MONOMETHYL FUMARATE MITIGATES ISCHEMIA REPERFUSION INJURY BY ACTIVATING NRF2/HO1 PATHWAY IN EXPERIMENTAL MODEL OF ISCHEMIC STROKE IN RAT

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Introduction

- Ischemic stroke still remains a major cause of mortality and morbidity, worldwide.
- Stroke-
 - Kills almost 130,000 people each year in USA; strikes in every 40 sec.
 - Causes 1 out of every 19 deaths with 1 death in every 4 minutes (Kochanek et al., 2011)
- Tissue plasminogen activator (t-PA) remains to be the only FDA approved drug with limitations of narrow therapeutic window and risk of bleeding.
- Activation of endogenous antioxidant pathway by stimulating nuclear factor erythroid-2-related factor 2 (Nrf2) plays a key role in cellular defense against oxidative stress in ischemic penumbra.
- Monomethyl fumarate (MMF), an active metabolite of dimethyl fumarate, has shown to be neuroprotective.
- Middle cerebral artery occlusion (MCAo) is a well-accepted model to study the pathophysiology and neuroprotection after ischemia/reperfusion (I/R) injury.

Aim

- To evaluate the neuroprotective effects of MMF in ischemia reperfusion injury model of ischemic stroke in rats.

Treatment schedule

Material and Methods

- Male SD rats (270±20 g) were taken from central animal facility, AIIMS, New Delhi.
- Middle cerebral artery was occluded for 90 min using 3.0 monofilament.
- Cerebral blood flow (CBF) assessed laser Doppler flow meter:

Fig. 1. Images showing CBF measurement using laser Doppler flow meter. a- showing site of CBF measurement using stereotaxic frame. b & c- showing Doppler probe attached over MCA segment. d- showing cerebral blood flow at baseline, during ischemia and post reperfusion.

Fig.2. a:- Drawing showing route of filament insertion from external carotid artery through internal carotid artery and finally advanced up to origin of middle cerebral artery (Ulluc et al., 2011). b:- Image showing site of surgery. c:- showing images of filament inserted to occlude middle cerebral artery.

- Rats showing occlusion more than 75 % to baseline were included in the study.
- After 24hr of MCAo, neurological deficit score was assessed (Longa et al., 1989). Effect on motor incoordination was assessed by time spent on rota rod.
- T2 images were acquired using 7.0T animals MRI (Bruker, Biospin MRI GmbH Biospec 70/20 US).
- Rats euthanized, perfusion fixed with paraformaldehyde followed by cryo preservation in sucrose.
- 10µm thick Cryo-sections from +1.2 to -0.4 with reference to bregma were taken.
- After blocking and permeabilization sections were incubated with first primary antibody.
 - Rabbit polyclonal anti-Nrf2 or Rabbit polyclonal to HO1 (abcam Plc)
- Sections were incubated with corresponding secondary antibody (Goat anti rabbit IgG H&L; Alexa Flour 488, FITC) followed by washing
- Sections were incubated with 2nd primary antibody for 1 hr
 - Mouse monoclonal to NeuN or Mouse monoclonal to GFAP (abcam Plc)
- Sections were washed and incubated with corresponding secondary antibody (Goat anti-mouse IgG H&L; Phycoerythrin).
- Nucleus counter stained with DAPI followed by mounting.
- Data were presented as mean ± SEM. One way ANOVA followed by Bonferroni post hoc analysis was used. p value of less than 0.05 considered statistically significant.

Results

Fig.3. a,b:- Degree of occlusion by LDF presented as % change in blood perfusion unit (BPU). c. showing effect of MMF on neurological deficit score (NDS), d. effect of MMF on motor incoordination. Data are presented as mean ± SEM (n=6). *p < 0.05, ***p < 0.001; * - as compared to sham, # - as compared to MCAo.

Effect of MMF on I/R damage using MRI:

Fig.4. Neuroprotective effect of MMF treatment 10 mg/kg as assessed after 24 hr of reperfusion by MRI. a. Representative MRI T-2 images, damaged area as hyper intense region in grey panel b. Neuroprotective effect of MMF on infarction as % of ipsilateral area. c. Effect of MMF on apparent diffusion coefficient represented as a ratio of ipsilateral ADC value vs ADC value of contralateral area. d. Change in signal intensity after MCAo and treatment with MMF. (n=4-8). *p < 0.05, ***p < 0.001; * as compared to Sham, # as compared to MCAo.

Nrf2 co-localization with NeuN

Fig.5. Representative double immunofluorescence stained images showing Nrf2 (green) co-localized with neurons (red) from peri-infarct cortex region of MCAo (upper panel) and MMF treated group (lower panel). DAPI (4,6-diamidino-2-phenylindole) was used for staining nucleus. Images e & j are merged images. Enlarged images f & k shows neuronal expression of Nrf2 from merged images.

HO1 co-localization with NeuN

Fig.6. Representative double immunofluorescence stained images showing HO1 (green) co-localized with neurons (red) from peri-infarct cortex region of MCAo (upper panel) and MMF treated group (lower panel). DAPI (4,6-diamidino-2-phenylindole) was used for staining nucleus. Images e & j are merged images. Enlarged images f & k shows neuronal expression of HO1 from merged images.

HO1 co-localization with GFAP

Fig.7. Representative double immunofluorescence stained images showing HO1 (green) co-localized with astrocytes (red) from peri-infarct cortex region of MCAo (upper panel) and MMF treated group (lower panel). DAPI (4,6-diamidino-2-phenylindole) was used for staining nucleus. Images e & j are merged images. Enlarged images f & k shows astrocytic expression of HO1 from merged images.

Conclusion

- I/R injury produced significant infarct, neurobehavioural changes and motor incoordination.
- MMF (10 mg/kg twice 30 min post ischemia and 5 min post reperfusion) produced significant improvement in I/R induced infarct, neurobehavioral deficit score and motor incoordination.
- Immunofluorescence data showed increased expression of Nrf2 in neurons and HO1 in neurons as well as astrocytes.
- The results showed that MMF acute treatment reduces the progression of ischemic damage, improves neurological deficit score and motor incoordination by activating endogenous antioxidant pathway via Nrf2
- Our results thus suggest the potential of MMF in ischemic reperfusion injury in rats.