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# Dimethyl fumarate (DMF) and Heparin Sulfate Mitigate Cerebral Ischemia-Reperfusion Injury in Male Rats

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## ABSTRACT

Ischemic cerebrovascular illness leads in oxygen deprivation to brain tissue, which can generate reactive oxygen species (ROS) and immune cell mediators are released after reperfusion, which can happen naturally through thrombus breakdown or through therapeutic recanalization. After a week of acclimatization, the rats were split into five groups as follows: Sham group: The animals got general anesthesia without having their unilateral carotid artery occluded. The animals in the ischemic-reperfused control group got general anesthesia, a 5-minute occlusion of the carotid artery, and an hour-long reperfusion without the administration of medication. Vehicle group: The animals underwent the same surgery as the control group, but they also received intraperitoneal (i.p.) dose of DSMO 5 min. following the ischemia. Group treated with heparin sulfate: 5 min. after the ischemia, the animals underwent the same surgical approach as the control group, but they additionally received 400IU/kg (i.p.). Group treated with dimethyl fumarate: 5 min. after the ischemia, the animals received the same surgical approach as the control group, plus they were given 50 mg/kg of dimethyl fumarate intraperitoneally (i.p.). ROS levels and brain tissue damage were significantly decreased ($P < 0.05$) in the treated groups. Histologically, the treated animals had less brain tissue damage than the control and vehicle groups ($P < 0.05$). Compared to the heparin sulfate group, the DMF group displayed a higher reduction in ROD and brain damage. Conclusion, DMF has a neuroproctive impact on cerebral I/R injury by downregulating oxidative stress and lowering the percentage of infarction area, while heparin also has this effect.

***Keywords:*** *DMF, heparin sulfate, reactive oxygen species, brain damage.*

## 1. INTRODUCTION

Ischemia-reperfusion injuries is defined by inadequate supply of oxygen and nutrients to tissues due to poor blood perfusion, followed by the restoration of blood flow. This process can lead to inflammation and the release of free radicals, which may increase local damage and limit the function of the affected organ (1). The most major risk factor for cerebral ischemia is excessive blood pressure (2). It accelerates atherosclerosis by boosting intraluminal pressure, which produces a wide alteration in smooth muscle function and endothelium in intracerebral courses, culminating in damage to the blood-cerebrum boundary and localized or multifocal mental edema. Furthermore, hypertension is obviously a risk factor for vascular dementia (3). Patients with diabetes mellitus (DM) and dyslipidemia are two to six times more likely to have cerebral ischemia, and this risk is amplified in younger individuals as well as those with hypertension and issues in other vascular beds. Severe cerebral ischemia is seen by DM patients, who may also have altered vascular tone and responsiveness, increased edema, neovascularization, and protease articulation (4).

During CIRI, activated microglia transition into an amoeboid-like state, marked by cellular swelling and the production of pro-inflammatory agents such as cytokines, chemokines, and reactive oxygen species (ROS) (5, 6), leading to BBB damage. Increased BBB permeability facilitates the infiltration of various immune cells into the ischemic area (7), including leukocytes and monocytes/macrophages, the cells can release neurotoxins or trophic factors, which have protective or harmful effects on brain tissue (8).

Following an ischemic attack, there is a marked increase in the levels of TNF-α within brain tissue, plasma, and cerebrospinal fluid, exacerbating cerebral edema, BBB damage, cellular apoptosis, and neuroinflammatory responses. Ischemia activates the local immune system, especially microglia, releasing a large amount of tumor necrosis factor-alpha (TNF-α), which stimulates other immune cells to produce more pro-inflammatory factors, further damaging neurons (9, 10). Additionally, TNF-α can upregulate the expression of adhesion molecules on endothelial cells, promoting leukocyte adhesion to the endothelium. Consequently, leukocytes adhere to damaged areas and release harmful agents such as free radicals and oxidants (11).

A significant mediator of the thrombotic and inflammatory reactions to ischemia, reperfusion, and vascular injury is thrombin. causes P-selectin and intercellular adhesion molecule-1 to be produced on endothelial cells, which enhances leukocyte adherence to damaged endothelium (12). Heparin reduces the synthesis of fibrin, thrombin's binding to endothelium, and P-selectin's binding to neutrophils (13), Recently, the FDA approved the fumarate ester dimethyl fumarate (DMF), which goes by the brand name Ticfedra, to treat psoriasis and multiple sclerosis (MS) (14, 15). With the same method of action as bardoxolone methyl, DMF is a potent antioxidant and anti-inflammatory medication that suppresses the NF-κB pathway and stimulates the Nrf2 pathway. Apart from controlling the glutathione system and boosting the cellular reactivity to oxidative stress, therefore, we reasoned that it would decrease the detrimental effects of cerebral artery air embolism (16).

## 2. AIM

Investigate the neuroprotective effects of dimethyl fumarate and heparin sulfate on ameliorating the damage induced by cerebral ischemia reperfusion.

## 3. MATERIALS AND METHOD

### *3.1 Animal Groups*

White albino Male rats (190–220 g; specified pathogen-free) were obtained from University of Karbala/ faculty of science Experimental Animals house. (License No. KUFS(Karbala) 2020–2030). Rats were housed in specific cages at the Laboratory Animal house of Al-Zahrawi University College, maintained under controlled environmental conditions of 22 ± 1 ℃, 40-55% humidity, and a 12-hour light-dark cycle. All experimental protocols were set up to comply with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee, Karbala University (Approval No.: 2023-KS- 40)

After a week of adapting, 20 white albino rats had been divided into the following six groups: The Sham group: The animals had general anesthesia without having their unilateral common carotid arteries blocked (17); The control group (ischemic-reperfused): The animals underwent general anesthesia, a half-hour-long common carotid artery blockage, and an hour-long reperfusion without the administration of medication (17); The vehicle group: The animals had the identical surgical technique as the control group, but 5 min. after ischemia, they were given DSMO intraperitoneally (i.p.) (18); Dimethyl fumarate group: The animals underwent the same surgical technique as the control group plus intraperitoneal (i.p.) administration of 50 mg/kg of dimethyl fumarate, five minutes following ischemia (19); and Heparin group: the animals underwent the same surgical technique as the control group in addition to receiving 400 IU/kg of heparin sulfate intraperitoneally (i.p.), five minutes after ischemia (20)

At the end of reperfusion, The animals were killed by decapitation under general anesthesia, brains were isolated for our analyses (21, 22)

### *3.2 Induction of brain ischemia*

Animals were given ketamine and xylazine intraperitoneally (i.p.) at doses of 80–100 mg/kg and 8–10 mg/kg, respectively, in order to induce general anesthesia (23). After the animals had got consciousness, the neck region was shaved and cleaned with 80% ethanol. The paratracheal muscles were then pulled by sterile artery forceps, and connective tissues were removed using spay forceps. Following the visualization of the carotid arteries and their separation from the vagal nerve, the left artery was blocked for thirty minutes using vascular clamps. The clamps were then released from the artery to initiate reperfusion for one hour, resulting in a unilateral cerebral ischemia/reperfusion damage (24, 25).

Rats were sacrificed by decapitation after one hour of reperfusion. The brains were removed and then immediately cold by placed in a pre-cold PBS solution. Then put it in the freezer for ten minutes. Each brain was then divided into three slices: one for histopathological analysis, one for TTC staining, and one for an ELISA investigation (26).

### *3.3 Measurement of Cerebral Infarction size*

Triphenyl tetrazolium chloride (TTC) staining was used to evaluate the brain tissues. TTC was dissolved in PBS at a concentration of 0.2% (w/v) before the brains were sliced. Following coronal slice cutting, the brain was immersed in TTC stain at 37 °C for 30 minutes in a glass petri dish covered with aluminum foil to block light exposure. This was followed by an immersion in PBS containing 10% paraformaldehyde (27). Digital imaging (digital camera) and image analysis software (image J system) were used to measure the infarct volume. Swanson's technique (28), which measures the infarct area, was

used. After measuring the total volumes of the red area, which is the valid area, and the white area, which is the infarction area, the infarct percentage (I) was computed as follows:

$$\% I = \frac{white\ area}{total\ area} \times 100\%$$

### *3.4 Histopathology*

Following reperfusion, brain tissues were embedded in a paraffin block, preserved in 10% formalin, sectioned into 5 µm thick slices, and seen under a microscope by a pathologist with expertise in the field. The following pathological grading system was employed in this investigation (29): Normal (0): edema, red blood cells, and eosinophilic neurons are absent. Slight (1): Edema or eosinophilic neurons are present. Moderate (2): edema, eosinophilic neurons, and a little amount of red blood cells. Severe (3): edema, necrosis, RBC, and eosinophilic neurons are present.

### *3.5 ROS tissue measure*

Brain tissues were sliced into very small pieces under cooling, then homogenized using a homogenization solution containing PBS, protease inhibitor cocktail, and Triton X-100 for 20 minutes (5 seconds each time) using a high-intensity ultrasonic liquid processor at -4°C. The samples were then centrifuged at 2,000–3,000 r.p.m. for 20 minutes at 4°C, and stored at −80°C until analysis (30).

### *3.6 Analytical statistics*

Mean ± standard error of the mean (SEM) is the data's expression. Following Tukey's post-test and one-way analysis of variance (ANOVA), a P value of < 0.05 was deemed statistically significant. Kruskal-Wallis non-parametric tests were used to analyze the histopathological parameters. The SPSS program update version 26.1 was used for the analyses, and Notepad Prism version 9.1 was used to create the graphs.

## 4. RESULTS

### *4.1 Heparin sulfate and DMF reduced inflammation in brain tissue.*

After an acute ischemic stroke, immune cells in the brain become rapidly activated and release significant concentration of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, leading to neuroinflammation and disruption to the blood-brain barrier. In our study, ELISA testing demonstrated higher levels of IL-1β, TNF-α, and MCP-1 in the control group, which were lowered by treatment with Heparin and DMF Table (1) and Figure (1). These data demonstrate that both Heparin and DMF efficiently inhibit MCAO activation and the production of proinflammatory mediators.

**Table 1: effect MCAO against pro-inflammatory levels of five groups.**

| Groups n=4 | Mean ± Std. Error | Std. Deviation | 95% CI | | P value |
|---|---|---|---|---|---|
| | | | Lower | Upper | |
| | IL-1β | | | | |
| **Sham** | 110 ± 30 | 21.3 | 45 | 65 | |
| **Control** | 222 ± 50 | 13.6 | 43 | 90 | # P< 0.05 |
| **DMSO (vehicle)** | 223 ± 50 | 11.2 | 33 | 35 | # P< 0.05 |
| **Heparin** | 170 ± 30 | 13.7 | 38 | 54 | * P< 0.05 |
| **DMF** | 154 ± 25 | 20.1 | 48 | 61 | * P< 0.05 |
| | TNF-α | | | | |
| **Sham** | 65 ± 11 | 3.2 | 25 | 39 | |
| **Control** | 163 ± 22 | 2.9 | 23 | 57 | # P< 0.05 |
| **DMSO (vehicle)** | 161 ± 18 | 3.1 | 26 | 48 | # P< 0.05 |
| **Heparin** | 100 ± 12 | 2.4 | 28 | 67 | * P< 0.05 |
| **DMF** | 83 ± 10 | 3.3 | 18 | 66 | * P< 0.05 |

| | MCP-1 | | | | |
|---|---|---|---|---|---|
| **Sham** | 215 ± 43 | 23.5 | 125 | 209 | |
| **Control** | 543 ± 68 | 22.9 | 223 | 447 | # P< 0.05 |
| **DMSO (vehicle)** | 551 ± 64 | 31.4 | 326 | 478 | # P< 0.05 |
| **Heparin** | 420 ± 42 | 29.4 | 227 | 367 | * P< 0.05 |
| **DMF** | 263 ± 10 | 32.3 | 118 | 206 | * P< 0.05 |

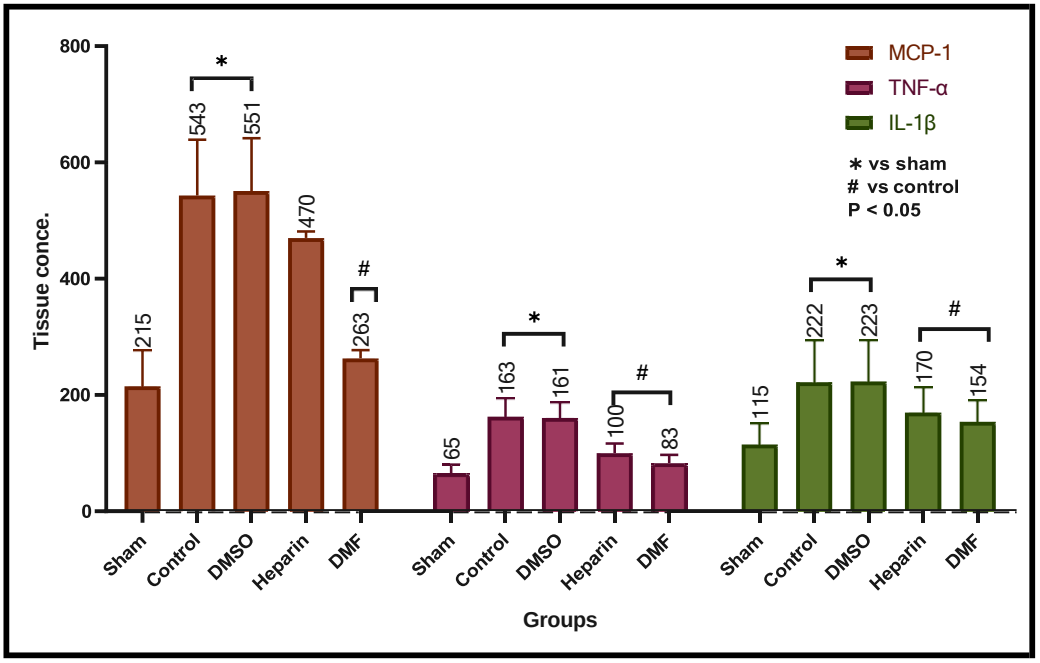


**Figure 1: pro-inflammatory levels (µmole/mg) of five experimental groups. The data expressed by one way ANOVA. # vs sham, * vs control.**

### *4.2 Heparin sulfate and DMF reduced oxidative stress in brain tissue.*

We used the ELISA method to examine the ROS concentrations in the brain tissue of each group after the experiment. Table (2) and Figure (2) illustrate that there is a significant ($p < 0.05$) reduction in ROS levels in the heparin group (0.71 ± 0.01) and DMF group (0.41 ± 0.01) as compared with the control group (1.22 ± 0.05). DMF reduced the ROS level significantly ($p < 0.05$) as compared with the heparin sulfate group. There were insignificantly different considerably ($p > 0.05$) in the ROS levels between the DMF group and the sham groups and between the vehicle group and the control groups.

**Table 2: effect MCAO against ROS levels of five groups.**

| **Groups n=4** | **Mean ± Std. Error** | **Std. Deviation** | **95% CI** | | **P value** |
|---|---|---|---|---|---|
| | | | Lower | Upper | |
| **Sham** | 0.44 ± 0.03 | 0.08 | 0.35 | 0.52 | |
| **Control** | 1.22 ± 0.05 | 0.13 | 1.09 | 1.36 | # P< 0.05 |
| **DMSO (vehicle)** | 1.23 ± 0.05 | 0.11 | 1.11 | 1.35 | # P< 0.05 |
| **Heparin** | 0.71 ± 0.01 | 0.03 | 0.48 | 0.54 | * P< 0.05 |
| **DMF** | 0.54 ± 0.03 | 0.06 | 0.48 | 0.61 | * P< 0.05 |

**Table (1): ROS levels (µmole/ml) of five experimental groups.** The data expressed by one way ANOVA. # vs sham, * vs control.

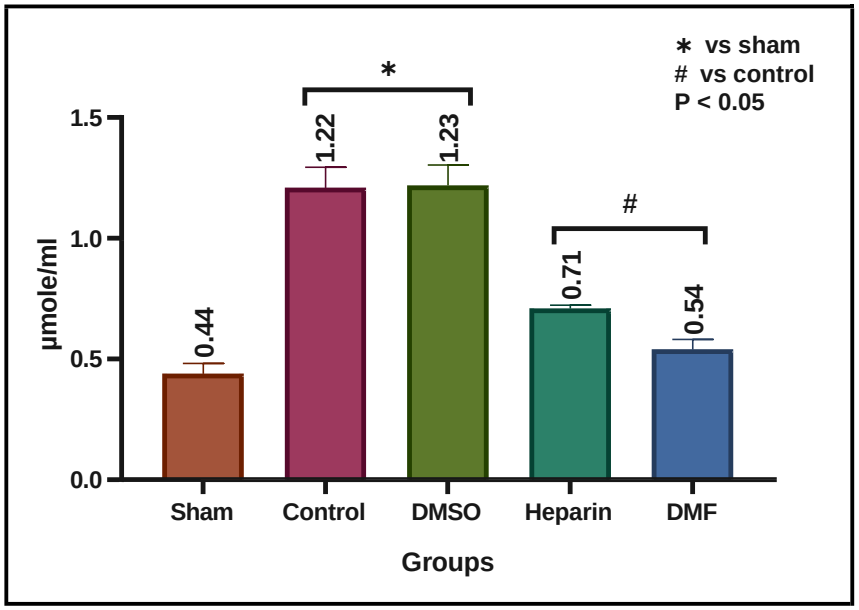


**Figure 2:ROS levels (µmole/mg) of five experimental groups.**

### *4.3 Effect of heparin sulfate and DMF on reduced Infarction area percent.*

An experimental stroke model was developed in mice to investigate the therapeutic effects of heparin sulfate and DMF on cerebral infarction. The experimental design is summarized in table (3) and figure (3). According to a photographic study, The mean area of infarction in the DMF group (6.36% ± 0.65%) and heparin group (13.81% ± 1.81%) in considerably significant ($p < 0.05$) comparison to the control group (43.15% ± 7.72%). DMF reduced the infarction area percentage significantly ($p < 0.05$) as compared with the heparin sulfate group. We found no statistically significant ($p > 0.05$) variation in the percentage of infarction area between the DMSO group and the control group.

**Table (3): brain damage score of five experimental groups. The data expressed by one-way ANOVA. # vs sham, * vs control.**

| Groups n=4 | Mean ± Std. Error | Std. Deviation | 95% CI | | P value |
|---|---|---|---|---|---|
| | | | Lower | Upper | |
| **sham** | 0.05 ± 0.00 | 0.00 | 0.00 | 0.00 | |
| **control** | 2.83 ± 0.17 | 0.41 | 2.40 | 3.26 | # P< 0.05 |
| **DMSO (vehicle)** | 2.83 ± 0.17 | 0.41 | 2.40 | 3.26 | # P< 0.05 |
| **Heparin** | 1.00 ± 0.26 | 0.63 | 0.34 | 1.66 | * P< 0.05 |
| **DMF** | 1.17 ± 0.17 | 0.41 | 0.74 | 1.60 | * P< 0.05 |

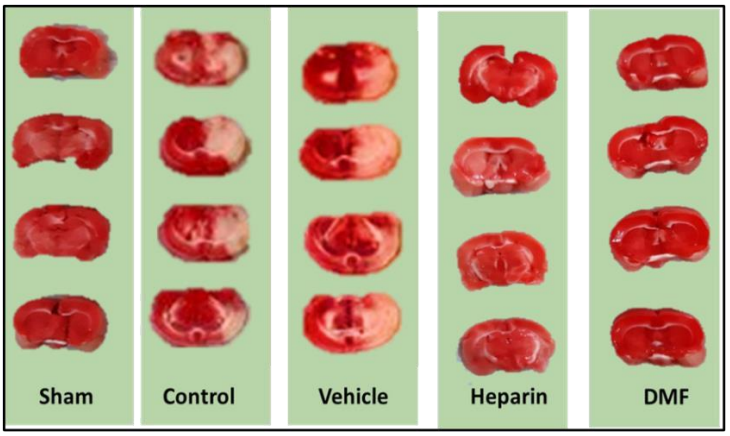


**Figure (3): Photograph of the coronal slices of rat brain stained with TTC of all groups.**

### *4.4 Effect of heparin and DMF on histopathological study.*

According to table (4) and figure (4), histopathological analysis revealed that the DMF group had the lowest brain tissue damage scores significantly ($p < 0.05$) as compared with the heparin group, respectively. The brain tissue damage score was considerably ($p < 0.05$) lower in the treated groups (DMF and heparin) when compared to the control group. There was no discernible change in the scores ($p > 0.05$) between the DMSO group and the control group.

**Table (4): brain damage score of five experimental groups. The data expressed by one-way ANOVA. # vs sham, * vs control.**

| Groups n=4 | Mean ± Std. Error | Std. Deviation | 95% CI | | P value |
|---|---|---|---|---|---|
| | | | Lower | Upper | |
| **Sham** | 0.05 ± 0.00 | 0.00 | 0.00 | 0.00 | |
| **Control** | 2.83 ± 0.17 | 0.41 | 2.40 | 3.26 | # P< 0.05 |
| **DMSO (vehicle)** | 2.83 ± 0.17 | 0.41 | 2.40 | 3.26 | # P< 0.05 |
| **DMF** | 1.17 ± 0.17 | 0.63 | 0.34 | 1.66 | * P< 0.05 |
| **Heparin** | 1.61 ± 0.17 | 0.41 | 0.74 | 1.60 | * P< 0.05 |

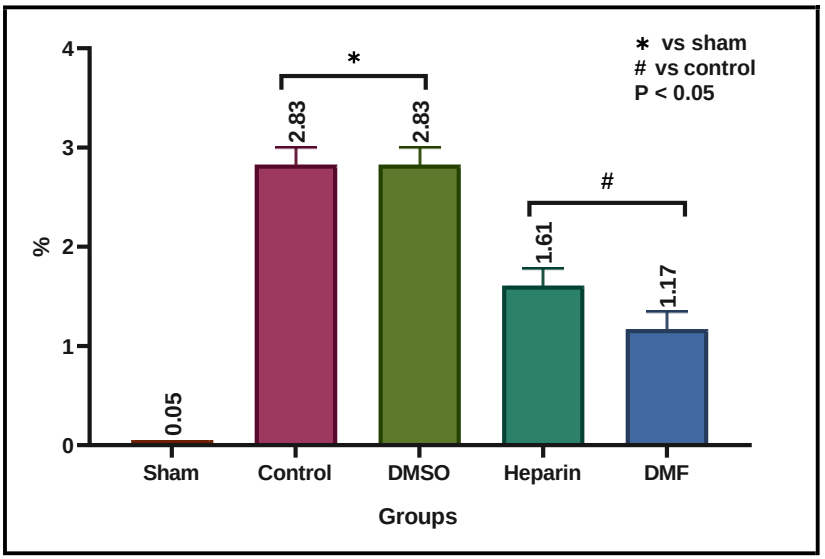


**Figure (4): the histopathological score among groups.**

## 5. DISCUSSION

ROS is a marker of oxidative stress since it is a byproduct of lipid peroxidation and is linked to cerebral ischemia/reperfusion injury. Oxidative stress impairs function and can occasionally result in neuronal death (31). Our experiment revealed that the sham group had low ROS levels, which suggested a basal level in non-ischemic brain slices. We also observed that the level of ROS increased dramatically in the untreated groups. when compared to untreated groups, the administration of heparin and DMF five minutes after ischemia dramatically decreased ROS levels. However, DMF was lowered ROS more powerful than heparin.

I/R injury causes oxidative stress, which can lead to lipid peroxidation, tissue damage, and disruption of cellular membranes. Peroxidation is reduced when treated with antioxidants following ischemia. Numerous investigations reveal that when compared to the treated group, control rats' brain tissue had higher concentrations of reactive oxygen species (ROS) (32).

Cerebral ischemia-reperfusion injury can increase oxidative mediators which cause impairment and death of neurons in the brain tissues via increase free radicals, mediators for cell injury and cell death, due to excessive generation of free radicals might deplete the store of these antioxidants that detoxified it (33).

The results of our study showed that the sham group had the lowest percentage of infarction area, indicating that there were fewer necrotic cells in non-ischemic brain slices. Additionally, we observed a significant increase in necrotic cells in untreated groups which indicated increased oxidative stress. Additionally, treatment with DMF and with less extend heparin 5 min. after ischemia significantly decreased the infarction area percent restore the neurological functions via lowering damage score that indicated the two drugs has anti-oxidant properties. I/R was interruption blood-brain barrier, edema formation (34), and induce pyknotic and dark eosinophilic neurons(35). Ischemia caused free radical generation, and the later induced neuronal damage and mitochondrial impairment (36). Ruihe Lin et al. (2016) who study the effect of DMF on middle cerebral artery occlusion and they found that DMF reduced the size of infarction about 30 % (37).

## 6. CONCLUSION

DMF reduces oxidative stress and reduces the percentage of infarction area that restores neurological functions by decreasing damage scores, which has a neuroproctive effect more powerful than heparin sulfate against cerebral I/R injury.

## 7. RECOMMENDATION

We recommended to use the DMF in combination with heparin to protect brain tissue against cerebral I/R injury.

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