Chapter 4

Pharmacological effects of Diindolylmethane in MCAO rats

4 Introduction

Diindolylmethane (DIM), a pivotal acid condensation product of indole-3-carbinol (I3C), is believed to be responsible for the pharmacological activities of I3C (Bradlow & Zeligs, 2010). I3C shows antiplatelet and antithrombotic activities (Park, 2008; Ramakrishna & Krishnamurthy, 2022; Ramakrishna, Singh, et al., 2022) and shows the antiischemic effect on middle cerebral artery occlusion injury mediated cerebral ischemia (P. Paliwal et al., 2018). Our recent findings showed that DIM exhibits antiplatelet and antithrombotic activities (objective 3) (Ramakrishna, Singh, et al., 2022). In a previous study, we found that I3C oral administration to middle cerebral artery occlusion (MCAO) rats produced high levels of DIM in cerebrospinal fluid and brains and ischemic injury mediated brain dysfunction was ameliorated with oral treatment of I3C than intravenous treatment of I3C (objective 2). These results indicate that DIM may be involved in neuroprotection conferred by I3C (Ramakrishna, Jain, & Krishnamurthy, 2022). In the recent findings, DIM has been reported to have neuroprotective effects against MCAO injury by ameliorating the inflammation and oxidative stress (Dhir et al., 2022), indicating that DIM can be used as a secondary treatment (neuroprotective agent) in managing stroke. Moreover, we have observed antiplatelet and antithrombotic properties of DIM (Ramakrishna, Singh, et al., 2022), suggesting that DIM could be used in the primary treatment of stroke. However, the antiplatelet and antithrombotic activities of DIM are beneficial to mitigating cerebral ischemia are unknown. Moreover, MCAO causes significant platelet adhesion to ischemic endothelium and subendothelial matrix resulting in secondary stroke (Kleinschnitz et al., 2007; Stegner, Klaus, & Nieswandt, 2019). Hence, ascertaining the DIM-mediated platelet aggregating inhibition property would help place the DIM in the primary or secondary stroke treatment. Therefore, in the present study, we hypothesized that DIM's antiplatelet mechanisms could be

responsible for brain protection following MCAO injury. To prove this, we have administered DIM to MCAO rats. The platelet function was assessed by measuring the platelet aggregation induced by ADP, collagen, thrombin, and arachidonic acid. The platelet aggregation inhibition mechanisms by DIM were assessed by measuring cAMP, COX-1, TXB_2, and PGE_2. Further, platelet oxidative stress (ROS and H_2O_2) and serum inflammatory markers (TNF-α and IL-6), and anti-inflammatory marker (IL-10) were estimated. Furthermore, we have evaluated the DIM neuroprotective role by measuring brain infarction, blood-brain barrier (BBB) permeability, brain water content, and histopathological abnormalities in MCAO rats. The proposed hypothesis is depicted in figure 4.1.

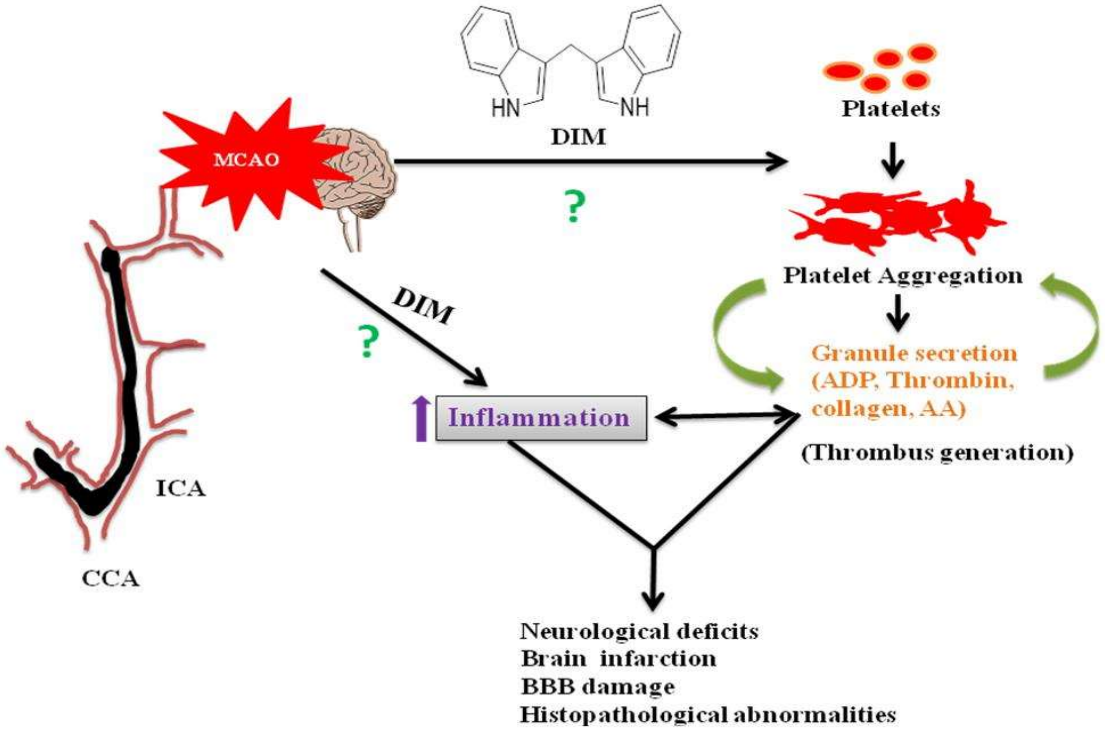

Figure 4.1. The objective of the study. DIM-diindolylmethane, CCA-common carotid artery, ICA-internal carotid artery, ADP-adenosine diphosphate, AA-arachidonic acid, MCAO-middle cerebral artery occlusion.

4.1 Materials and methods

Indole-3-carbinol (CAS no: 700-06-1, Sigma Aldrich, USA), diindolylmethane (CAS no:1968-05-4, TCI Chemicals, Japan), collagen (CAS no:9007-34-5, Sigma Aldrich, USA), adenosine diphosphate (ADP) (CAS no:16178-48-6, Sigma Aldrich, USA), arachidonic acid (CAS no:506-32-1, Sigma Aldrich, USA), thrombin (CAS no: 9002044, Sigma Aldrich, USA), dichlorodihydrofluorescein diacetate (CAS no:4091-99-0, DCFH-DA)(Sigma Aldrich, USA), and thiopentone sodium (Neon Labs, India) were purchased. Clopidogrel (CLOP) was obtained from Dr. Reddys' lab, India, as a gift sample. Cyclic adenosine monophosphate (cAMP) ELISA kit (K11-0299, KinesisDx, USA), thromboxane B2 (TXB_2) ELISA kit (501020, Cayman Chemical, USA), cyclooxygenase 1 (COX-1) ELISA kit (K11-0909, KinesisDx, USA), and prostaglandin E2 (PGE_2) (K11-0505, KinesisDx, USA) ELISA kits were obtained. Tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-10 (IL-10) were purchased from Krishgen Biosystems, India. Amplex Red kit for the estimation of hydrogen peroxide (H_2O_2) was purchased from Thermo Fisher, USA (A22188).

4.1.1 4.1.1 Experimental animals

Male Wistar rats (240-260 gm) were procured from the central animal house of the Institute of Medical Sciences, Banaras Hindu University, and Varanasi, India. Rats were housed under standard laboratory conditions, like free access to food, water, and constant light and dark cycle (12/12hr). Before experiments, two weeks acclimatization period was given to experimental animals. Approval of the Institutional animal ethics committee (Protocol no. Dean/2021/IAEC/2548) was granted before the beginning of the experiment.

4.1.2 Experimental design

Male Wistar rats (220-240 grams) were randomly divided into the following experimental groups using Microsoft Excel (standard = RAND () function), group 1: Sham, group 2:

MCAO, group 3: MCAO + DIM 12.5 mg/kg, group 4: MCAO + DIM 25 mg/kg, and group 5: MCAO + DIM 50 mg/kg, group 6: MCAO + CLOP (15 mg/kg). DIM and CLOP doses were chosen from previous studies (Ramakrishna, Singh, et al., 2022). MCAO was occluded and reperfused after two hours. DIM was orally administered just before the withdrawal of nylon monofilament, and treatment was continued for 72 hours (once daily). A total of 120 animals were used in the study. At the end of the study, the following number of animals survived; sham (n=19), MCAO (n=16), MCAO + DIM 12.5 mg/kg (n=17), MCAO + DIM 25 mg/kg (n=17), MCAO + DIM 50 mg/kg (n=18), and MCAO + CLOP (15 mg/kg) (n= 18). At the end of the experiment, blood was collected from the cardiac puncture, serum, platelet-rich plasma (PRP), and platelets were collected. PRP was investigated for platelet aggregation studies. Serum COX-1, TXB_2, PGE_2, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10) and platelet cAMP levels were measured. Neurologic deficits, brain infarction (n=4), BBB leakage (n=4), brain water content (n=4), and histopathology (n=4) were evaluated at the end of the experiment. The remaining animals were euthanized and excluded from the study..

4.1.3 MCAO surgery and drug treatment

The middle cerebral artery was occluded, as reported in **section 2.1.8**. DIM was administered just before the reperfusion and continued for three days (72 hrs). After 30 min of the final dose administration of DIM, blood was collected to separate the serum, platelets (Irfan et al., 2020), platelet-rich plasma (PRP) (Xue Li et al., 2020b), and platelet-poor plasma (PPP) (Xue Li et al., 2020b). PRP was used for platelet aggregation studies (T.-F. Cheng et al., 2020). The dosing schedule and parameters evaluated in the present study are depicted in figure 4.2.

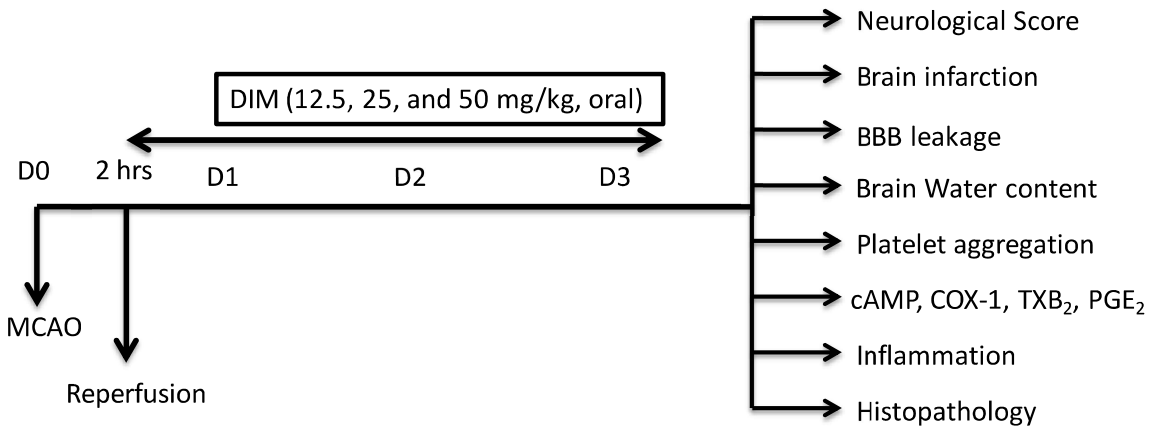

Figure 4.2. The experimental design. DIM-diindolylmethane, MCAO-middle cerebral artery occlusion, cAMP-cyclic adenosine monophosphate, COX-1-cyclooxygenase 1, TXB_2-thromboxane B2, and PGE_2- prostaglandin E2.

4.1.4 Cerebral blood flow (CBF) measurement

Alterations in CBF after MCAO was recorded as described earlier (P. Paliwal et al., 2018). CBF was recorded using a laser Doppler-speckle blood flow imaging system (Omegazone OZ-2 STD, Japan). The imaging system consists of a black sponge sheet, armstand, LSI software for recording blood flow, and LSI software for blood flow image analysis. An armstand grasps the CCD camera, lenses (ZM 10-18, MF12), and the laser unit (780 nm for measurement and 650 nm for positioning). Skull was opened by midline scalp incision and rats were positioned on a black-sponge sheet placed under the arm stand. LSI software (LSI ver.3.3, Omegawave, Inc., Tokyo, Japan) was used to record the raw speckle skull and average CBF was evaluated using LIA Software (LIA ver.3.3, Omegawave, Inc., Tokyo, Japan). The black sponge sheet does not reflect the laser light, producing clear blood flow images (Guo, Wang, & Namura, 2010; P. Paliwal et al., 2018).

4.1.5 Behavioral assessment

Neurological deficits: Neurological deficits were evaluated as mentioned in **section 2.1.9.** (Longa et al., 1989). Rotarod and grip strength analysis was performed on day 3 and day 4 between 9 am to 4 pm. ***Rotarod:*** The rotarod test assesses motor coordination abilities. Rats were trained twice daily on the rotarod (IKON Instrument, New Delhi, India) for two days with the rotation speed set at 8 revolutions per minute (rpm) on D-1 and 10 rpm on D-2 to achieve steady results. In the test session, the rotating speed on D-3 was increased to 15 rpm. Each rat's time spent keeping its balance on the moving rod was noted. Counting is stopped as soon as the rat falls from the rod and data were reported as retention time on the rotarod during three test trials (Mishra, Chandravanshi, Trigun, & Krishnamurthy, 2018). ***Grip strength:*** The animal's neuromuscular strength is measured by suspending it from a 90 cm long metal wire (1 cm diameter) with its forepaws in the middle. Two vertical supports of 50 cm in height held the horizontally fastened metal wire in place. Within 5 seconds, the control animals grasped the wire and climbed up. Grip strength was scored as follows: 0- fall off; 1- hangs onto string by two fore-paws; 2- as for 1 but also attempts to climb on string; 3- hangs onto string by two fore-paws plus one or both hind-paws; 4- hangs onto string by all four paws plus tail wrapped around the string and 5- escape from the apparatus and fall on the flat surface (cut-off time=60 s) (Meyer, Tilson, Byrd, & Riley, 1979; Mishra et al., 2018).

4.1.6 Brain infarction

Brain infarction in MCAO rats was assessed as described in section 2.1.10

4.1.7 Evaluation of blood-brain barrier integrity

Blood-brain barrier integrity was determined using the Evans blue (EB) extraction method as reported in section 2.1.11

4.1.8 Brain water content

Following MCAO ischemic reperfusion injury, brain water content was determined as described in section 2.1.12

4.1.9 Platelet aggregation

Platelet aggregation studies were performed as mentioned in section 3.1.5.

4.1.10 Platelet oxidative stress

Platelet ROS levels were determined using DCFH-DA fluorescence dye. DCFH-DA (10 μM) was added to platelets and incubated for 30 min at 37°C in the dark. Then, fluorescence was measured at 485 nm/520 nm, respectively (C. Liu et al., 2019). Platelet H_2O_2 levels were determined using an Amplex Red kit.

4.1.11 ELISA

Serum COX-1, TXB_2, PGE_2, TNF-α, IL-6, IL-10, and platelet cAMP levels were measured.

4.1.12 Histopathology

At the end of the experiment, animals were anesthetized with thiopentone sodium (40 mg/kg) and chilled saline (0.9%) was perfused through the left ventricle. Then, chilled formalin (4%, in PBS) was passed. After that, the brains were dissected, dehydrated, and paraffin-embedded. Further, brain sections (5μm) were made using a microtome (Leica Biosystems, Germany) (Pan et al., 2016). The coronal sections were then stained with hematoxylin and eosin. Cortical images were captured under 40X magnification using a bright field microscope (Olympus microscope, Japan). Viable and damaged cortical neurons of ischemic core regions were counted in a blinded fashion. Round and intact neurons are considered viable neurons represented in the black arrow (healthy neurons) and neurons that appeared in fragmented (yellow arrow), condensed (green arrow), and vacuoles (red arrow) are

considered damaged neurons. The percentage ratio of healthy neurons was calculated as follows: *healthy neurons/total neurons X 100*.

4.2 Results

4.2.1 DIM improved cerebral blood flow

Changes in CBF after treatment with DIM were depicted in figure 4.3. DIM treatment significantly improved the cerebral blood flow in the brain as revealed by two-way ANOVA and post hoc analysis among groups [$F(4, 125) = 21.68$; $P < 0.001$], time [$F(4,125) = 494.2$; $P < 0.001$], and interaction between groups and time [$F(16, 125) = 3.73$; $P < 0.001$]. We have observed a significant improvement in CBF after DIM treatment in dose-dependent manner.

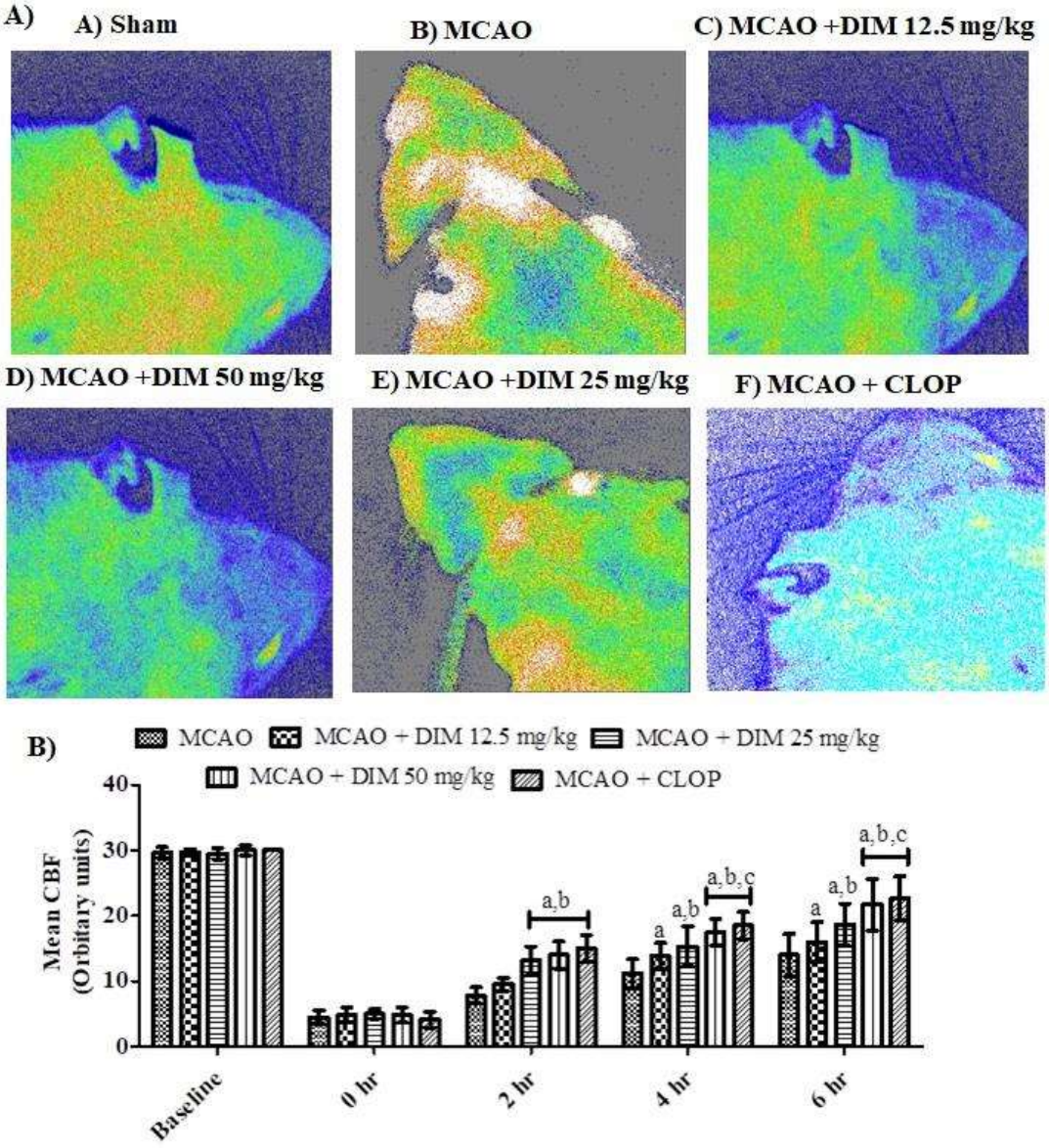

Figure 4.3. Effect of DIM treatment on CBF in MCAO rats. Figure 4.3A indicates the representative images of CBF and Figure 4.3B indicates the mean CBF. $^{a}p<0.001$ *vs. sham,* $^{b}p<0.001$ *vs. MCAO,* $^{c}p<0.001$ *vs. MCAO+DIM 12.5 mg/kg, and* $^{c}p<0.001$ *vs. MCAO+DIM 25 mg/kg. All the results are expressed as mean ± SD (n=6). Two-way ANOVA followed by Bonferroni post hoc test.*

4.2.2 DIM improved the neurobehavior in MCAO rats

DIM-mediated behavioral improvement in MCAO animals is depicted in figure 4.4. One-way ANOVA revealed significant differences among groups; neurological deficits [$F(5, 119) = 99.12$; $P < 0.001$], rotarod [$F(5, 119) = 187.1$; $P < 0.001$], and grip strength [$F(5, 119) = 81.21$; $P < 0.001$]. Post hoc analysis revealed that MCAO rats significantly increased neurological deficits and impaired rotarod and grip strength performances than sham-operated animals. DIM treatment in MCAO rats significantly reduced neurological deficits and improved rotarod and grip strength performances. Further, we did not observe significant differences between DIM 50 mg/kg and CLOP-treated MCAO rats.

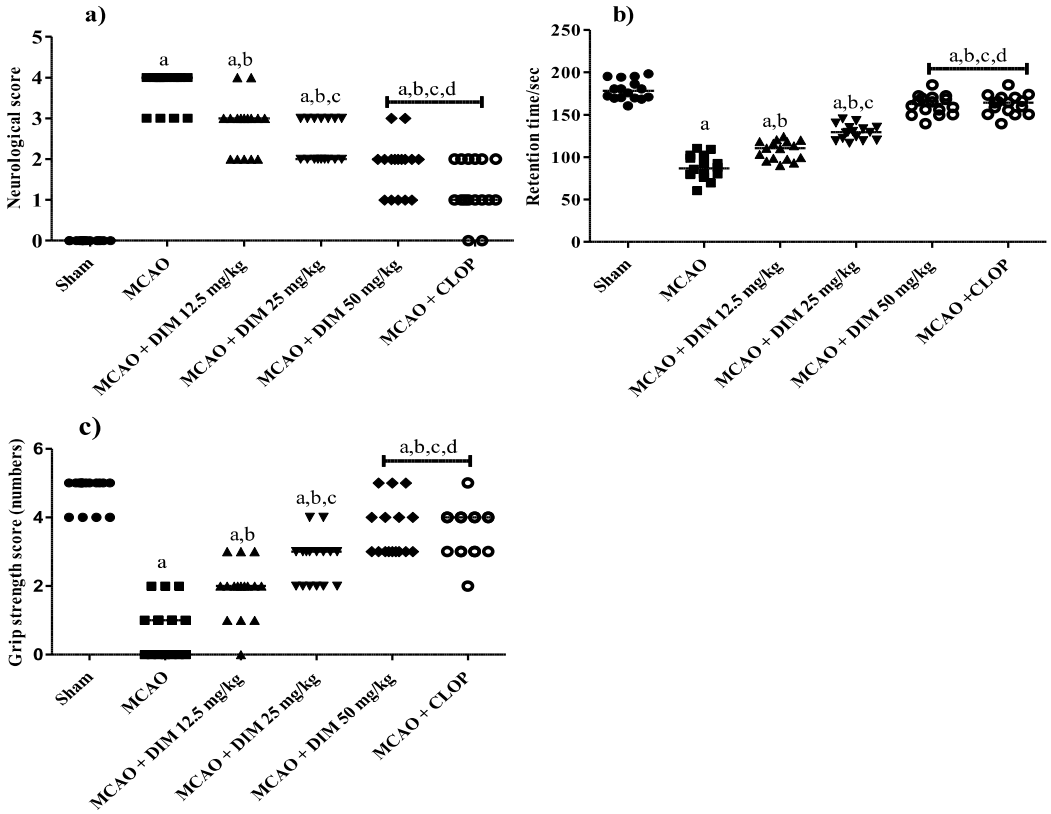

Figure 4.4. Effect of DIM treatment on MCAO-induced neurological deficits. Figures 4.4a, 4.4b, and 4.4c represent the changes in neurological deficits, rotarod, and grip strength

performances in MCAO rats. [a]p<0.001 vs. sham, [b]p<0.001 vs. MCAO, [c]p<0.001 vs. MCAO+DIM 12.5 mg/kg, and [c]p<0.001 vs. MCAO+DIM 25 mg/kg. All the results are expressed as Median (n=16). One-way ANOVA followed by Tukey's post hoc test.

4.2.3 DIM ameliorated brain infarction

DIM treatment-induced changes in brain infarction in MCAO rats are illustrated in figure 4. One-way ANOVA observed the significant differences among groups: infarct size (%) [F (5, 23) = 188.5; P < 0.001] and infarct volume [F (5, 23) = 347.3; P < 0.001]. There was no infarction identified in sham-operated animals. We found that DIM treatment in MCAO rats significantly decreased brain infarction and infarct volume than in untreated MCAO rats. DIM 50 mg/kg treated MCAO rats did not show a significant difference compared to CLOP-treated MCAO rats.

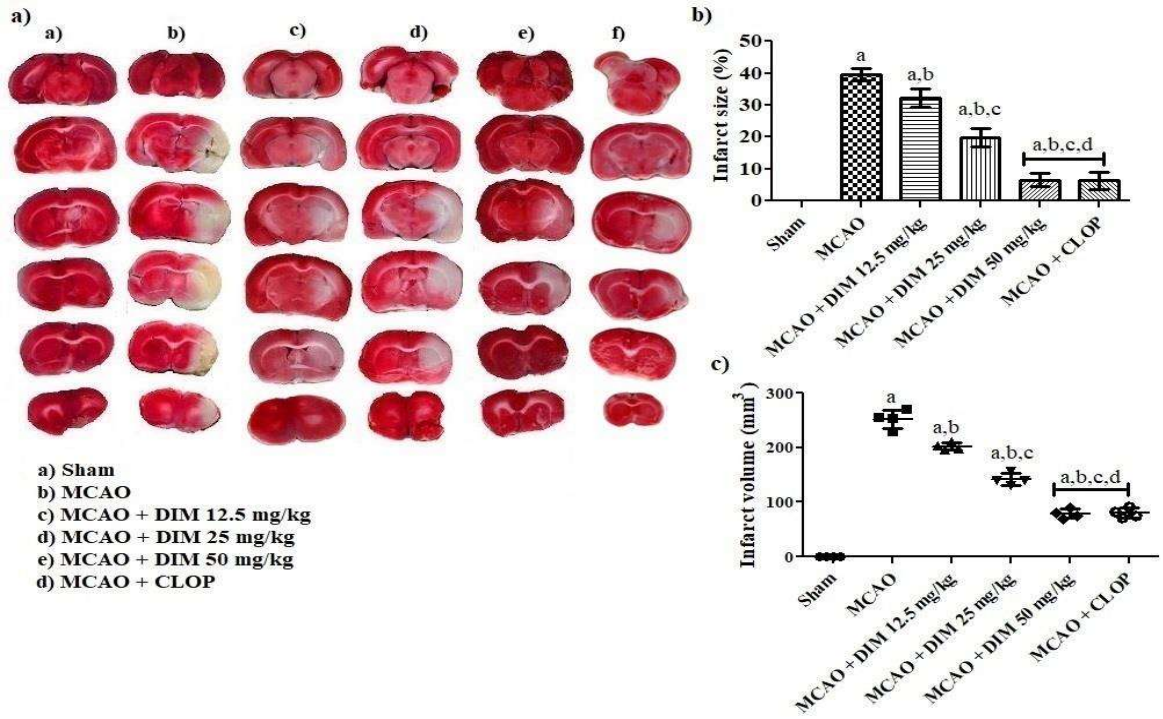

Figure 4.5. Effect of DIM treatment on brain infarction. Figures 4.5a, 4.5b, and 4.5c indicate the representative brain infarction images, infarction size (%), and infarct volume.

[a]p<0.0001 vs. sham, [b]p<0.001 vs. MCAO, [c]p<0.001 vs. MCAO+DIM 12.5 mg/kg, and [c]p<0.001 vs. MCAO+DIM 25 mg/kg. All the results are expressed as mean ± SD (n=4). One-way ANOVA followed by Tukey's post hoc test.

4.2.4 **DIM improved BBB** integrity

DIM-induced changes in BBB permeability in MCAO rats are depicted in figure 4.6. One-way ANOVA identified that there were significant differences among groups: [F (5, 23) = 137.8; P < 0.001]. Post hoc analysis revealed that MCAO rats significantly increased BBB permeability of EB than sham animals. DIM dose-dependently reduced the BBB permeability of EB in MCAO treated rats. DIM 50 mg/kg rats did not show significant differences compared to CLOP treated rats.

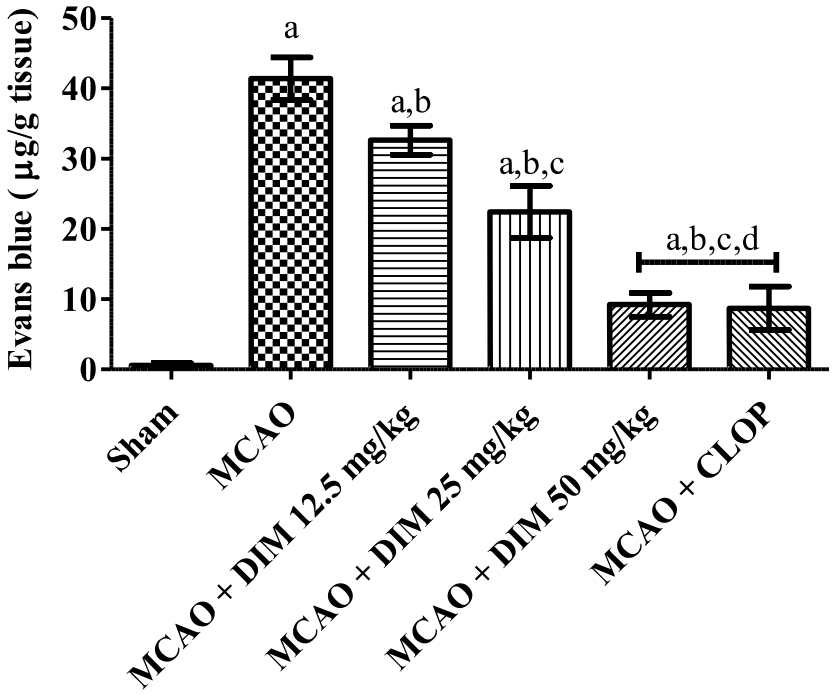

Figure 4.6. Effect of DIM treatment on BBB permeability of EB. Figures 4.6a represent the EB concentration in the brain. [a]p<0.001 vs. sham, [b]p<0.001 vs. MCAO, [c]p<0.001 vs.

MCAO+DIM 12.5 mg/kg, and [c]p<0.001 vs. MCAO+DIM 25 mg/kg. All the results are expressed as mean ± SD (n=4). One-way ANOVA followed by Tukey's post hoc test.

4.2.5 DIM decreased brain water content

Changes in the brain water levels after the treatment with DIM in MCAO rats were illustrated in figure 4.7. One-way ANOVA observed the significant differences among groups: [F (5, 23) = 70.70; P < 0.001]. We have observed a significant increase in brain water content in MCAO rats than in sham-operated animals. DIM treatment dose-dependently ameliorated the brain water levels in MCAO treated rats. Moreover, we did not observe significant differences between DIM 50 mg/kg and CLOP treated MCAO rats.

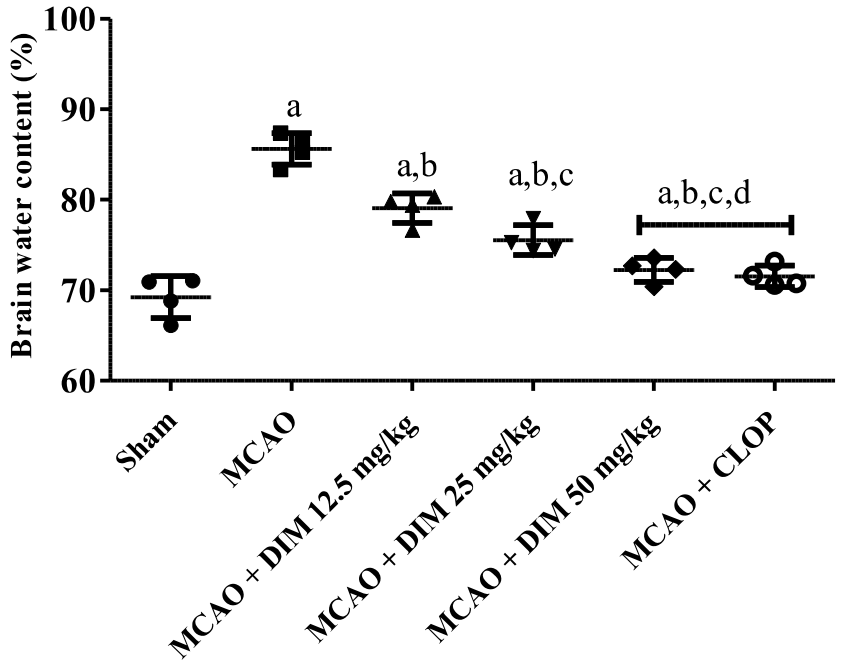

Figure 4.7. Effect of DIM treatment on brain water content. [a]p<0.001 vs. Sham, [b]p<0.001 vs. MCAO, [c]p<0.001 vs. MCAO + DIM 12.5 mg/kg, [d]p<0.001 vs. 25mg/kg, and [e]p<0.001 vs. 50 mg/kg. All the results are expressed as Mean ± SD (n=4). One-way ANOVA followed by Tukey's post hoc test.

4.2.6 DIM inhibited platelet aggregation

Antiplatelet aggregation properties of DIM in MCAO rats were depicted in figure 4.8. We have observed significant differences in platelet aggregation among groups: ADP [$F(4, 29) = 371.9$; $P < 0.001$], collagen [$F(4, 29) = 168.9$; $P < 0.001$], thrombin [$F(4, 29) = 258.2$; $P < 0.001$], and arachidonic acid [$F(4, 29) = 162.7$; $P < 0.001$]. Post hoc analysis revealed that DIM ameliorated the ADP, collagen, thrombin, and AA-induced platelet aggregation in a dose-dependent manner. Further, we found no significant differences between DIM 50 mg/kg and CLOP-treated MCAO rats against ADP-induced platelet aggregation. Moreover, DIM 50 mg/kg treatments showed a significant decrease in collagen, thrombin, and arachidonic acid-induced platelet aggregation in PRP of MCAO rats.

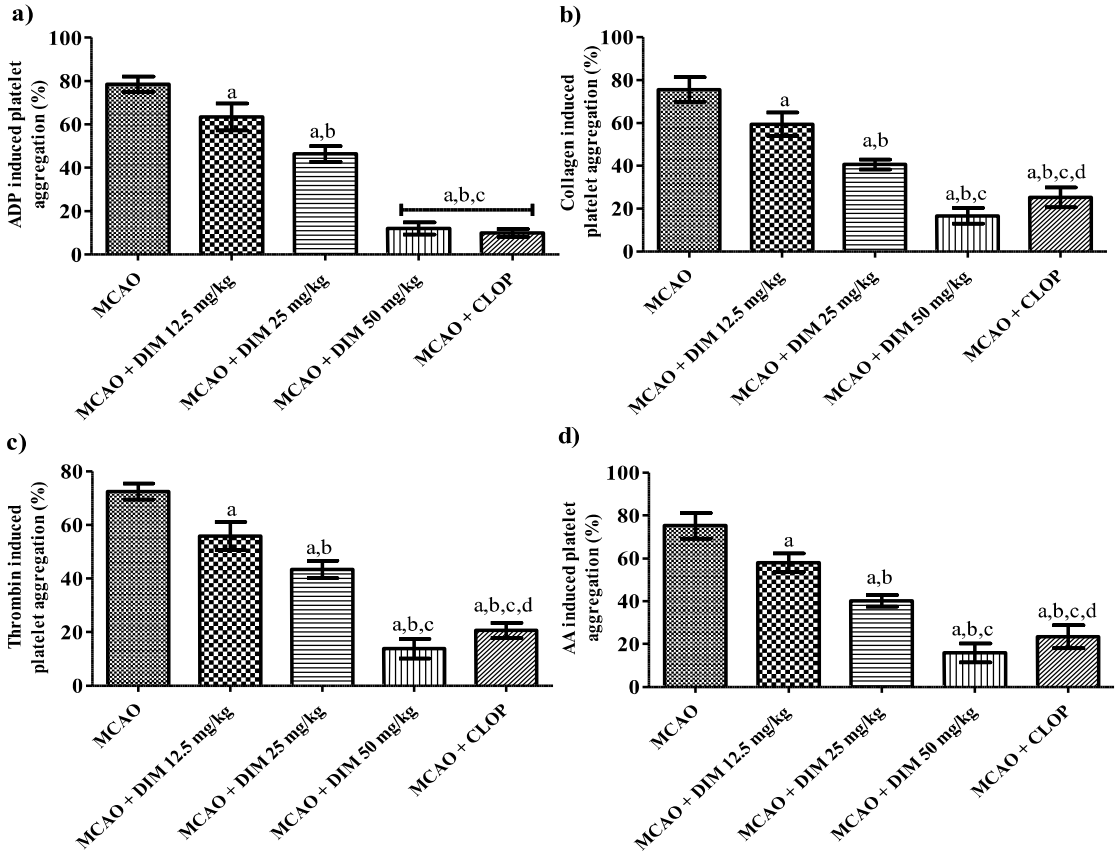

Figure 4.8. Effect of DIM treatment on platelet aggregation. Figures 4.8a, 4.8b, 4.8c, and 4.8d represent ADP, thrombin, collagen, and AA-induced platelet aggregation. [a]p<0.001 vs. MCAO, [b]p<0.001 vs. MCAO+DIM 12.5 mg/kg, and p<0.001 vs. MCAO+DIM 25 mg/kg, and [d]p<0.001 vs. MCAO+DIM 50 mg/kg. All the results are expressed as mean ± SD (n=6). One-way ANOVA followed by Tukey's post hoc test.

4.2.7 DIM improved cAMP and inhibited COX-1, TXB_2, and PGE_2

Changes in cAMP, COX-1, TXB_2, and PGE_2 levels after treatment with DIM in MCAO rats are depicted in figure 4.9. One-way ANOVA observed that there were significant differences among groups: cAMP [$F(5, 35) = 79.08$; $P < 0.001$], COX-1 [$F(5, 35) = 173.8$; $P < 0.001$], TXB_2 [$F(5, 35) = 72.88$; $P < 0.001$], and PGE_2 [$F(5, 35) = 227.8$; $P < 0.001$]. Post hoc studies revealed that MCAO rats significantly decreased cAMP and increased the COX-1, TXB_2, and PGE_2 as compared to sham-operated animals. DIM-treated MCAO rats significantly increased the cAMP and decreased the COX-1, TXB_2, and PGE_2 than MCAO rats. However, DIM 50 mg/kg treated rats did not significantly differ in cAMP levels as compared to CLOP treated MCAO rats. But, DIM 50 mg/kg treated rats show significantly lower COX-1, TXB_2, and PGE_2 than CLOP treated MCAO rats.

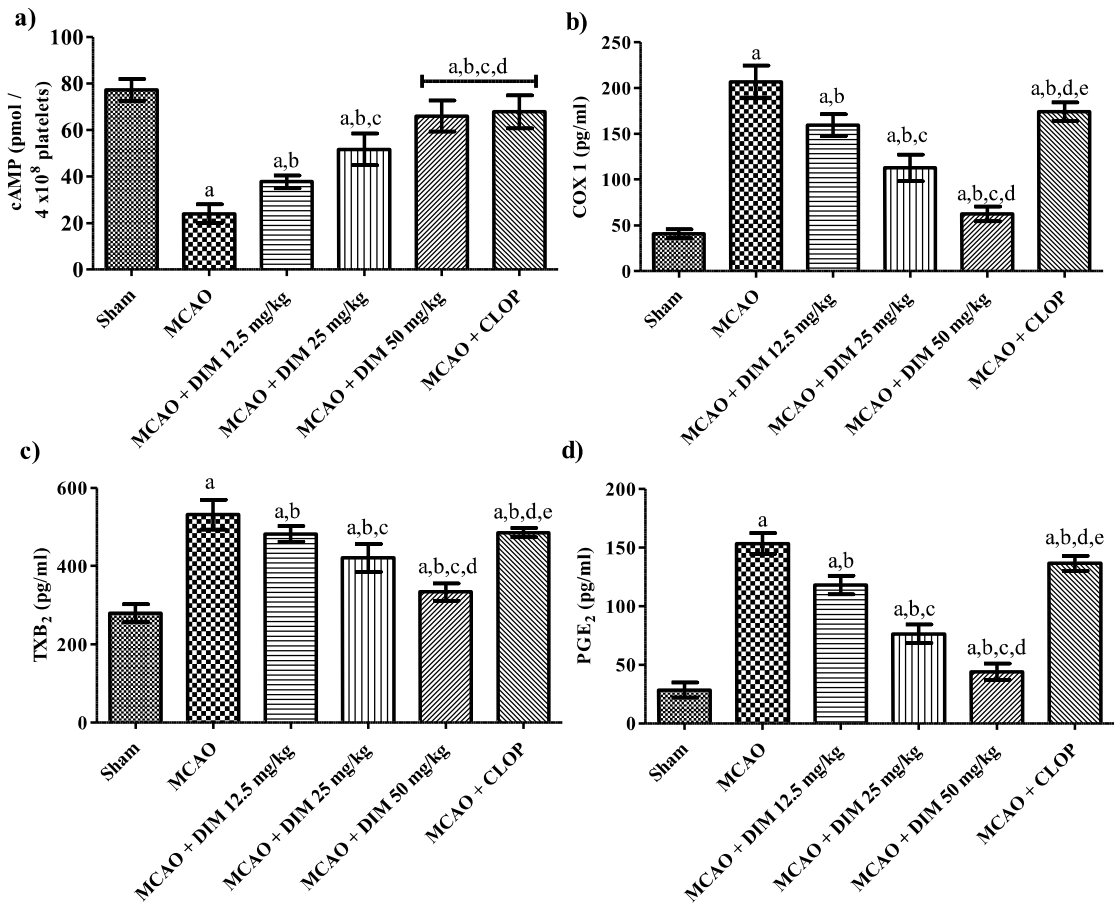

Figure 4.9. Effect of DIM treatment on cAMP, COX-1, TXB_2, and PGE_2 levels. *Figures 4.9a, 4.9b, 4.9c, and 4.9d represent the cAMP, COX-1, TXB_2, and PGE_2 levels. [a]$p<0.001$ vs. Sham, [b]$p<0.001$ vs. MCAO, [c]$p<0.001$ vs. MCAO + DIM 12.5 mg/kg, [d]$p<0.001$ vs. 25mg/kg, and [e]$p<0.001$ vs. 50 mg/kg. All the results are expressed as mean ± SD (n=6). One-way ANOVA followed by Tukey's post hoc test.*

4.2.8 DIM mitigated platelet oxidative stress

Alterations in platelet ROS and H_2O_2 were reported in figure 4.10. One-way ANOVA studies observed substantial differences among groups; ROS [$F(5, 35) = 157.5$; $P < 0.001$], H_2O_2 [$F(5,35) = 72.64$, $P < 0.001$]. MCAO rats significantly had higher platelet ROS and H_2O_2 levels than sham animals. DIM treatment in MCAO rats dose-dependently alleviated the platelet

ROS and H_2O_2 levels. DIM 50 mg/kg significantly mitigated the platelet ROS and H_2O_2 levels than CLOP-treated MCAO rats.

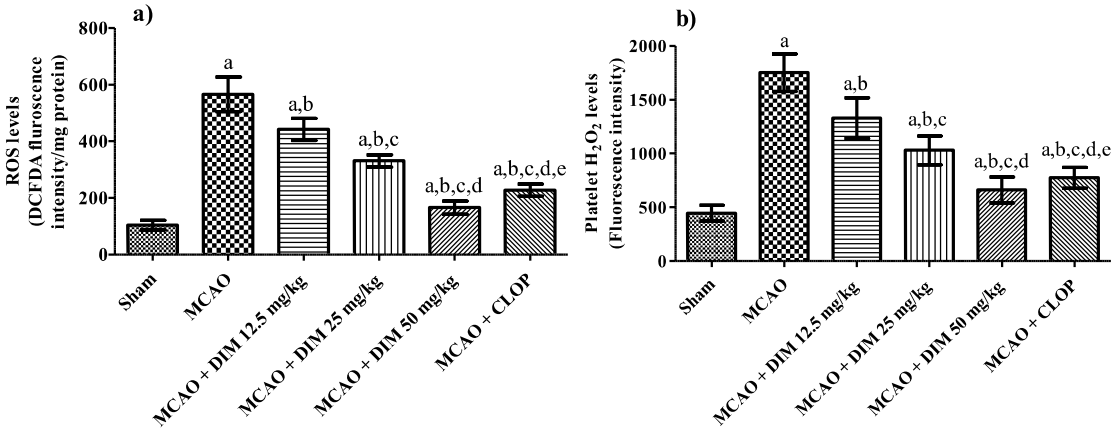

Figure 4.10. Effect of DIM treatment platelet ROS and H_2O_2 levels. Figures 4.10a and 4.10b represent the platelet ROS and H_2O_2 levels, respectively. $^{a}p<0.001$ vs. Sham, $^{b}p<0.001$ vs. MCAO, $^{c}p<0.001$ vs. MCAO + DIM 12.5 mg/kg, $^{d}p<0.001$ vs. 25mg/kg, and $^{e}p<0.001$ vs. 50 mg/kg. All the results are expressed as Mean ± SD (n=6). One-way ANOVA followed by Tukey's post hoc test.

4.2.9 DIM alleviated inflammation

Alterations in TNF-1α, IL-6, and IL-10 in MCAO rats are depicted in figure 4.11. One-way ANOVA observed that there were significant differences among groups: TNF-1α [F (5, 35) = 88.99; P < 0.001], IL-6 [F (5, 35) = 146.1; P < 0.001], and IL-10 [F (5, 35) = 149.3; P < 0.001]. MCAO rats had significantly increased serum TNF-1α, IL-6, and decreased IL-10 compared to sham-operated animals. DIM-treated MCAO rats significantly decreased TNF-1α, IL-6 and increased IL-10 than untreated MCAO rats. DIM 25 and 50 mg/kg treated rats significantly decreased TNF-1α, IL-6, and increased IL-10 than CLOP-treated MCAO rats.

There were no significant differences in TNF-1α, IL-6, and IL-10 between DIM 12.5 mg/kg and CLOP-treated MCAO rats.

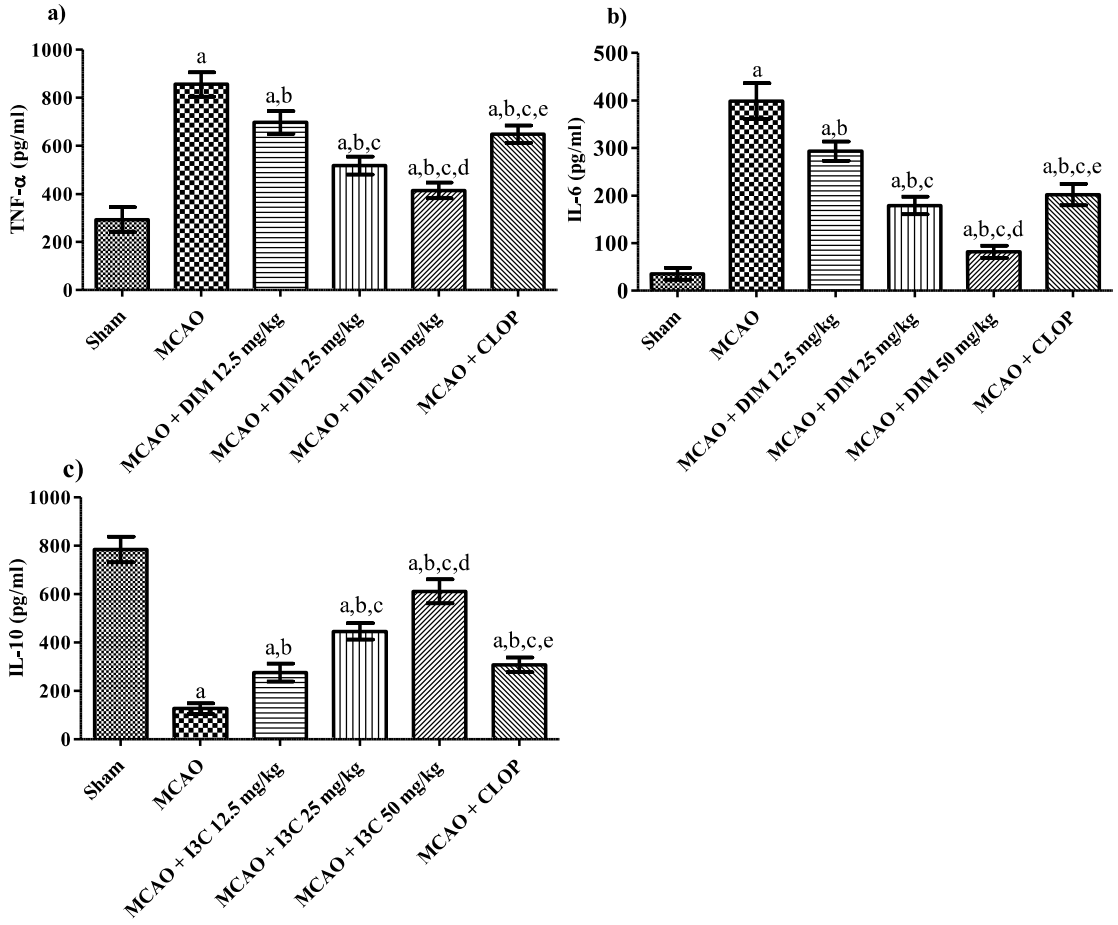

Figure 10. Effect of DIM treatment on inflammation. Figures 10a,10b, and 10c represent the TNF-1α, IL-6, and IL-10 levels. [a]p<0.001 vs. Sham, [b]p<0.001 vs. MCAO, [c]p<0.001 vs. MCAO + DIM 12.5 mg/kg, [d]p<0.001 vs. 25mg/kg, and [e]p<0.001 vs. 50 mg/kg. All the results are expressed as mean ± SD (n=6). One-way ANOVA followed by Tukey's post hoc test.

4.2.10 DIM ameliorated histopathological abnormalities

Changes in histopathological abnormalities after treatment with DIM in MCAO rats are depicted in figure 4.12. One-way ANOVA reported significant differences among groups: [F (5, 23) = 41.30; P < 0.001]. We have found that MCAO rats significantly decreased the

percentage ratio of healthy neurons than sham-operated neurons. Further, DIM-treated MCAO rats significantly increased the healthy neuron percentage than untreated MCAO rats. However, DIM 50 mg/kg and CLOP treated rats show a similar percentage of healthy neurons.

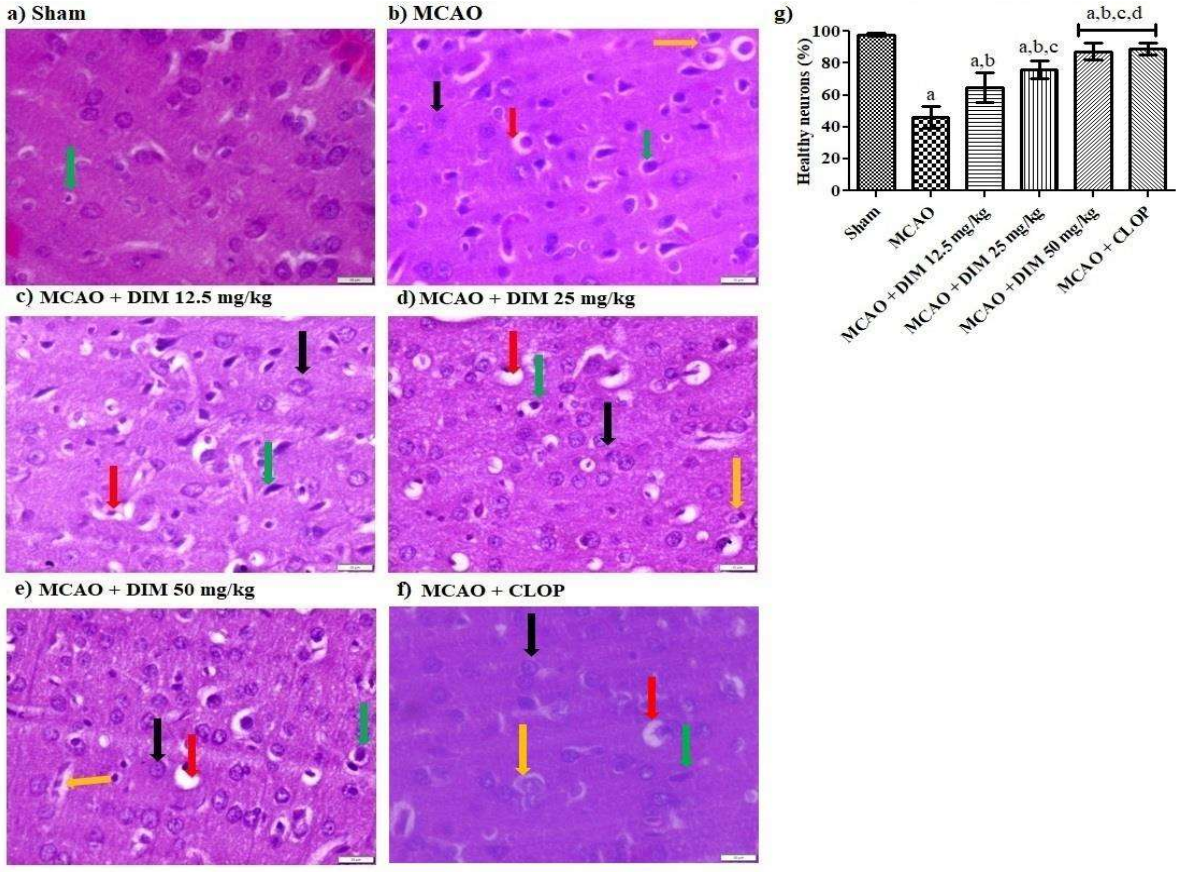

Figure 4.12. Effect of DIM treatment on histopathological abnormalities in MCAO rats. Figures 4.12a, 4.12b, 4.12c, 4.12d, 4.12e, and 4.12f indicate the cortex's representative H and E staining images. Figure 11g represents the percentage ratio of healthy neurons. Healthy neurons (black arrow), *fragmented neurons (yellow arrow), condensed neurons (green arrow), and vacuolated neurons (red arrow) are indicated in representative images.* $^{a}p<0.001$ *vs. sham,* $^{b}p<0.001$ *vs. MCAO,* $^{c}p<0.001$ *vs. MCAO+DIM 12.5 mg/kg, and*

[c]p<0.001 vs. MCAO+DIM 25 mg/kg. All the results are expressed as mean ± SD (n=4). One-way ANOVA followed by Tukey's post hoc test.

4.3 Discussion

The present study reports the DIM protective role against ischemic stroke. DIM's ability to suppress platelet aggregation could be attributed to an increase in platelet cAMP and the inhibition of the COX-1 enzyme-mediated increase of TXB_2 and PGE_2. DIM confers neuroprotection by inhibiting platelet aggregation, platelet-mediated oxidative stress, and inflammation could alleviate neurological deficits, brain infarction, BBB leakage, brain water content, histopathological abnormalities, and restore the cerebral cortex blood flow. Therefore, the present study reported that the DIM protected the brain from MCAO and reperfusion injury.

MCAO and reperfusion injury model is a widely used preclinical animal model which shares the clinical features of stroke in humans (Longa et al., 1989). Since MCAO deprives the brain of glucose, oxygen, and other nutrients, it develops a persistent infarction as a result of necrosis (F. Liu et al., 2018; Shah et al., 2019) and the stroke therapy academic industry roundtable (STAIR) is recommended to alleviate the brain infarction by neuroprotective compounds (Fisher et al., 2009; STAIR, 1999). We found that DIM reduced brain infarction in MCAO rats, implying that DIM prevents necrosis produced by MCAO injury. MCAO causes BBB disruption leading to loss of CNS homeostasis (Abdullahi, Tripathi, & Ronaldson, 2018; T. Liu, Zhang, Yu, Shen, & Xia, 2014). The EB levels are directly proportional to BBB damage (Goldim, Della Giustina, & Petronilho, 2019). We found that DIM reduced EB levels in MCAO rats, suggesting that DIM maintains BBB integrity in MCAO rats. MCAO causes BBB failure, and subsequent leakage around the BBB increases cerebral vascular permeability, leading to brain edema (Jin et al., 2008; Sandoval & Witt,

2008), leading to a rise in intracranial pressure, causing tissue and blood vessel compression and nerve function impairment (Ioannides, Tadi, & Naqvi, 2017). DIM treatment significantly reduced brain edema in MCAO rats, indicating that DIM prevents excessive water accumulation in the brain and lowers intracranial pressure. Furthermore, decreased water levels following DIM treatment could be attributable to the inhibition of BBB leakage. Moreover, in MCAO rats, we found a significant increase in the percentage of intact neurons in cortical regions of the ischemic core part of the brain, implying that DIM can reduce the structural abnormalities caused by MCAO injury. Because MCAO frequently compromises neuronal function, determining neurological deficits following MCAO is critical for assessing the level of neuronal damage, sensory-motor function, and MCAO occlusion success (Srinivasan & Sharma, 2011). We found that DIM treatment alleviated neurological impairments and improved rotarod and grip strength performance in MCAO rats, suggesting that DIM can restore sensory motor neurological function after a stroke. These results suggesting that DIM can be a potential neuroprotective compound to treat ischemic stroke.

Platelets bind to damaged endothelium via glycoprotein VI (GPVI) receptors, resulting in the formation of a platelet plug (Onselaer et al., 2020). Previous reports found that DIM reduces collagen-induced platelet aggregation by modulation of GPVI receptors (Ramakrishna, Singh, et al., 2022). Similarly, in the present study, DIM treatment in MCAO rats ameliorated collagen-induced platelet aggregation, indicating that DIM inhibited the MCAO injury that caused platelet adhesion to endothelial cells due to reducing the contact between collagen and platelets. Platelet aggregation is mediated by the P_2Y_{12} receptors, which are stimulated by ADP produced by active platelets. The activation of the ADP-P_2Y_{12} receptor complex-mediated platelet aggregation is evidenced by P_2Y_{12} reduction in adenylyl cyclase activity and decreased platelet cAMP (Xiaohua Li et al., 2020). In our previous

findings, DIM significantly inhibited ADP-induced platelet aggregation and enhanced cAMP by modulating the interaction between ADP and P_2Y_{12} receptors (Ramakrishna, Singh, et al., 2022). Similar to previous findings, we found that DIM mitigated the ADP-induced platelet aggregation in MCAO rats. In support of these observations, we also found a significant elevation of platelet cAMP with DIM treatment in MCAO rats, suggesting that the DIM impedes the ADP-P_2Y_{12} receptors complex interaction. Platelet aggregation is also regulated by arachidonic acid metabolites such as COX-1, TXB_2, and PGE_2 (Sakata et al., 2013). Furthermore, after MCAO ischemic injury, activation of the arachidonic pathway raises TXB_2 levels leading to thrombus generation (Xu et al., 2021). *Ex vivo* data from this study suggested that DIM ameliorates the AA-induced platelet aggregation and inhibits the COX-1 enzyme and its products such as TXB_2 and PGE_2. Furthermore, thrombin synthesis and its induced platelet aggregation are terminal processes involved in thrombus development (Shin, Kwon, Irfan, Rhee, & Lee, 2020). Thrombin stimulates the platelet granule release such as ADP and collagen, which further activates the GP IIb/IIIa receptor to enhance fibrinogen binding to platelets in clot retraction (Jiansong Huang et al., 2019; Nieswandt, Varga-Szabo, & Elvers, 2009; Seiffert et al., 2002). We found that DIM significantly reduced thrombin-induced platelet aggregation, implying that DIM inhibited thrombin and its downregulation of proteins involved in platelet aggregation and thrombus formation. P_2Y_{12} receptors, in particular, play a critical role in platelet recruitment to the site of damage, potentiating platelet aggregation and promoting granule release, which eventually helps to stabilize clots (S. Kim & Kunapuli, 2011).

Moreover, P_2Y_{12} receptors are stimulated by several platelets aggregating agents such as thrombin, collagen, and TXA_2 (Dorsam & Kunapuli, 2004; S. Kim & Kunapuli, 2011). In previous findings, we found that DIM modulates the interaction between ADP and P_2Y_{12}

receptors and collagen and GP VI receptors (Ramakrishna, Singh, et al., 2022). The current investigation found that DIM decreased TXB_2 production and increased platelet cAMP levels, which is consistent with previous findings and suggests that DIM reduces platelet aggregation in MCAO rats in multiple ways.

Platelets produce more reactive oxygen species (ROS) during the platelet activation phase, which promotes platelet aggregation and activation (Qiao et al., 2018). Moreover, MCAO reperfusion injury elevates the ROS, might cause the acceleration of platelet aggregation and contributes to thrombus generation (Fu et al., 2021). As a result of scavenging lipid peroxides and free radicals that damage vascular endothelium, antioxidants may diminish the platelet activity. We have seen a significant decrease in platelet ROS and H_2O_2 after treatment with DIM in prior studies (Ramakrishna, Singh, et al., 2022). Similarly, DIM significantly reduced ROS and H_2O_2 levels in MCAO rats' platelets, implying that DIM's antioxidant properties are also advantageous for platelet aggregation and neuroprotection. Furthermore, theoretically, antioxidants may have antiplatelet aggregation and anticoagulant properties (Jang et al., 2014). As a result of these observations, we assume that DIM's antiplatelet effect is mediated via its antioxidant activity. Moreover, platelet activation and aggregation promote inflammation, further aggravating platelet aggregation and microvascular dysfunction, leading to thrombus generation (Stokes & Granger, 2012). TNF-α, a pro-inflammatory cytokine, stimulates platelet activation factor (Rabinovici et al., 1990), arachidonic acid pathway, and promotes collagen-induced platelet aggregation (Pignatelli et al., 2005). Another pro-inflammatory cytokine, IL-6, causes platelet hyperactivation and accelerates thrombosis (Mutlu et al., 2007; Peng, Friese, George, Dale, & Burstein, 1994). MCAO injury also significantly elevates the inflammation process, further accelerating ischemic brain injury (Zhu et al., 2022). Therefore, inhibition of these

inflammatory markers and increasing anti-inflammatory markers are essential to mitigate platelet aggregation, thrombosis, and ischemic injury. Previous findings reported that DIM mitigated the lipopolysaccharide-induced inflammation by reducing the TNF-α and IL-6 (H. W. Kim et al., 2014; Luo, Yang, Cao, & Guan, 2018). Similar to these findings, we found a significant decrease in serum inflammatory markers like TNF-α and IL-6, as well as an increase in the anti-inflammatory marker IL-10, implying that DIM reduces inflammation in MCAO rats, thereby inhibits the platelet aggregation and thrombus generation. At the same time, DIM ameliorated the inflammation, arachidonic acid and collagen-induced platelet aggregation, indicating that DIM was involved in ameliorating platelet mediated inflammation and vice versa.

Previous findings indicate that DIM treatment exerts neuroprotection against MCAO injury by ameliorating oxidative stress and inflammation (Dhir et al., 2022). Despite many mechanisms, platelet aggregation and activation are the fundamental events in the development of thrombus formation that lead to ischemic stroke (Powers et al., 2019). Earlier, we found that DIM exhibited antiplatelet and antithrombotic activities (Ramakrishna, Singh, et al., 2022). Similarly, we have observed platelet aggregation inhibition with the treatment of DIM. Therefore, DIM's neuroprotectivity is attributed to platelet aggregation and thrombosis mitigation.

In stroke patients, ischemic stroke recurrence is common, and early blocking of the spread of damage from injured neurons to surrounding neurons could protect the brain against subsequent ischemia injury (Chang & Ho, 2019; Del Brutto et al., 2019). Antiplatelet and antithrombotic therapies are widely recommended to mitigate the recurrence of ischemic stroke, thereby protecting the brain from ischemic damage (Fisher et al., 2009; Powers et al., 2019). Moreover, intraperitoneal administration of DIM protected the brain from MCAO

injury (Dhir et al., 2022). However, clinically, stroke is treated with intravenous or oral administration of antiplatelet and antithrombotic drugs (Bansal et al., 2013). We found the oral treatment of DIM exhibited platelet aggregation in MCAO rats. Therefore, these findings indicate that oral therapy is an advantage over i.v. treatment. As reported earlier, DIM did not cause thrombolysis and cannot be a substitute for rtPA (Ramakrishna, Singh, et al., 2022). As a result, DIM can be used as a primary treatment in stroke management. Additionally, DIM's antioxidant and anti-inflammatory mechanisms are advantageous for mitigating ischemic injury. As a result, our preclinical findings suggest that DIM can be used in primary treatment as an antiplatelet and antithrombotic agent and used as a neuroprotectant in secondary treatment of stroke.

4.4 Summary

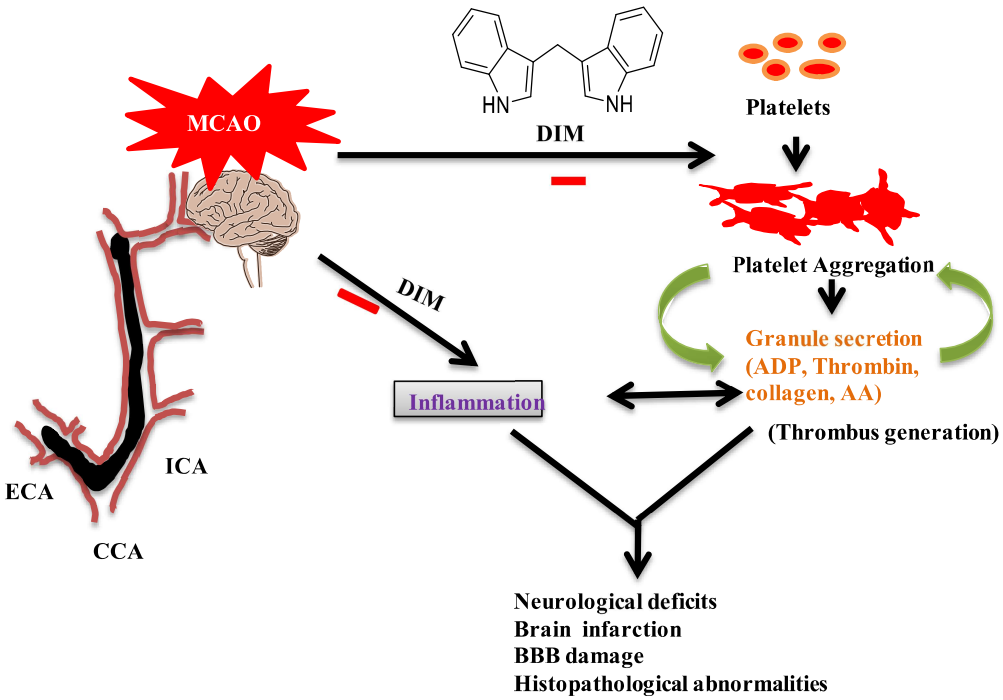

Figure 4.13. The pharmacological effects of DIM in MCAO rats.

- Repeated dose of DIM ameliorated the brain infarction, BBB permeability, and brain edema in MCAO rats.

- DIM improved the sensory-motor reflexes in MCAO rats.
- DIM inhibited the platelet aggregation by ameliorating COX-1, TEB2, and PGE2 and improved cAMP levels in MCAO rats.
- DIM treatment reduced oxidative stress, inflammation, and restore cortical architecture of MCAO rats.
- Therefore, the present study demonstrates that DIM antiplatelet and antithrombotic activities are responsible for neuroprotection in MCAO rats.