MRI

MRI studies were carried out after 24 h of MCAo using a 7.0 T small animal scanner (Bruker, Biospin MRI GmbH Biospec 70/20 US). 72 mm transmit and receive coil (112/72 mm, outer/inner diameter) was used. The animals were anaesthetized with chloral hydrate and placed in supine position in animal holder with headlock to avoid any movement. Body temperature was maintained using water bath. Cerebral infarct was identified by acquiring T-2 weighted images indicating vasogenic edema using rapid acquisition and rapid enhancement sequence with parameters: slice thickness = 1 mm, slices = 14, echo time (TE) = 33 ms, repetition time (TR) = 2500 ms, averages = 2, matrix size = 256 × 256 mm, field of view = 35 × 35 mm. Diffusion weighted images (DWI) representing cytotoxic edema were acquired using following parameters; TR/TE = 3000/32.5 ms, slices = 14, slice thickness = 1 mm, average = 2, matrix size = 96 × 96 mm and six b values as 0, 200, 400, 600, 1000, and 1500 s/mm^2.

For image analysis, Paravision 6.0 software was used and contrast of T2 images was adjusted using the grey scale. A blinded observer selected region of interest (ROI) and the infarct area was calculated for each slice. This was reported as percentage of ipsilateral area calculated by dividing infarcted area by total area of ipsilateral hemisphere multiplied by 100. Signal intensity was calculated and presented as ratio of infarct area and contralateral area using DWI. Four different circular regions of interest (0.02 cm^2) were selected to calculate ADC value in ipsilateral and contralateral hemispheres. ADC ratios were presented as ratio of ADC value of ipsilateral/contralateral hemisphere. Data was analyzed by an observer blinded to different groups (Chauhan et al., 2011; Gupta et al., 2017).

2.10. Expression of Nrf2/HO1 by western blot

Cytoplasmic and nuclear fractions were separated and Bradford's assay was used to determine protein. A denatured aliquot containing equivalent amount of protein was subjected to SDS-polyacrylamide gel electrophoresis. Consecutively, resolved proteins were transferred onto a nitrocellulose membrane which was blocked in nonfat milk for 1 h and incubated overnight dipped in primary antibody against rabbit polyclonal anti-Nrf2 (1: 1000; Abcam Plc, UK), rabbit polyclonal anti-HO1 (1: 1000; Abcam Plc, UK), rabbit polyclonal anti-beta actin (1: 4000; Abcam Plc, UK) and rabbit polyclonal anti-Histone H3 (1: 4000; Abcam Plc, UK). Secondary antibody goat-anti-rabbit IgG H&L HRP (1: 4000; Abcam Plc, UK) was used for 2 h. Finally, membrane was washed and visualized using enhanced chemiluminescence reagent (Luminata Forte; Millipore, Germany) using gel documentation system (FluorChem E; Cell Biosciences, USA). The optical densities of bands were analyzed using image-J software (NIH).

2.11. Dual immunofluorescence staining

After 24 h of occlusion, the rats were euthanized; brains were first perfused transcardially with normal saline followed by perfuse fixation with 4% ice cold paraformaldehyde. The brains were fixed further in 4% paraformaldehyde for 24 h followed by cryo-protection by keeping in sucrose gradients. Thereafter, OCT blocks were prepared and 10 μm thick coronal sections were taken using cryotome.

The sections were taken on poly-l-lysine coated slides, washed, incubated with 10% normal goat serum for blocking and permeabilized with 0.2% Triton-X100 in PBS. The sections were incubated overnight with primary antibody for Nrf2 (1:100, rabbit polyclonal anti-Nrf2, Abcam Plc) or for 2 h with primary antibody to HO1 (1:200, rabbit polyclonal anti-HO1). Sections were incubated with goat anti-rabbit IgG H&L (Alexa Flour 488, FITC, Abcam Plc) for 1 h. Thereafter, the sections were again washed and incubated with second primary antibody against NeuN (1:500; mouse monoclonal anti-NeuN) or GFAP (1:500; mouse monoclonal anti-GFAP) for 2 h. Slides were washed and incubated with the corresponding secondary antibody, goat anti-mouse IgG H&L (Phycoerythrin), for 1 h. Finally, the sections were counterstained with DAPI and mounted. Immunofluorescent images were acquired from peri-infarct cortex (Fig. 6C) using Nikon-Ti microscope with the help of NIS-Element software. For analysis, four sections from each rat brain were analyzed by counting immune-positive cells in 3–4 fields of peri-infarct region of cortex. Cell counting was performed at x200 magnification image using Image-J (NIH, USA) software. Results were presented as percentage of dual immunofluorescence positive to NeuN or GFAP positive cells per field. During analysis, the investigator was blinded to the groups.

2.12. Statistical analysis

Experimental data presented as mean ± SEM and p value < 0.05 was considered statistically significant. Statistical tests were performed using GraphPad Prism-5 software. One way analysis of variance followed by Bonferroni post-hoc test for multiple comparisons between the groups was applied. Unpaired *t*-test was utilized for comparing infarct volume of cortex and striatum of MCAo and MMF group in MRI. Finally, non-parametric Kruskal-Wallis followed by Dunn's multiple comparison with Bonferroni's correction was applied to compare groups for NDS and data presented as median (range).

3. Results

3.1. General observation

A total of 96 SD rats were included in the present study, out of which 76 rats were analyzed for various parameters, 5 rats were excluded and 15 rats died. No mortality was observed in sham group. Exclusion criteria included occlusion less than 70% of baseline (2 rats), operation time more than 30 min (1 rat), excessive bleeding during surgery (1 rat) and subarachnoid hemorrhage (1 rat). The survival rate of MCAo rats after exclusion included: MCAo control group 21/29 (72.4%); MMF 10 mg/kg 7/9 (77.7%); MMF 20 mg/kg 21/25 (84%) and MMF 40 mg/kg 7/8 (87.5%).

3.2. Change in cerebral blood flow

Baseline cerebral blood flow measured before MCAo surgery was considered as 100%. Overall, after MCAo, more than 80% reduction in BPU (of baseline) was achieved. However, after removal of filament i.e. post reperfusion, more than 70% blood flow (of baseline) was found to be restored (Fig. 2A). Hence, changes in cerebral blood flow showed a remarkable occlusion and reperfusion with no significant intergroup differences (supplementary data, Table S1).

3.3. Neurobehavioral parameters

3.3.1. Effect of MMF on motor coordination

There was a significant ($p < 0.001$) decrease in time spent on rotarod in MCAo group (26.7 ± 6.4 s) when compared with sham group indicating a severe motor incoordination as a result of I/R injury. Treatment with MMF at 10 mg/kg (42 ± 6.5 s) did not produce significant improvement in motor incoordination compared to MCAo group (Fig. 2B). However, in MMF 20 mg/kg (64.5 ± 7.1 s, $p < 0.05$) and 40 mg/kg (72 ± 9.3 s, $p < 0.01$) groups, rats spent significantly more time on rotating spindle than MCAo groups, indicating improvement in motor performance with MMF treatment after ischemia. Time spent on rotarod did not differ significantly between MMF 20 mg/kg and 40 mg/kg.

3.3.2. Effect of MMF on neurological deficit score

Sham rats did not show any neurological deficits. Non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison with Bonferroni correction revealed significantly increased NDS in MCAo group [4 (2–4), $p < 0.001$] rats as compared to sham indicating I/R induced severe neurological damage (Fig. 2C). Treatment with MMF significantly reduced the NDS in 20 mg/kg [2 (2–3), $p < 0.05$] and 40 mg/kg [2 (1–3), $p < 0.01$] when compared with MCAo group. However, treatment with MMF 10 mg/kg [3 (2–4), $p > 0.05$] did not reduce NDS when compared with MCAo group.

3.4. Effect of MMF on infarct damage by TTC

Sham group did not show any infarcted region; while there was extensive infarct (39.4 ± 2.7% of ipsilateral area, $p < 0.001$) involving striatum and cortex in MCAo group (Fig. 2E). One way ANOVA followed by Bonferroni post hoc analysis revealed that in MMF treatment groups, the infarct was significantly ($p < 0.05$) less at the dose of 20 mg/kg (26.1 ± 4%) and 40 mg/kg (24.9 ± 2.1%) when compared with MCAo group. Treatment with MMF 10 mg/kg (34.1 ± 4%) did not show significant reduction in infarct when compared with MCAo group.

As evidenced by the results of TTC staining and neurobehavioral parameters, MMF 20 and 40 mg/kg produced significant improvement when compared to MCAo. Since, there was no significant difference in the effects produced by 20 and 40 mg/kg doses, 20 mg/kg was selected for further studies on oxidative stress parameters, inflammatory cytokines, and MRI followed by protein expression study using western blot and immunofluorescence.

3.5. Effect of MMF on oxidative stress parameters

One way ANOVA followed by Bonferroni post hoc test revealed the levels of GSH in MCAo group to be significantly ($p < 0.001$) lower when compared with sham group in both cortex and striatum of ipsilateral hemisphere (Table 1). However, treatment with MMF significantly increased MCAo induced decrease in the levels of GSH in both cortex ($p < 0.01$) and striatum ($p < 0.05$) when compared with MCAo group.

Similarly, levels of superoxide dismutase were significantly ($p < 0.01$) decreased in cortex and striatum ($p < 0.001$) by I/R in MCAo group when compared with sham group. Treatment with MMF significantly ($p < 0.05$) improved I/R induced decrease in SOD levels in cortex when compared with MCAo; however the effects were not statistically significant in striatum.

I/R injury caused significant ($p < 0.01$) increase in the levels of malondialdehyde and the levels of nitrite, a marker for nitric oxide in cortex and striatum of MCAo group when compared with sham group. Treatment with MMF brought the levels of MDA and NO significantly ($p < 0.05$) towards normal in cortex when compared with MCAo group. However, in the striatum increased levels of MDA and NO were

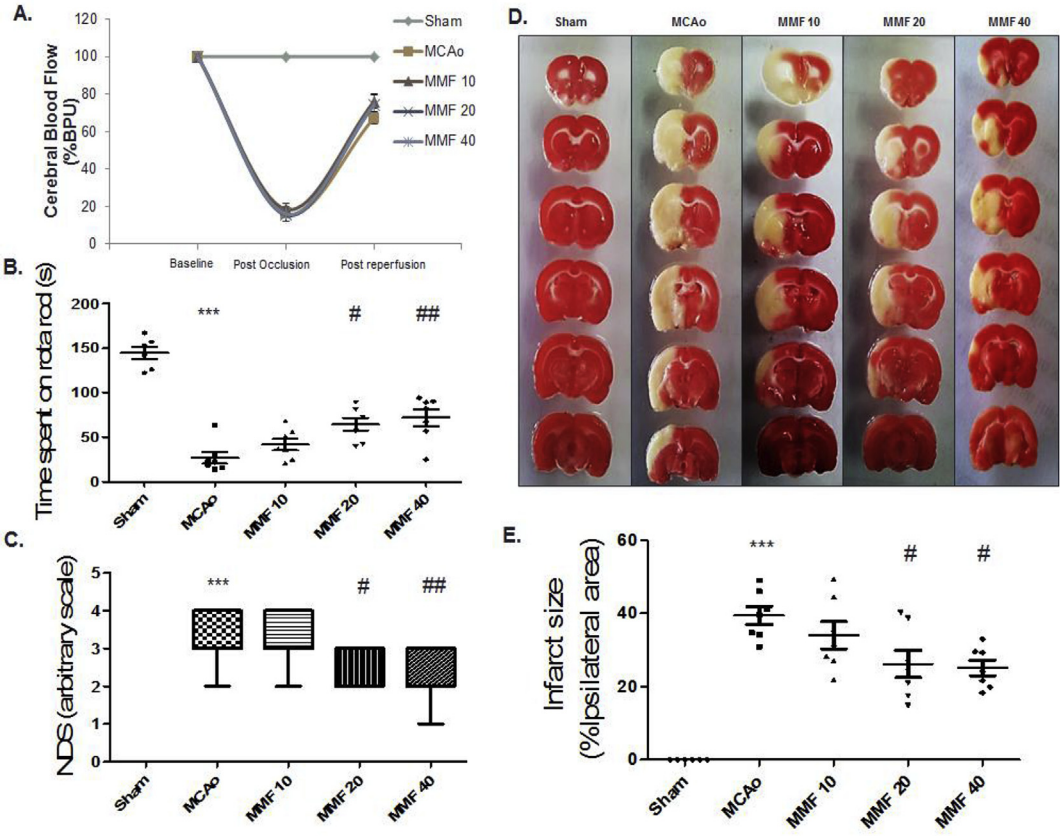

Fig. 2. Effect of MMF treatment at different doses on ischemic damage and selection of effective dose. (A) Change in cerebral blood flow after occlusion and after reperfusion as measured by laser Doppler flow meter; (B) Effect of MMF on motor coordination; (C) Effect of MMF on neurological deficit score. (D) Representative TTC stained sections of different groups indicating viable tissue as red colored and damaged tissue as unstained; (E) Effect of MMF on infarct size represented as % of ipsilateral area (n = 6–7). Each point represents the raw data of each rat, horizontal line represents mean and error bars represent standard error of mean. For NDS, data presented as median (range) using box and whiskers plot. MMF 10 - MMF 10 mg/kg, MMF 20 – MMF 20 mg/kg, MMF 40– MMF 40 mg/kg * - as compared to sham, # - as compared to MCAo. MMF - monomethyl fumarate. *p < 0.05, **p < 0.01, ***p < 0.001.

not significantly reduced by MMF treatment when compared with MCAo.

3.6. Effect of MMF on I/R induced changes in the levels of inflammatory cytokines

One way ANOVA followed by Bonferroni post hoc test revealed that I/R induced significant increase in the levels of pro-inflammatory cytokine, TNF-α, in the ipsilateral cortex (115.2 ± 10.4 pg/mg of protein, $p < 0.01$) as well as striatum (133.4 ± 10.7 pg/mg of protein, $p < 0.001$) when compared with the levels in sham group (66.4 ± 9.7 and 68.2 ± 6.1 pg/mg of protein in cortex and striatum, respectively) (Fig. 3A). Further, I/R also increased the levels of IL-1β significantly from 86.7 ± 8.4 and 92.4 ± 12.6 pg/mg of protein in sham to 130.6 ± 8.4 and 149 ± 18.7 pg/mg of protein in cortex ($p < 0.01$) and striatum ($p < 0.05$) respectively, in MCAo group (Fig. 3B). However, MMF treatment significantly reduced I/R induced increase in cortical levels of TNF-α and IL-1β ($p < 0.05$). In striatum, MMF showed no improvement when compared with MCAo group. Moreover, the level of an anti-inflammatory cytokine, IL-10 was not increased significantly both in cortex and striatum after MMF treatment when compared with MCAo group (Fig. 3C).

3.7. Effect of MMF on signal intensity, ADC value and infarct size by magnetic resonance imaging

T-2 weighted images revealed no infarct in sham group. MCAo group rats showed significant ($p < 0.001$) infarct (37.4 ± 1.4% of ipsilateral hemisphere) when compared with sham group (Fig. 4D). Treatment with MMF (20 mg/kg) significantly ($p < 0.05$) reduced infarct damage (26.0 ± 3.6% of ipsilateral hemisphere) when compared with MCAo group. Further, we also calculated changes in cortical and striatal infarct volume in MCAo and MMF treated groups. Infarct volume was significantly ($p < 0.05$, student t-test, unpaired) less in

Table 1
Effect of MMF on parameters of oxidative stress. (n = 6). Results are presented as mean ± SEM. * - as compared to sham, # - as compared to MCAo. *p < 0.05, **p < 0.01, ***p < 0.001.

Parameters/Groups	GSH (μg/mg of protein)		MDA (ηmol/mg of protein)		SOD (U/mg of protein)		NO (μM/mg of protein)	
	Cortex	Striatum	Cortex	Striatum	Cortex	Striatum	Cortex	Striatum
Sham	6.1 ± 0.9	6.4 ± 0.3	2.9 ± 0.2	2.5 ± 0.3	89.9 ± 10.7	107.4 ± 8.4	1.6 ± 0.1	2.0 ± 0.2
MCAo	1.3 ± 0.2***	1.4 ± 0.2***	6.3 ± 0.7**	5.6 ± 0.7**	32.2 ± 2.6**	43.6 ± 6.4***	3.7 ± 0.5**	4.4 ± 0.5**
MMF	4.7 ± 0.4##	3.3 ± 0.7#	3.7 ± 0.6#	4.9 ± 0.7	77.9 ± 12.6#	66.7 ± 9.5	2.3 ± 0.3#	3.4 ± 0.5

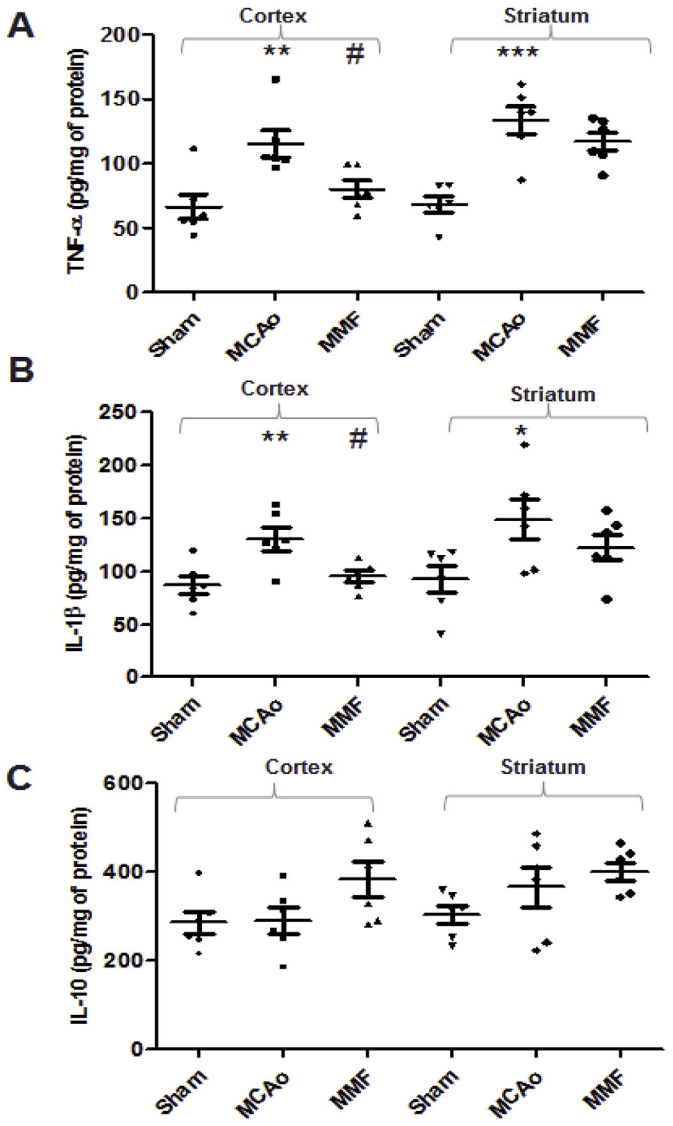

Fig. 3. Effect of MMF on levels of inflammatory cytokines in cortex and striatum. (A) Effect on levels of TNF-α; (B) Effect on the levels of IL-1β; (C) Effect on the levels of IL-10 (n = 6). Each point represents the raw data of each rat, horizontal line represents mean and error bars represent standard error of mean. MMF - monomethyl fumarate 20 mg/kg * - as compared to sham, # - as compared to MCAo. *p < 0.05, **p < 0.01, ***p < 0.001.

cortex of MMF treated group (94.2 ± 18.6 mm^3) when compared with MCAo group (151.2 ± 15.6 mm^3). However in striatum, no reduction in infarct volume was observed after MMF treatment (Fig. 4E).

Similarly, signal intensities of ipsilateral and contralateral hemispheres did not differ in sham group. Signal intensity ratios were calculated for each group as ratio of hyperintense infarct area with contralateral area. Signal intensity ratio in MCAo group was significantly ($p < 0.001$) higher than sham group showing more hyperintense regions indicating more damage (Fig. 4F). Signal intensity ratio was improved significantly ($p < 0.01$) in MMF treated group when compared with MCAo group.

ADC values of ipsilateral area ($6.3 \pm 0.1 \times 10^{-4}$ mm^2/s) and contralateral area ($6.4 \pm 0.07 \times 10^{-4}$ mm^2/s) were almost similar in sham group rats. In MCAo group, ADC values of ipsilateral hemisphere ($4.0 \pm 0.2 \times 10^{-4}$ mm^2/s) were significantly lower than the contralateral hemisphere ($6.9 \pm 0.3 \times 10^{-4}$ mm^2/s). MMF (20 mg/kg) treated group also showed reduced ipsilateral ADC value ($5.5 \pm 0.6 \times 10^{-4}$ mm^2/s) when compared with contralateral hemisphere ($7.0 \pm 0.6 \times 10^{-4}$ mm^2/s). However, ADC ratio (ipsilateral/contralateral ADC) of MCAo group was significantly ($p < 0.001$) reduced when compared with sham group indicating restriction of cellular water molecule movement and hence progression of injury (Fig. 4G). Treatment with MMF significantly ($p < 0.001$) improved the ADC ratio when compared with MCAo group.

3.8. Effect of MMF on expression of Nrf2 and HO1 by western blot and dual immunofluorescence

We investigated the effect of MMF on expression of Nrf2 and HO1 after I/R injury. Separation of cytoplasmic and nuclear fraction was confirmed by western blot (Supplementary Fig. S4). Nuclear Nrf2 expression was not increased significantly in peri-infarct cortex of MCAo group when compared with sham. However, treatment with MMF showed significant ($p < 0.05$) increase in Nrf2 expression in peri-infarct cortex when compared with sham. Notably, the levels of Nrf2 were decreased in MCAo as well as MMF treatment groups in striatum when compared with sham indicating permanently damaged cells (Fig. 5B and C). The cytoplasmic HO1 levels were increased in peri-infarct cortex region of MCAo group significantly ($p < 0.01$) when compared with sham group. Treatment with MMF significantly ($p < 0.05$) increased the expression of HO1 when compared with MCAo. Further, there was no increase in HO1 expression in striatum of MCAo whereas, MMF treatment showed significantly higher HO1 expression when compared to sham group (Fig. 5D and E).

To determine which cell types express Nrf2 and HO1, we performed double immunofluorescence staining with specific cellular markers for neurons (NeuN) and astrocytes (GFAP). In the sham group, the results revealed expression of Nrf2 in neurons. Co-localization data revealed, Nrf2 was overexpressed primarily in neurons in MCAo rats (Fig. 6A and B). Treatment with MMF increased the Nrf2 expression significantly ($p < 0.05$) in neurons as compared to sham. In sham, HO1 expression was evidenced in very few neurons or astrocytes. Similarly, western blot results also revealed low expression of HO1 in sham. Co-localization data showed significantly ($p < 0.01$) increased HO1 expression in MCAo group in neurons and many non-neuronal cells as well. Therefore, to see specific non neuronal cell, we further co-localized HO1 with GFAP. HO1 was significantly ($p < 0.001$) increased in astrocytes as well, in MCAo group when compared with sham. Further, treatment with MMF increased HO1 expression in neurons ($p < 0.05$) (Fig. 7 A, B) and in astrocytes ($p < 0.01$) (Fig. 8 A, B) when compared with MCAo group.

4. Discussion

In the present study, we demonstrated the neuro-protective effect of MMF in I/R model of experimental stroke in rats. MMF administration at two time points; post ischemia and post reperfusion elicited a significant neuro-protection as evidenced by improved neurobehavioral parameters, decreased infarct damage, normalized oxidative stress and inflammatory cytokines. Few experimental studies have been conducted to evaluate the efficacy of fumaric acid esters in stroke (Hjelm et al. 2017; Lin-Holderer et al., 2016; Yao et al., 2016). Most of these studies demonstrated that DMF or MMF has favorable effects on functional outcome. However, neuro-protection in specific part of the brain like cortex or striatum in rats has not been studied earlier. In the present study, we for the first time showed a significant reduction in cortical infarct after MMF treatment in rats, with no effect in the striatum indicating restricted progression of infarct with MMF. Also, effect of MMF on expression of Nrf2 and HO1 in specific cell type has not been studied earlier.

The rationale of selecting MMF and time of administration was based on the evidence that dimethyl fumarate takes around 2–2.5 h to get converted to its active molecule MMF (Litjens et al., 2004). Stroke being an acute condition, intervention cannot be delayed to lose the

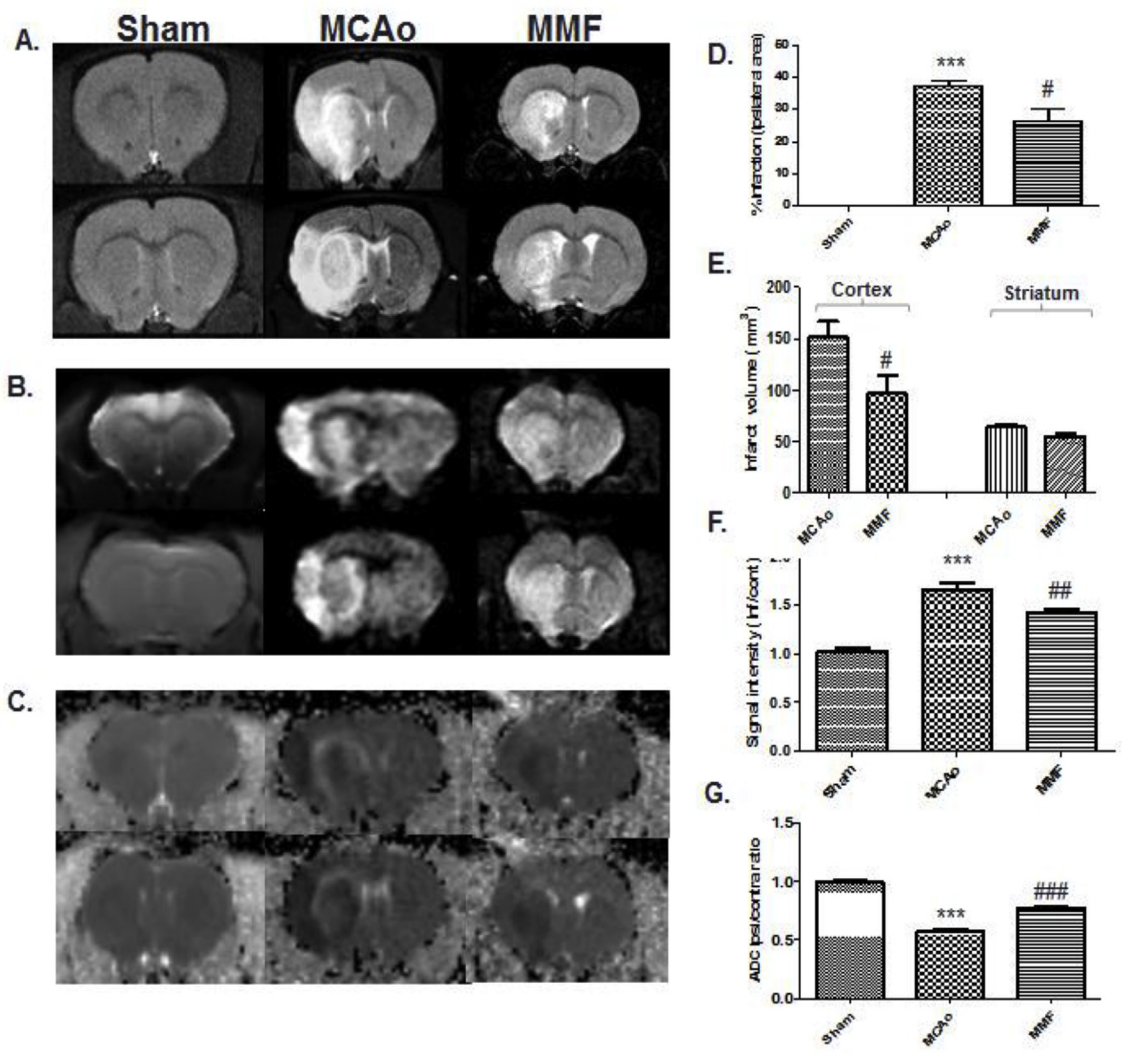

Fig. 4. Effect of MMF on infarct size, signal intensity and ADC values. A, B and C represent the two consecutive slices taken from T2 images, DWI images and parametric images of diffusion, respectively. Hyperintense region in images A and B represents the infarct region whereas hypointense region in C shows restricted water molecule movement across the cell and further implies progression of injury; (D) Represents quantitative estimation of infarct size as assessed by T2 images; (E) Represents change in infarct volume of cortex and striatum separately after MMF treatment as compared with MCAo. Student t-test revealed significantly less cortical infarct volume in MMF treated group; (F) Represents change in signal intensity ratio after MMF treatment: inf - infarct region, cont - contralateral region; (G) Represents change in ADC ratio after MCAo and MMF treatment. (n = 8). Results are presented as mean ± SEM. MMF - monomethyl fumarate 20 mg/kg * - as compared to sham, # - as compared to MCAo. *p < 0.05, **p < 0.01, ***p < 0.001.

critical golden period. Further, looking at half-life of MMF i.e. 57 ± 13.6 min (Litjens et al., 2004), and our target of post reperfusion ROS-induced damage, we wanted MMF to be available in tissue at the time of reperfusion. So, MMF was administered 30 min post ischemia and a booster dose at 5–10 min post reperfusion. Moreover, administration of MMF at two time points after reperfusion (5–10 min and 2 h post reperfusion) did not reduce the infarct and motor incoordination significantly as compared to MCAo group (Supplementary Fig. S1). Since, MMF administration has been reported to be associated with cutaneous flushing (Hanson et al., 2010), we used naïve rats to assess its effect on systolic blood pressure. Results did not show any significant difference in the systolic blood pressure (Supplementary Fig. S2).

Although, restoration of blood supply is expected to reverse ischemia induced changes, yet it carries risk too. Reperfusion may exacerbate the clinical outcome by increasing ROS, mitochondrial dysfunction, leukocyte infiltration, platelet adhesion and release of pro-inflammatory cytokines, etc. (Chen et al., 2017; Pundik et al., 2012). High metabolic activity including high oxygen demand, abundance of redox active metals like iron and copper, high levels of polyunsaturated fatty acid and low levels of GSH makes brain more vulnerable to oxidative damage during ischemia reperfusion (Wang and Michaelis, 2010). We observed increased oxidative stress in MCAo group as evidenced by reduced levels of GSH, SOD and increased levels of MDA and NO in cortex and striatum. GSH is a tripeptide that either reacts with peroxide and hydroxyl ions or acts as electron donor for peroxides (Kim et al., 2015). Further, after reperfusion, superoxide radicals produced are dismutated by SOD to less potent H_2O_2 (Chen et al., 2011). MMF induced improvement in levels of GSH may be attributed to the ability of fumarates to facilitate astrocyte dependent glutathione synthesis in neurons (Steele et al., 2013). Moreover, Nrf2 downstream pathway is associated with up regulation of enzymes involved in synthesis of glutathione and SOD in neurons (Shih et al., 2003). Intracellular NO, produced by over activation of NMDA receptor during ischemia converts superoxide to a potent protein and lipid oxidant, peroxynitrile ($ONOO^-$) leading to apoptosis in ATP deficient cell (Shirley et al., 2014). MMF treatment decreased ischemia induced increase in NO levels. Further, levels of MDA, a marker reflecting rate and extent of lipid peroxidation were significantly decreased in cortex in MMF treated

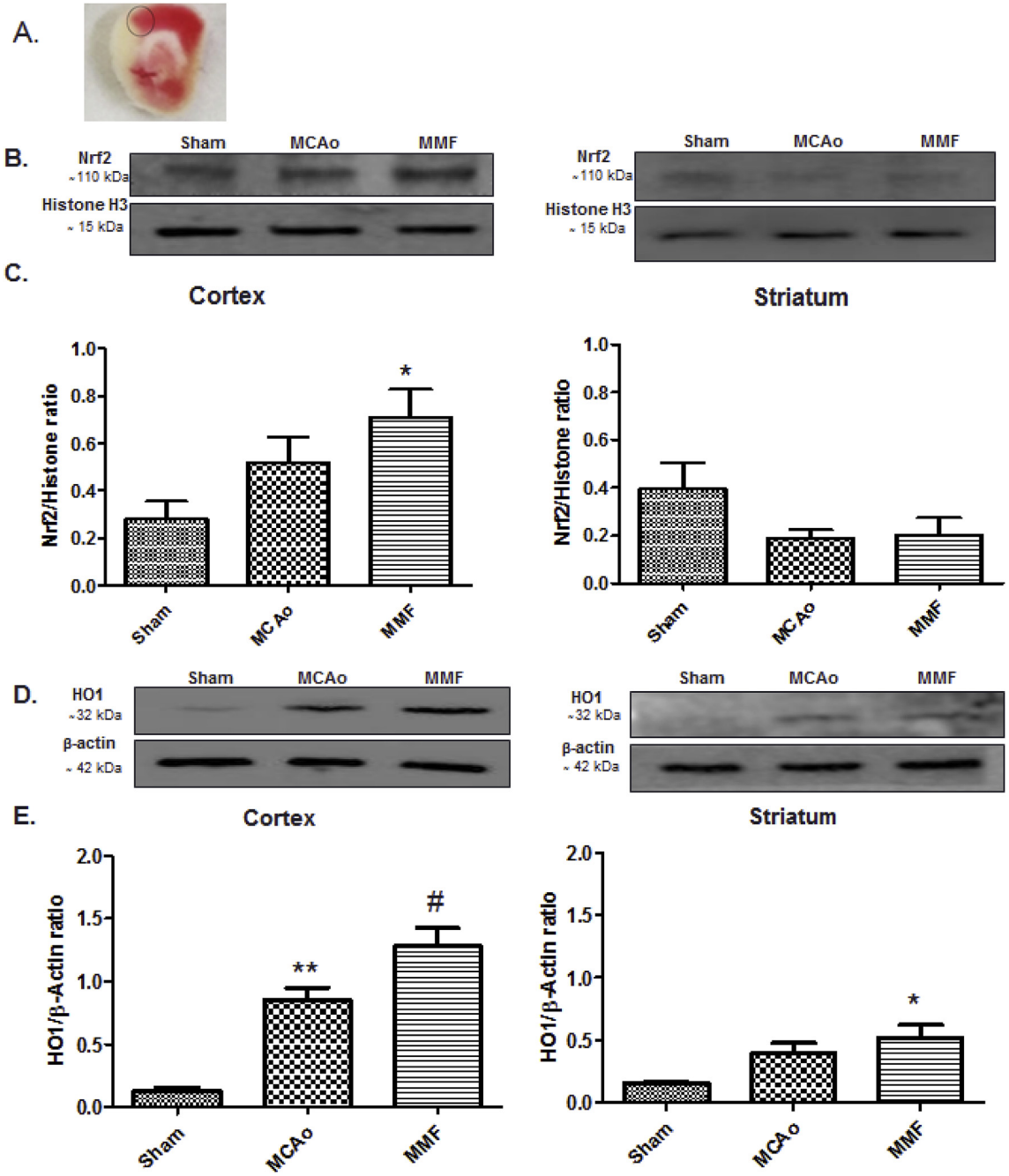

Fig. 5. Effect of MMF on expression of Nrf2 and HO1. (A) Representative TTC stained image showing peri-infarct region (circled). Adjacent region from the next section was taken and processed for cytoplasmic and nuclear fractionation; B & D. Representative blots showing change in expression of nuclear Nrf2 and cytoplasmic levels of HO1, respectively; C & E. Quantitative change in expression of Nrf2 and HO1 expression when normalized with histone H3 and β-Actin respectively. (n = 4); Results are presented as mean ± SEM. MMF - monomethyl fumarate. * - as compared to sham, # - as compared to MCAo. *p < 0.05, **p < 0.01, ***p < 0.001.

group. Here, our results point towards the ability of MMF in normalizing I/R induced disturbed redox status mainly in cortex.

Along with oxidative stress, neuro-inflammation is another important factor that exacerbates ischemic damage causing dysfunction of BBB, edema and intensifies neuronal damage (Lakhan et al., 2009). While TNF-α and IL-1β are the key cytokines in the inflammatory mechanism contributing to the progression of ischemic damage, IL-10 facilitates the resolution of inflammatory cascade (Garcia et al., 2017; Spera et al., 1998). Evidence suggests that soon after ischemia, the levels of TNF-α and IL-1β starts increasing, reach peak in few hours and remain elevated for days (Lambertsen et al., 2012; Liu et al., 1993). In agreement with this notion, our data indicate that I/R significantly increased TNF-α and IL-1β in cortex and striatum which were effectively reversed by MMF in cortex. Anti-inflammatory cytokine, IL-10, down regulates cytokine receptor expression/activation and also inhibits TNF-α and IL-1β (Strle et al., 2001). Evidence suggest that administration of IL-10 was associated with reduced infarct damage (Spera et al., 1998). The levels of IL-10 were moderately increased by MMF. This moderate increase after ischemia may be explained by late microglial activation dependent IL-10 release which initiates after 12 h and reaches peak after 2 days (Zhao et al., 2017; Li et al., 2001). A growing body of evidences both in pre-clinical and clinical fields suggests anti-inflammatory property of fumarates. Clinically, fumarates have well accepted anti-inflammatory effects in multiple sclerosis

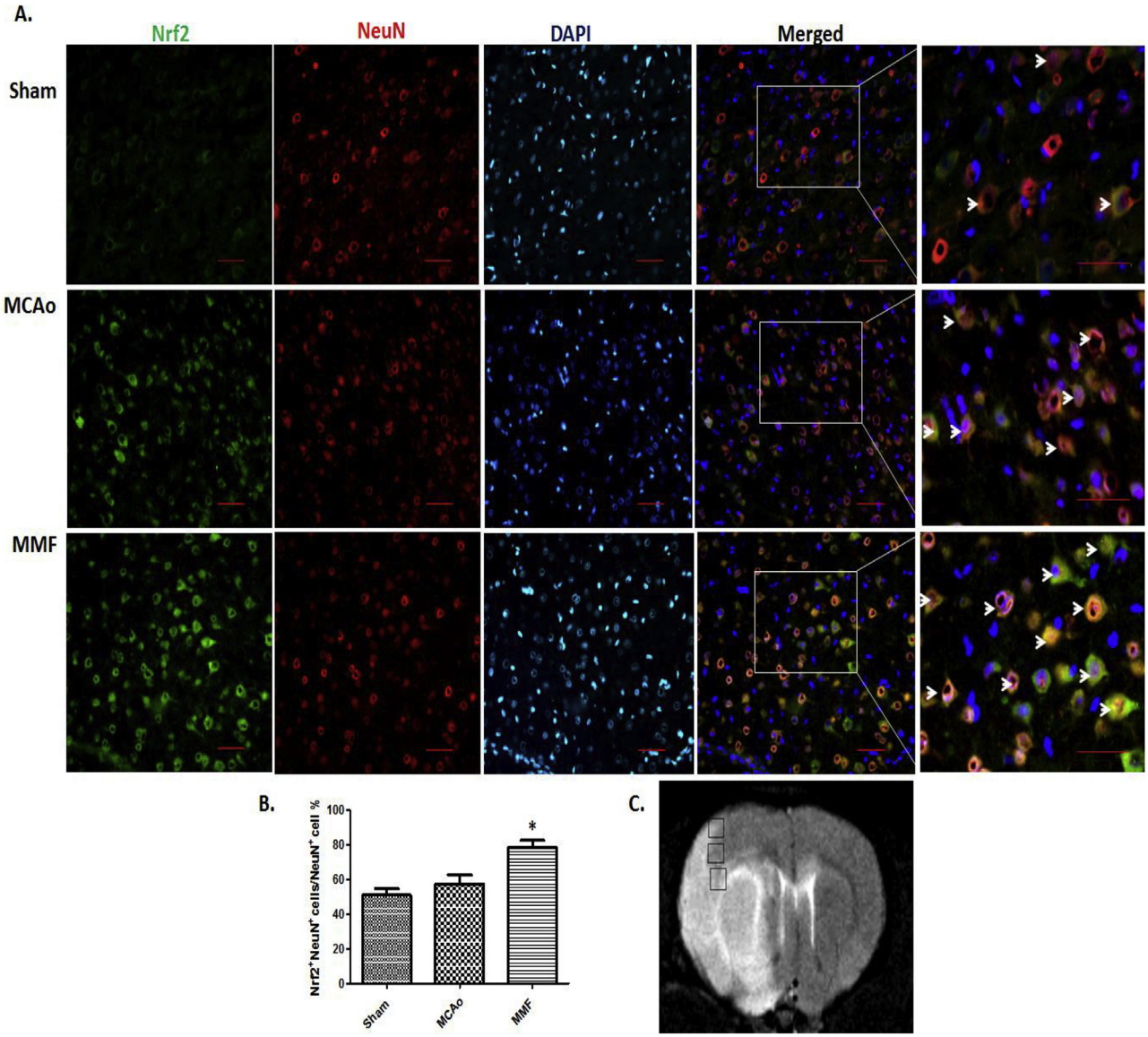

Fig. 6. (A) Representative photomicrographs from peri-infarct region of the rat brain 24 h after I/R injury in sham, MCAo and MMF groups. Sections were double stained with anti-Nrf2 (green), NeuN (red) followed by counterstaining with DAPI (blue) to mark nucleus. Scale bar = 100 μm. Arrow heads indicate Nrf2 co-localized with NeuN; (B) Bar diagram shows quantitative change as percentage of $Nrf2^+NeuN^+$ vs $NeuN^+$ immunoreactive cells, there was slight increase in number of $Nrf2^+NeuN^+$ cells in MCAo group when compared with sham. However, treatment with MMF significantly increased the expression of $Nrf2^+NeuN^+$ cells, mainly in peri-infarct neurons. (n = 4); (C) Small square on T2 image showing peri-infarct region used to acquire microphotographs. Results are presented as mean ± SEM. MMF - monomethyl fumarate 20 mg/kg * - as compared to sham, # - as compared to MCAo. *p < 0.05, **p < 0.01, ***p < 0.001.

(Bomprezzi, 2015; Li et al., 2018). Various preclinical evidence suggests that dimethyl fumarate and MMF inhibits microglia and astrocytic inflammation (Wilms et al., 2010). Moreover, there are reports of a cross talk between Nrf2 and NF-κB wherein Nrf2 inhibits NF-κB pathway (Wakabayashi, 2010). Thus taken together, Nrf2 and Nrf2 dependent decreased NF-κB pathway may presumably be the mechanism by which MMF reduces pro-inflammatory cytokines.

Further, T2 weighted image derived MRI data also revealed a significant reduction in infarct damage, complementary to TTC results but only in cortex. Diffusion weighted images used to calculate signal intensity and ADC value also revealed significant improvement in MMF treated group indicating reversal of ischemia induced restricted cellular water molecule movement. We observed significant reduction in infarct volume only in cortex of MMF treated group whereas striatal infarct was almost identical to MCAo group indicating ability of MMF to preserve the penumbral cortical tissue from damage.

Nrf2, a member of the cap "n" collar transcription factor family, is a master regulator of antioxidant defense gene and plays a protective role against exogenous and endogenous stresses (Kaspar et al., 2009; Kensler et al., 2007; Nguyen et al., 2009). In quiescent conditions, Nrf2 resides in cytoplasm attached to repressor protein Kelch like ECH-associated protein-1 (Keap-1), only to be ubiquitinized by cullin-3 (Turpaev, 2013; Zhang, 2006). It is noteworthy that α/β unsaturated carbonyl group of fumaric acid esters including MMF, or other electrophiles may interact with thiol group of Keap-1, leading to dissociation and nuclear translocation of Nrf2 (Schmidt et al., 2007). In the nucleus, Nrf2 transcribes many neuro-protective genes having antioxidant response element (ARE) in their promoter region. These include mainly heme oxygenase-1, nicotinamide adenine dinucleotide phosphate, quinone oxido-reductase, SOD and gama-glutamyl cysteine ligase etc. These downstream pathways together, maintain redox status of a cell by forming a pleiotropic cellular defense (Itoh et al., 1999; Satoh et al., 2006).

Finally, expression of nuclear Nrf2 and cytoplasmic HO1 were assessed using western blot followed by dual immunofluorescence to

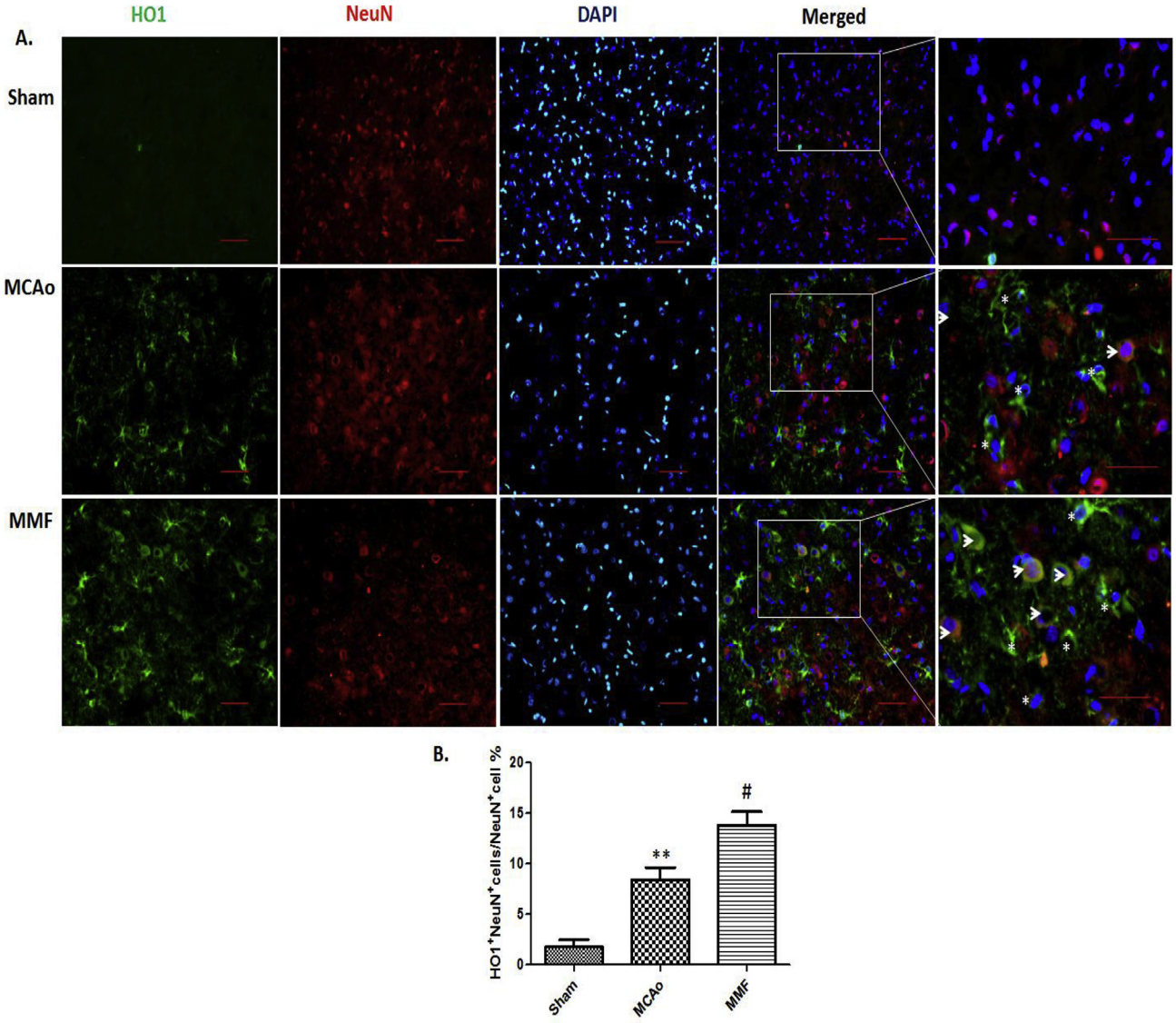

Fig. 7. (A) Representative photomicrographs from peri-infarct region of rat brain 24 h after I/R injury in sham, MCAo and MMF groups. Sections were double stained with anti-HO1 (green), NeuN (red) followed by counterstaining with DAPI (blue) to mark nucleus. Scale bar = 100 μm; (n = 4). Arrow heads indicate HO1 co-localized with NeuN whereas asterisk indicate HO1 expression in non-neuronal cells; (B) Bar diagram shows quantitative change as percentage of $HO1^+NeuN^+$ vs $NeuN^+$ immunoreactive cells, there was significant increase in number of $HO1^+NeuN^+$ cells in MCAo group when compared with sham. However, treatment with MMF significantly increased ($p < 0.05$) the expression of HO1. Results are presented as mean ± SEM. MMF - monomethyl fumarate 20 mg/kg * - as compared to sham, # - as compared to MCAo. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

check change in their expression in neurons or astrocytes. Our western blot results showed increased nuclear expression of Nrf2 in peri-infarct cortex region of MCAo group. Treatment with MMF further increased the nuclear expression of Nrf2 indicating increased nuclear translocation by MMF. Dual immunofluorescence study also revealed increased number of neurons expressing Nrf2. Results were also in accordance with previously published literature wherein MMF exerted neuronal protection against retinal I/R injury via modulating Nrf2 expression (Cho et al., 2015). In contrast to peri-infarct cortex, Nrf2 levels were decreased in striatum as evidenced by western blot data and dual immunofluorescence (Supplementary Fig. S3). These results were also supported by other parameters studied like oxidative stress, inflammatory cytokines and infarct volume assessed by T2-MRI images; which showed no improvement in striatal region. In striatum, decreased expression of Nrf2 can be explained most likely by the fact that cell starts dying soon after minutes to hours of ischemia in core region leading to degradation of RNA and protein, making transcription almost undetectable (Dang et al., 2012).

Free heme, a known pro-oxidant and pro-inflammatory has been deleterious to endothelial cells and brain cells (Tsuchihashi, 2004). HO1, also known as heat shock protein (hsp-32), is an inducible cyto-protective gene that converts free heme to biliverdin, carbon monoxide (CO) and Fe^{2+}. Subsequently, Fe^{2+} increases anti-oxidant ferritin level, biliverdin gets converted to bilirubin and CO produces anti-apoptotic, anti-inflammatory effect and vasodilation making it a promising target (Doré, 2002; Zeynalov et al., 2009). Previously published reports have suggested increased HO1 expression post stroke (Nimura et al., 1996). HO1 overexpression was associated with reduced infarct (Zhang et al., 2012) whereas HO1 knock out mouse showed larger infarct (Shah et al., 2011). Our results of western blot were also in accordance with previous reports where in the expression of HO1 was increased prominently in peri-infarct cortex of MCAo group when compared with sham. MMF treatment further increased HO1 expression compared to MCAo in peri-infarct cortex. Notably, dual immunofluorescence data revealed that MMF increased HO1 expression in both neurons and astrocytes of peri-infarct region. We found that Nrf2 was expressed predominantly in

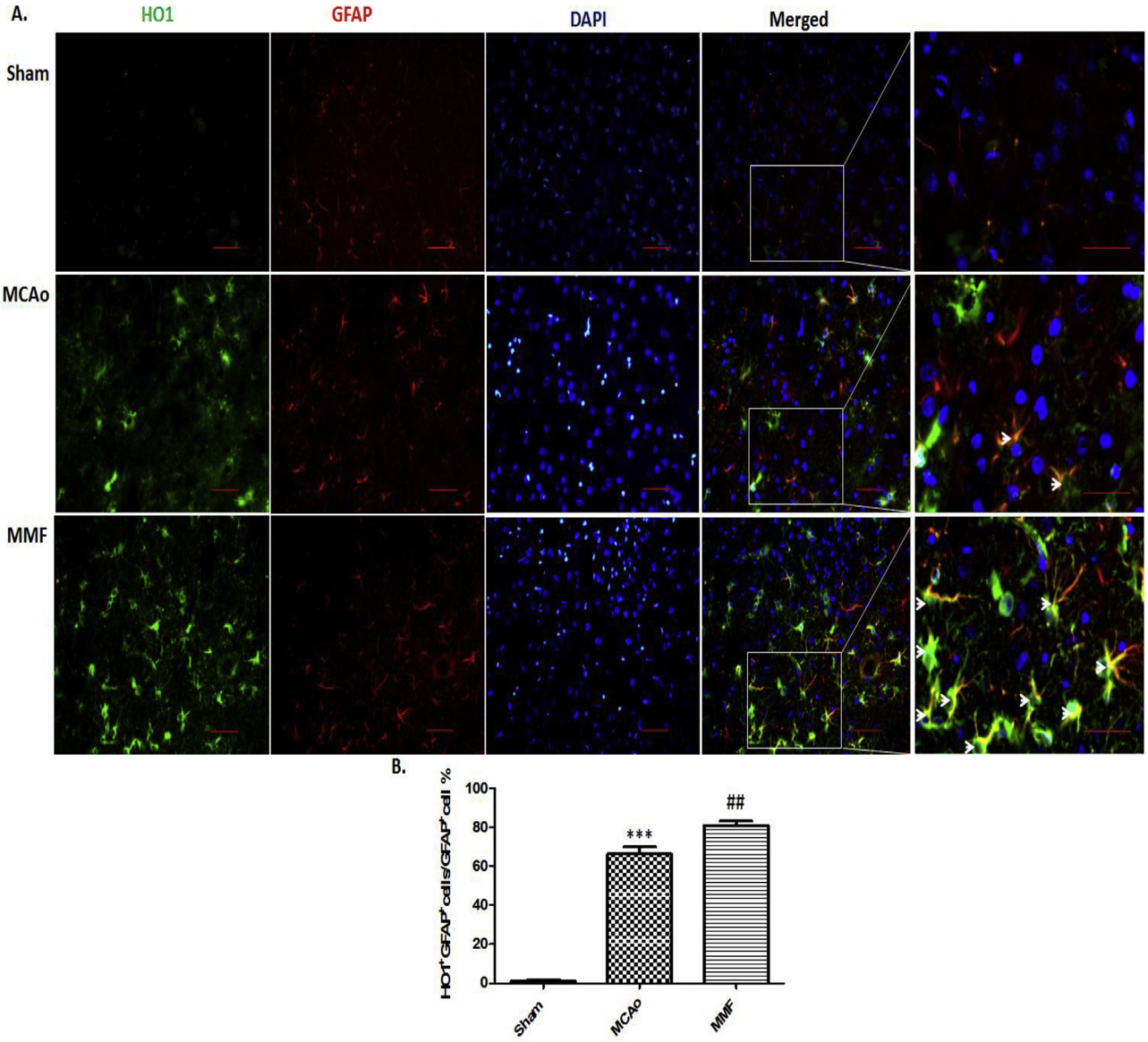

Fig. 8. (A) Representative photomicrographs from peri-infarct region of the rat brain 24 h after I/R injury in sham, MCAo and MCAo along with MMF groups. Sections were double stained with anti-HO1 (green), GFAP (red) followed by counter staining with DAPI (blue) to mark nucleus. Scale bar = 100 μm. Arrow heads indicate HO1 co-localized with GFAP; (B) Bar graph shows quantitative change as percentage of $HO1^+GFAP^+$ vs $GFAP^+$ immunoreactive cells. There was increase in number of $HO1^+NeuN^+$ cells in MCAo group when compared with sham. However, treatment with MMF significantly increased ($p < 0.01$) the expression of HO1, mainly in peri-infarct neurons ($n = 4$); Results are presented as mean ± SEM. MMF - monomethyl fumarate 20 mg/kg * - as compared to sham, # - as compared to MCAo. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

neurons whereas HO1 was expressed in neurons and non-neuronal cells like astrocytes. Although, similar findings of HO1 induction in predominantly functionally active astrocytes were also reported in peri-infarct region post stroke (Alfieri et al., 2013). However, the mechanism of how neuronal Nrf2 expression influence non-neuronal HO1 expression still remains to be explored. Also, MMF has been reported to produce neuro-protection through some unknown, Nrf2 independent manner (Lin-Holderer et al., 2016) like activating AMPK/SIRT1 axis in microglia through hydroxycarboxylic acid receptor 2 (Parodi et al., 2015). Hence, other currently unknown molecular mechanism cannot be excluded.

There are few limitations in the present study. First, we used only male rats in our study for uniformity of the data and to avoid interference of protective effects of the female hormone estrogen (Hurn and Macrae, 2000). Young males were included in this study because stroke in the young has a disproportionately high economic impact by leaving victims disabled before their most productive years. However, further studies using old male and female rats are needed in the future. Moreover, early administration of MMF in the study is good to start with but has less translational value and hence require sub-acute or chronic treatment study in future.

It is worth noting that as evidenced by T2 image data, the MMF treatment was associated with improvement predominantly in cortex than in striatum. The results of oxidative stress parameters, inflammatory cytokines and protein expression were also complementary, showing improvement in cortex after MMF treatment. Notably, the expression of Nrf2 in striatum was decreased after stroke, indicating an irreversible damage in that area. There is an evidence suggesting spatiotemporal evolution of infarct, rapidly after 6–12 h of I/R from striatum to include cortex (Liu and Mccullough, 2011). Hence, the neuro-protective effects of MMF may be postulated to stimulation of Nrf2/HO1 pathway in peri-infarct region that leads to control of the spatiotemporal progression of damage from striatum to cortex.

5. Summary

Taken together, we conclude that MMF treatment exhibits neuroprotection in I/R injury. The results of our study along with previous literature further explains that the neuro-protective effects of MMF may be orchestrated through activation of Nrf2 in neurons and HO1 in neurons and astrocytes in peri-infarct region of the cortex. Finally, data also demonstrate the improvement in cortex region indicating the role of MMF in halting the progression of I/R damage from striatum to cortex. Our results thus suggest the potential of MMF in the management of stroke.

Conflicts of interest

There is no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.03.010.

Abbreviations

ADC	apparent diffusion coefficient
AMPK/SIRT1	AMP-activated protein kinase and the histone/protein deacetylase sirtuin 1
BBB	blood brain barrier
BPU	blood perfusion unit
DALY	disability adjusted life years
DMF	dimethyl fumarate
DTNB	5, 50-dithiobis 2-nitrobenzoic acid
DWI	diffusion weighted image
ECA	external carotid artery
GFAP	glial fibrillary acidic protein
GSH	reduced glutathione
HCAR2	hydroxycarboxylic acid receptor 2
HO1	heme oxygenase
MMF	monomethyl fumarate
ICA	internal carotid artery
IL-10	interleukin 10
IL-1β	interleukin 1 beta
Keap1	Kelch like ECH-associated protein-1
LDF	laser Doppler flow meter
MCAo	middle cerebral artery occlusion
MDA	malondialdehyde
MRI	magnetic resonance imaging
NDS	neurological deficit score
NeuN	neuronal marker
NF-κB	nuclear factor-kappa B
Nrf2	nuclear factor erythroid 2 related factor 2
OCT	optimum cutting temperature
ONOO	peroxynitrile
PBS	phosphate buffer saline
rCBF	regional cerebral blood flow
ROS	reactive oxygen species
SOD	superoxide dismutase
STZ	streptozotocin
TNF-α	tumor necrosis factor α
tPA	tissue plasminogen activator
TTC	tri-phenyl tetrazolium chloride.

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