Chapter 5

Evaluation of mitochondrial based mechanisms by Indole-3-carbinol in MCAO rats

5 Introduction

I3C is known to inhibit platelet aggregation, thrombus generation, and improve the neurobehavioral outcomes in MCAO rats (P. Paliwal et al., 2018). Our earlier findings suggest that I3C can cross the blood-brain barrier (BBB) in both sham and MCAO rats, implying that I3C can have central effects (objective 2) (Ramakrishna, Jain, et al., 2022). Moreover, MCAO I/R injury causes severe neuronal damage. Therefore, centrally, neuronal damage due to MCAO has to minimize. However, it is unclear how I3C acts centrally to protect neurons from MCAO injury. Further, MCAO injury produces significant brain mitochondrial damage, resulting in energy deficiency, redox imbalance, structure and morphology of mitochondrial membrane, oxidative stress, and mitochondrial death (S. Chen et al., 2017; Y. Gao et al., 2016; Mu et al., 2020). Therefore, restoring the mitochondria function in ischemic stroke (Guan et al., 2018; J.-L. Yang, Mukda, & Chen, 2018) may have pharmacological effects.

AMPK is the crucial regulator of cellular energy, mitochondrial function, oxidative stress, inflammation, and cell survival (Jiang et al., 2018). AMPK activation enhances the mitochondrial biogenesis proteins, including peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α) (Ashabi, Khodagholi, Khalaj, Goudarzvand, & Nasiri, 2014). Activation of AMPK/PGC1-α has been reported to protect the brain from cerebral ischemia by stimulating mitochondrial biogenesis (MB) (Ashabi et al., 2014; Jiang et al., 2018; L. Li et al., 2016; B. Liu et al., 2019). Stimulation of PGC-1α promotes the upregulation of the nuclear respiratory factors (NRF1 and NRF2) (L. Li et al., 2016; Wen, Hu, Tang, & Hu, 2020). NRF1 activates the mitochondrial transcriptional factor A (tFAM), which promotes the mitochondrial DNA (mtDNA) transcription and replication (L. Li et al., 2016). Simultaneously, NRF 2 activation causes the upregulation of antioxidant enzyme levels (Gureev et al., 2019). Moreover, in cerebral ischemia, the elevation of oxidative stress (L. Li et al., 2016) and inflammation (Fan et al., 2020; Kes, Simundic, Nikolac, Topic, & Demarin, 2008) impairs mitochondrial biogenesis (Cherry & Piantadosi, 2015). Therefore, cerebral ischemia-mediated deleterious effects on mitochondria (S. Chen et al., 2017) can be hampered by increasing mitochondrial biogenesis (Mu et al., 2020; Popov, 2020) and alleviating oxidative stress and inflammation (Youngshim Choi et al., 2012).

I3C is reported to activate the AMPK/PGC-1α pathway (H.-S. Choi et al., 2014; Youngshim Choi et al., 2012; Y Choi et al., 2013), improve the AMPK-α dependent energy metabolism (W. Deng et al., 2014), and exhibit antioxidant and anti-inflammatory activities (Ramakrishna & Krishnamurthy, 2022). Activation of the AMPK/PGC1-α pathway stimulates mitochondrial biogenesis. Based on the above reports, we presume that I3C-mediated activation of the AMPK/PGC-1α pathway will stimulate mitochondrial biogenesis thereby alleviating mitochondrial dysfunction, oxidative stress, and inflammation associated with cerebral ischemia.

Therefore, the current study investigated the neuroprotective mechanisms of I3C protection against ischemic brain injury. We administered I3C to focal cerebral ischemic rats for 7 days, and neuropathological abnormalities, mitochondrial biogenesis, oxidative stress, inflammation, and apoptosis were determined. The proposed hypothesis is illustrated in figure 5.1

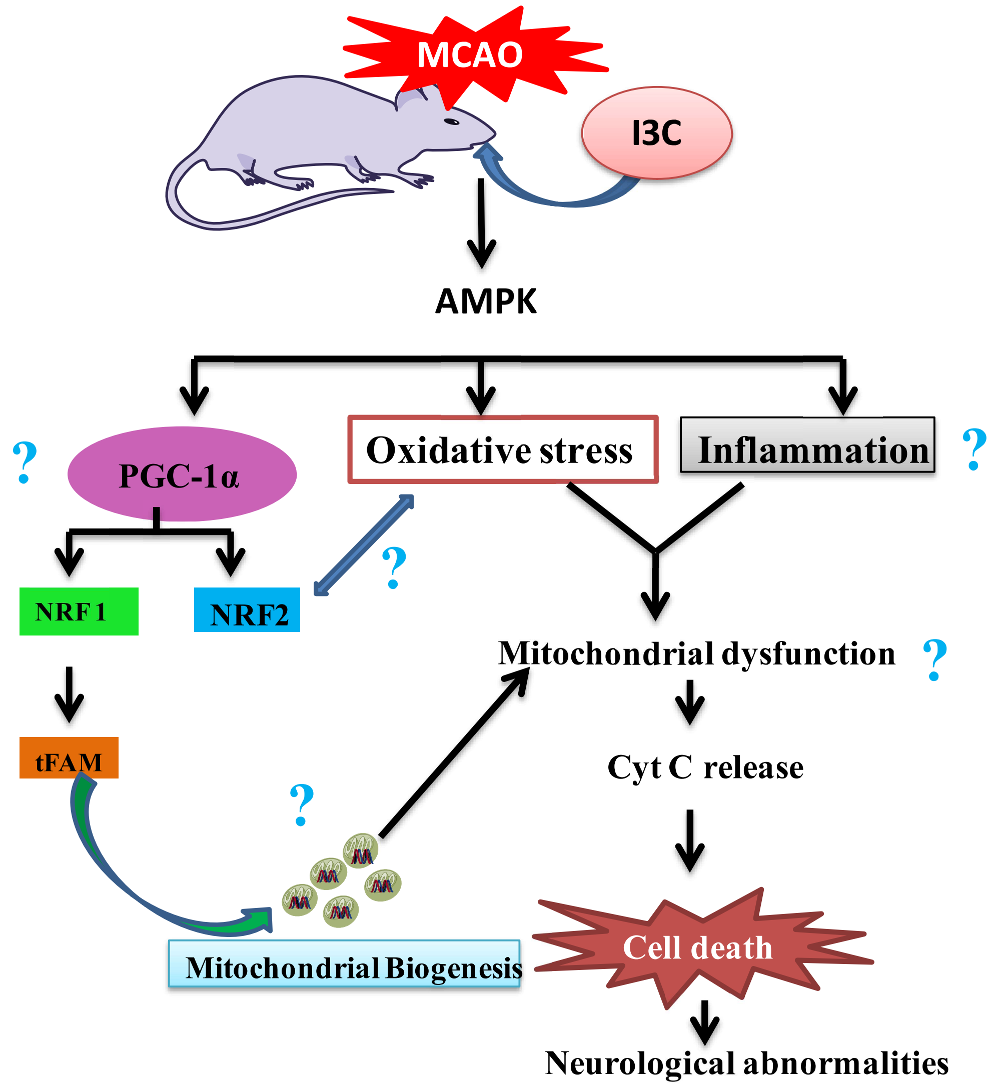

Figure 5.1. The proposed hypothesis.

5.1 Materials & Methods

5.1.1 Animals

Adult male Wistar rats weighing 220-240 grams were obtained from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University (IMS-BHU). All animals were housed in clean polypropylene cages and maintained standard environmental conditions (temperature of 25±1 °C, 45–55% relative humidity, and 12:12-h light/dark cycle). The experimental protocol was approved by Institutional Animal Ethical Committee (Protocol

No. Dean/2019/IAEC/1635). All experiments were conducted as per CPCSEA guidelines and strictly adhered to National Research Council US Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2019) guidelines.

5.1.2 Chemicals

Indole -3 carbinol (Sigma Aldrich), Evans blue (Sigma Aldrich, USA), Thiopentone sodium (Neon Labs, India), Betadine solution (Cipla, India), dimethylsulfoxide (DMSO), (Sigma Aldrich, USA), 2,3,5-Triphenyl tetrazolium chloride (TTC) (Sigma Aldrich, USA), Hemotoxylin (SRL chemicals, India), Eosin (Sigma Aldrich, USA), 2′,7′-Dichlorofluorescin Diacetate (DCFDA, Sigma Aldrich, USA), phosphorylated AMPK (p-AMPK), PGC-1α, TNF-1α, IL-6, IL-10, cytochrome C, and caspase 9 ELISA kits were procured from Krishgen Biosystems (India). Caspase-3 colorimetric kit was purchased from Abcam, USA. TRIzol® reagent Chloroform, PCR-Grade Water, Isopropanol, Ethanol, cDNA Synthesis kit, SYBR Green Master Mix, and other PCR chemicals and material were obtained from Applied Biosystems (USA). All other chemicals related to oxidative stress and mitochondrial complexes used in the study were obtained from higher analytical grades.

5.1.3 Experimental design Animal grouping

A total number of 135 rats were randomly divided into the following groups using Microsoft Excel (standard = RAND () function); sham (1), MCAO (2), MCAO + I3C (12.5 mg/kg) (3), MCAO + I3C (25 mg/kg) (4), MCAO + I3C (50 mg/kg) (5). The doses of I3C used in the present study was selected from previous studies in a geometric progression (P. Paliwal et al., 2018). MCAO and the ischemic reperfusion (I/R) injury method were used to produce the ischemic stroke. I3C was prepared in DMSO (0.5%) and orally administered to respective groups for 7 days, once daily. Sham group of animals received equal volumes of DMSO (0.5%). After 7 days, after completing behavioral experiments (neurological deficits, rotarod, and grip strength), rats were euthanized and brains were collected. Brain infarction (n=4), brain water content (n=4), blood-brain barrier permeability (BBB) (n=4), and histopathological studies (n=4) were determined. Mitochondria was isolated and mitochondrial electron transport chain (ETC) enzymes and oxidative stress markers were evaluated (n=4). Brain inflammatory markers (TNF-α, IL-6, and IL-10), apoptosis markers (cytochrome C, caspase 9, and caspase 3), and RT-PCR analysis carried out in the same samples (n=4). At the end of the experiment, the following animals survived: sham (n=26), MCAO (n=24), MCAO + I3C (12.5 mg/kg) (n=25), MCAO + I3C (25 mg/kg) (n=25), and MCAO + I3C (50 mg/kg) (n=25). All experimental groups have an equal number of animals for behavioral experiments, biochemical, and molecular studies (n=24). The remaining animals were euthanized and brains were stored at -80 ^{o}C. The study design is depicted in figure 5.2.

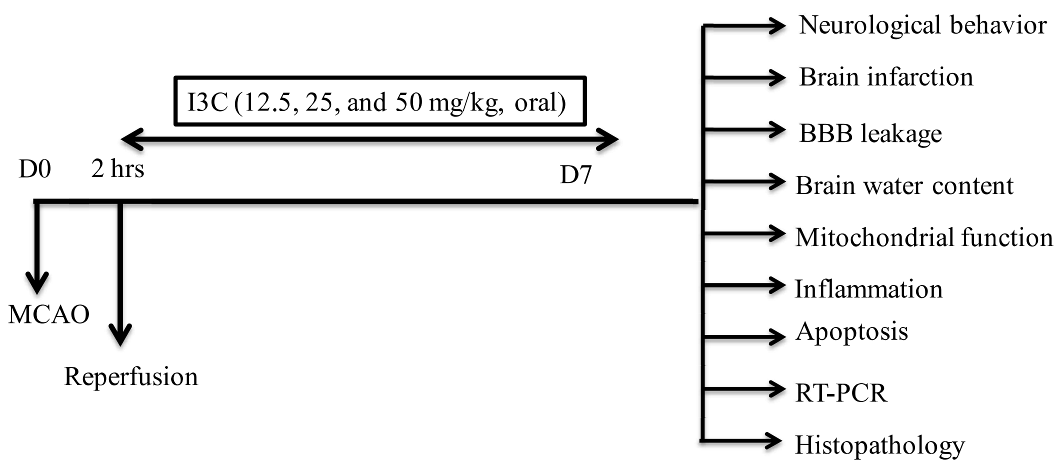

Figure 5.2: Study design. MCAO-middle cerebral artery occlusion, D-day, I3C-indole-3-carbinol, BBB-blood-brain barrier, RT-PCR-reverse transcriptase-polymerase chain reaction.

5.1.4 MCAO surgery

MCAO injury was induced as described in section 2.1.8.

5.1.5 Assessment of neurobehavior

Neurological deficits, rotarod, and grip strength tests were performed as mentioned in section 4.1.5.

5.1.6 Measurement of Brain infarction

Brain infarction was measured by the TTC staining method as described in section 2.1.10.

5.1.7 BBB integrity

Evans blue (EB) leakage into the brain was measured to evaluate the BBB integrity as reported in section 2.1.11.

5.1.8 Brain water content

Brain water content was evaluated by using the wet and dry weight method, as mentioned in section 2.1.12.

5.1.9 Mitochondrial isolation

Mitochondria were isolated from the cortex of the ischemic hemisphere as mentioned earlier (Samaiya & Krishnamurthy, 2015). Precisely, the cortex of the ischemic brain was blended in a mitochondrial isolation buffer (mannitol (215 mM), sucrose (75 mM), bovine serum albumin (0.1% w/v), HEPES buffer (20 mM), and EGTA (1 mM) in 100 ml of distilled water pH 7.2). Then homogenates were centrifuged at 1300g for 5 min at 4 °C. The mitochondrial pellet was obtained by centrifuging the supernatant at 14,000g for 10 min at 4 °C. Further, EGTA was removed by washing the mitochondrial pellet in mitochondrial isolation buffer (without EGTA) by centrifuging at 14,000g for 10 min. A dense mitochondrial pellet sedimented at the bottom of centrifuged tubes and was immediately used or stored at -80 °C till further analysis. Mitochondrial protein levels were measured using the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

5.1.10 Measurement of mitochondrial complex enzyme activities

NADH dehydrogenase (complex I) activity was evaluated as described earlier (Shapiro, Feigal, & Lam, 1979). The activity of NADH dehydrogenase was expressed as nmol NADH oxidized/min/mg protein. Succinate dehydrogenase (Complex II) activities were evaluated as per Old et al. (Old & Johnson, 1989). Cytochrome C oxidase (complex-IV) activity was measured as per the method of Storrie (Storrie & Amadden, 1990). Mitochondrial F_oF_1-ATP synthase (Complex V) enzyme activities were evaluated as described earlier (Griffiths, 1974). Fisk and subbarow method was used to find the inorganic phosphate (Fiske & Subbarow, 1925).

5.1.11 Mitochondrial membrane potential (MMP) and MPTP opening

Mitochondrial membrane potential (MMP) was evaluated using the tetramethylrhodamine methyl ester (TMRM) dye method. The fluorescence intensity of mitochondria incubated with TMRM was measured at 535nm (excitation) and 580nm (emission) (S.-G. Huang, 2002). Mitochondrial swelling was measured as mentioned previously (Tedeschi & Harris, 1958). Mitochondrial absorbance at 520 nm was normalized with the protein concentration (nm/mg protein).

5.1.12 ROS, LPO, SOD, and CAT

Reactive oxygen species (ROS) levels were evaluated using dichloro-dihydro-fluorescein diacetate (DCFH-DA) fluorescence dye. DCFH-DA (10 μM) was incubated with mitochondria for 30 min at 37°C in the dark. Then, fluorescence was measured at 485 nm/520 nm (excitation and emission), respectively (C. Liu et al., 2019). Mitochondrial lipid peroxidation was measured as described earlier. Malondialdehyde (MDA) levels are expressed as moles of MDA/mg protein (Sunderman, Marzouk, Hopfer, Zaharia, & Reid, 1985). Superoxide dismutase (SOD) enzyme activities were evaluated by nitroblue tetrazolium (NBT) reduction in the presence of phenazine methosulphate (PMS) and NADH. Blue-colored formazan was measured at 560 nm (Kakkar, Das, & Viswanathan, 1984). Catalase (CAT) activity was evaluated by reduction in H_2O_2 (6%) absorbance at 240 nm for 3 min at 30 s intervals (Beers & Sizer, 1952).

5.1.13 Enzyme-linked immunosorbent assay (ELISA)

Brain tissue homogenates were prepared, and p-AMPK, TNF-α, IL-6, IL-10, PGC-1α, cytochrome C, and caspase 9 levels were estimated as per the manufacturer's protocol (Krishgen Biosystems, India). Caspase 3 levels were determined as per user guidelines (Abcam, USA).

5.1.14 RT-PCR

TRIzol reagent method of RNA isolation was performed as per manufacturing guidelines (Applied biosystems, USA). Further, Nanodrop (Thermofischer, USA) was used to quantify the RNA at 260 nm (OD260) and nucleic tainting was observed at 260/280 and 260/230 ratios. Then, cDNA was synthesized by using the cDNA Reverse Transcription Kit (Applied Biosystems, USA). RNA (10 µL) was kept in a thermal cycler and reverse transcription commenced under the following conditions 25 °C for 10 min, 37°C for 120 min and 85 °C for 5min. cDNA was then stored at -20 ^{o}C, until further analysis. The primer sequence is illustrated in table 1. Further, PGC-1α, NRF 1, and tFAM gene expressions were evaluated by the addition of the reverse and forward primers, SYBR premix (Applied Biosystems, USA) to cDNA. After that, the master mix (20 µL) was placed on a thermocycler with the following PCR conditions; initial denaturation at 95 °C for 1 min for 40 cycles, denaturation at 95^{o}C for 30 sec, annealing at 60.1°C for 45 sec, and extension 72°C for 45 min. GAPDH was used as a housekeeping gene. All the samples were run in duplicates. RT-qPCR data were quantitatively analyzed by using the formation of $2^{-\Delta\Delta Ct}$.

S.No.	Primer	Sequence
1	PGC-1α	Forward: 5’ TGAAGTGGTGTAACCAATC 3’
		Reverse: 5’ GCAAGTTTGCCTCATTCTCTCC 3’
2	NRF1	Forward: 5’CTATGCGAAAGAGACAGCAGAC 3’
		Reverse: 5’ GGGTGAGATGCAGAGAACAA 3’
3	NRF2	Forward: 5’ GATTCGTGCACAGCAGCA3’
		Reverse: 5’ GCCAGCTGAACTCCTTAGAC 3’
4	tFAM	Forward: 5’ GCCTGTCAGCCTTATCTGTAAT 3’
		Reverse: 5’ TGCATCTGGGTGTTTAGCTTTA 3’
5	GAPDH	Forward: 5’ GGGTGGTCCAGGGTTTCTTACT 3’
		Reverse: 5’ AGGTTGTCTCCTGCGACTTCA 3’

Table 5.1: Primer sequence of mitochondrial genes.

5.1.15 Histopathology

Hematoxylin and eosin staining was performed to evaluate the histopathological abnormalities as described in section **4.1.12**.

5.1.16 Statistical analysis

All the data are expressed as mean ± standard deviation (SD). One-way ANOVA followed by Tukey's post hoc test was used to analyze the data. Statistical calculations were performed using GraphPad Prism, version 5, USA). A level of $p<0.05$ was considered to be statistically significant.

5.2 Results

5.2.1 I3C improved the neurobehavior in MCAO rats

Changes in the sensory-motor function after treatment with I3C in MCAO rats are depicted in figure 5.3. One-way ANOVA revealed significant differences among groups; neurological deficits [$F(4, 119) = 114.4$; $P < 0.0001$], rotarod [$F(4, 119) = 232.9$; $P < 0.001$], and grip strength [$F(4, 119) = 95.64$; $P < 0.001$]. MCAO rats significantly had increased neurological deficits and impaired rotarod and grip strength abilities than sham-operated animals. I3C treatment significantly reduced the neurological deficits and improved the rotarod and grip strength abilities in a dose-dependent manner. Further, there were significant differences in sensory-motor reflexes between I3C-treated MCAO and sham-operated rats. These findings suggest that I3C treatment improved the sensory-motor functions in MCAO rats.

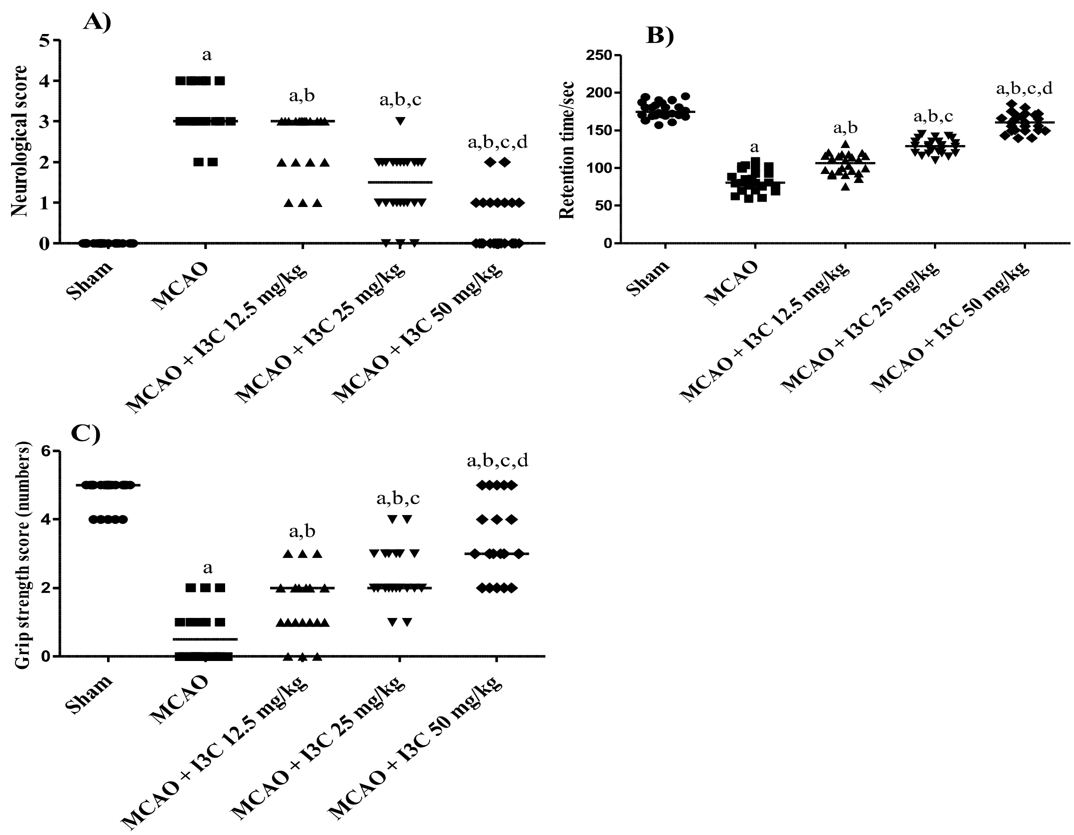

Figure 5.3. Effect of I3C treatment on sensory-motor functions in MCAO rats. Figures 5.3A, 5.3B, and 5.3C indicate the changes in neurological deficits, rotarod, and grip strength performances in MCAO rats. $^{a}p<0.0001$ vs. Sham, $^{b}p<0.001$ vs. MCAO, $^{c}p<0.001$ vs. MCAO + I3C 12.5 mg/kg, and $^{d}p<0.001$ vs. MCAO + I3C 25mg/kg. All the results are expressed as Median (n=24). One-way ANOVA followed by Tukey's post hoc test.

5.2.2 I3C ameliorated the brain infarction

Changes in the brain infarction size and infarction volumes after treatment with I3C are depicted in figure 5.4. One-way ANOVA observed that there were significant differences observed among the groups: brain infarction (%) [F (4, 29) = 445.5; P < 0.0001] and infarct volume [F (4, 29) = 537.6.0; P < 0.001]. MCAO rats had a significant increase in brain infarction (%) and infarction volume than sham animals. I3C dose-dependently attenuated the brain infarction (%) and infarction volume in MCAO rats than in untreated MCAO rats and sham animals. These results indicate that I3C significantly ameliorated brain infarction.

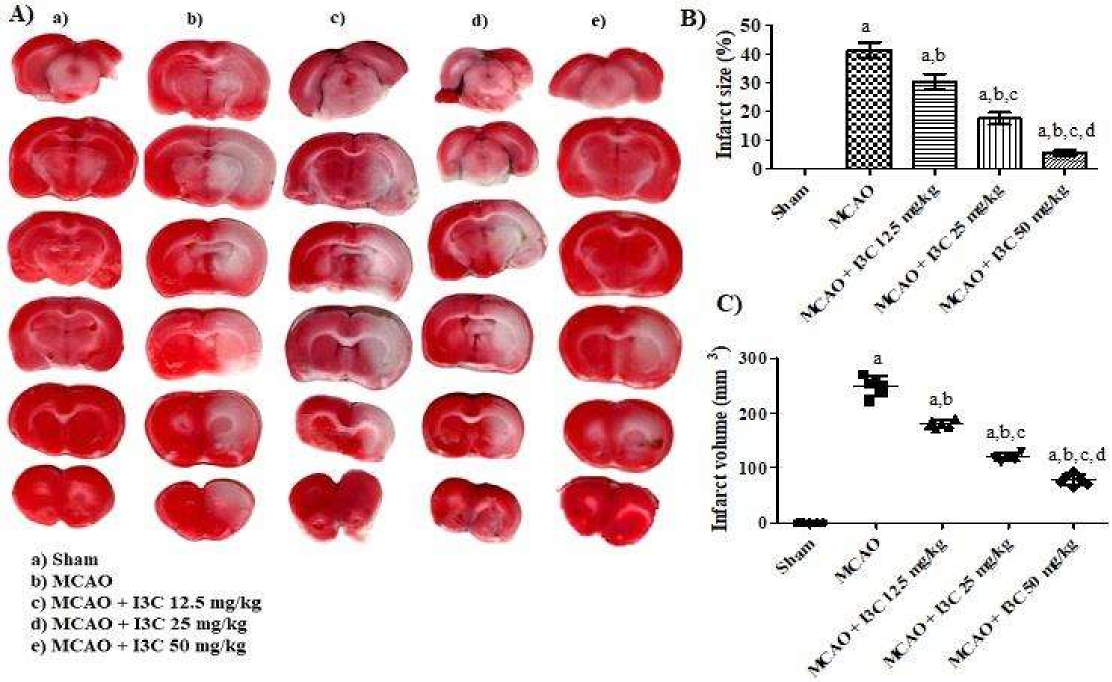

Figure 5.4. The effect of I3C treatment on brain infarction. Figure 5.4A indicates the representative TTC images of respective groups, figure 5.4B represents the changes in infarction size (%), and figure 5.4C indicates the alterations in infarct volume. [a]p<0.0001 vs. Sham, [b]p<0.001 vs. MCAO, [c]p<0.001 vs. MCAO + I3C 12.5 mg/kg, and [d]p<0.001 vs. MCAO + I3C 25mg/kg. All values are expressed as Mean ± SD (n = 6). One-way ANOVA followed by Tukey's post hoc test.

5.2.3 I3C alleviated the BBB leakages and brain water content

Alterations in BBB permeability and brain water levels in MCAO rats are illustrated in figure 5.5. One-way ANOVA observed the significant differences among groups: EB levels [F (4, 29) = 552.2; P < 0.001] and brain water levels [F (4, 29) = 115.1; P < 0.001]. Post hoc analysis indicated that MCAO rats significantly increased the EB content and brain water levels than sham animals. I3C treatment ameliorated the EB content and water content levels in MCAO rats in a dose-dependent manner than in untreated MCAO rats and sham-operated rats. These findings indicate that I3C treatment preserves the BBB integrity by restricting the excessive water accumulation in the brains of MCAO rats.

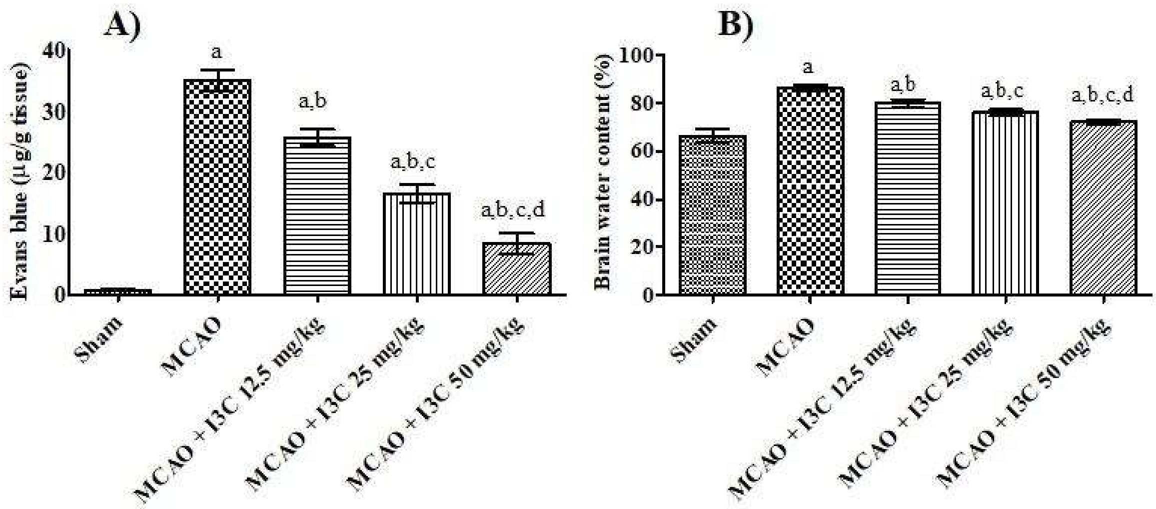

Figure 5.5. The effect of I3C treatment on BBB integrity and brain water content. Figures 5.5A and 5.5B indicate the changes in EB and brain water content, respectively. $^{a}p<0.0001$ vs. Sham, $^{b}p<0.001$ vs. MCAO, $^{c}p<0.001$ vs. MCAO + I3C 12.5 mg/kg, and $^{d}p<0.001$ vs. MCAO + I3C 25mg/kg. All values are expressed as Mean ± SD (n = 6). One-way ANOVA followed by Tukey's post hoc test.

5.2.4 I3C improved the ETC complex enzyme activities

Figure 5.6 represents changes in mitochondrial complex enzyme activities in MCAO rats. One-way ANOVA revealed that there were significant differences observed among groups: complex I [$F(4, 19) = 107.4$, $P < 0.001$], complex II [$F(4, 19) = 79.58$, $P < 0.001$], complex IV [$F(4, 19) = 150.7$, $P < 0.001$], and complex V [$F(4, 19) = 107.8$, $P < 0.001$]. MCAO rats significantly decreased mitochondrial complex enzyme activities than sham animals. I3C treatment dose-dependently enhanced the ETC complex enzyme activities in MCAO rats than in untreated MCAO rats and sham rats. These findings indicate that I3C treatment improved the ETC enzyme activities in MCAO rats, thereby maintaining energy homeostasis.

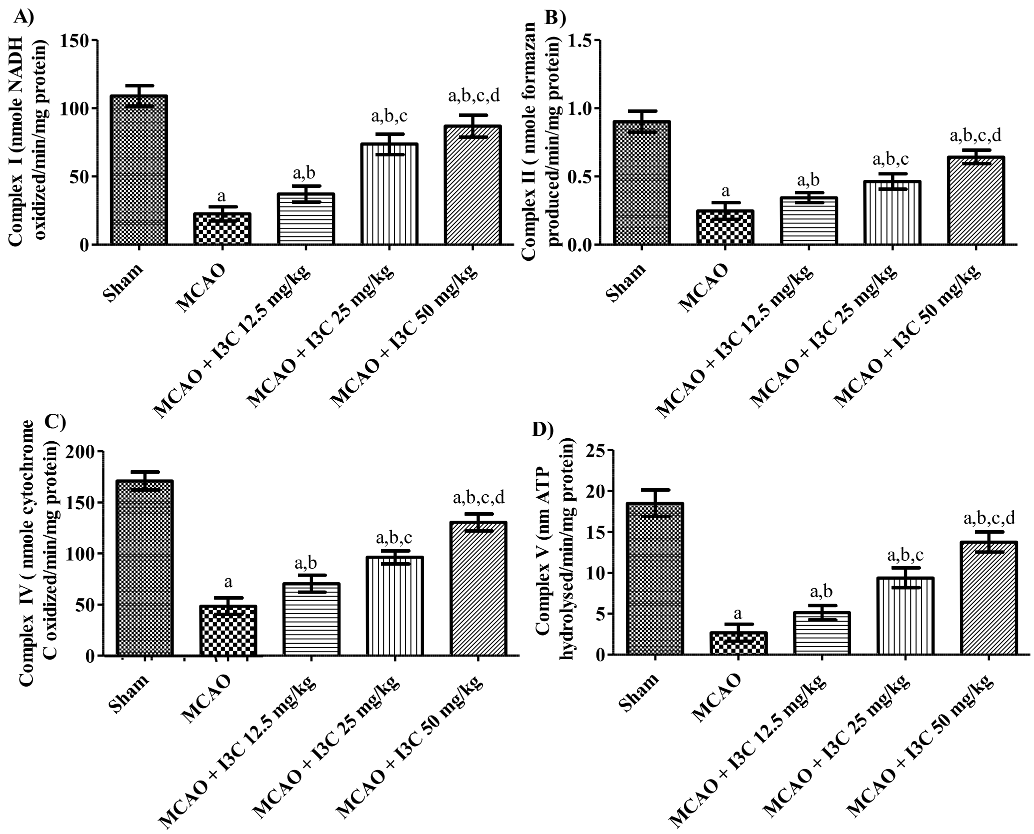

Figure 5.6. The effect of I3C treatment on ETC complexes enzyme activities. Figures 5.6A, 5.6B, 5.6C, and 5.6D indicate complex I, complex II, complex IV, and complex V, respectively. $^{a}p<0.001$ vs. Sham, $^{b}p<0.001$ vs. MCAO, $^{c}p<0.001$ vs. MCAO + I3C 12.5 mg/kg, and $^{d}p<0.001$ vs. MCAO + I3C 25mg/kg. All values are expressed as Mean ± SD (n = 4). One-way ANOVA followed by Tukey's post hoc test.

5.2.5 I3C improved the MMP and inhibited the MPTP opening

I3C-induced alterations in MMP and MPTP levels are illustrated in figure 5.7. One-way ANOVA revealed significant differences among the groups: MMP [$F(4, 19) = 173.8$, P <

0.001] and MPTP opening [F (4, 19) = 123.6, P < 0.0001]. Post hoc analysis revealed that MCAO rats had decreased MMP and increased MPTP opening than sham animals. I3C treatment increased the MMP and decreased MPTP in MCAO rats than in untreated MCAO rats and sham-operated rats. These results indicate that I3C treatment restored the MMP and MPTP function, maintaining mitochondrial structural and functional integrity.

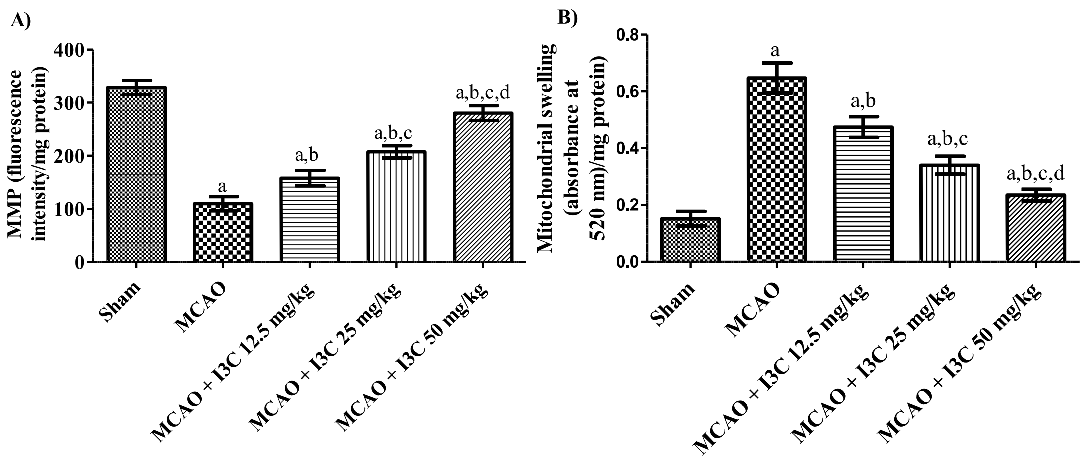

Figure 5.7. The effect of I3C treatment on MMP and MPTP opening. Figures 5.7A and 5.7B indicate the levels of alterations in MMP and MPTP. [a]p<0.0001 vs. Sham, [b]p<0.001 vs. MCAO, [c]p<0.001 vs. MCAO + I3C 12.5 mg/kg, and [d]p<0.001 vs. MCAO + I3C 25mg/kg. All values are expressed as Mean ± SD (n = 4). One-way ANOVA followed by Tukey's post hoc test.

5.2.6 I3C mitigated the oxidative stress

Figure.5.8 represents changes in mitochondrial oxidative stress with I3C treatment in MCAO rats. One-way ANOVA revealed that there were significant differences observed among groups: ROS [F (4, 19) = 191.3, P < 0.001], MDA [F (4, 19) = 145.9, P < 0.001], SOD [F (4, 19) =205.3, P < 0.001], and CAT [F (4, 19) = 46.70 , P < 0.001]. Post hoc analysis revealed that MCAO rats significantly had higher levels of ROS and MDA levels and lower levels of

SOD and CAT enzymes than sham animals. I3C treatment dose-dependently decreased the ROS and MDA levels and elevated the SOD and CAT levels in MCAO rats than in untreated MCAO rats and sham rats. Therefore, these findings indicate that I3C treatment maintains the redox balance in MCAO rats.

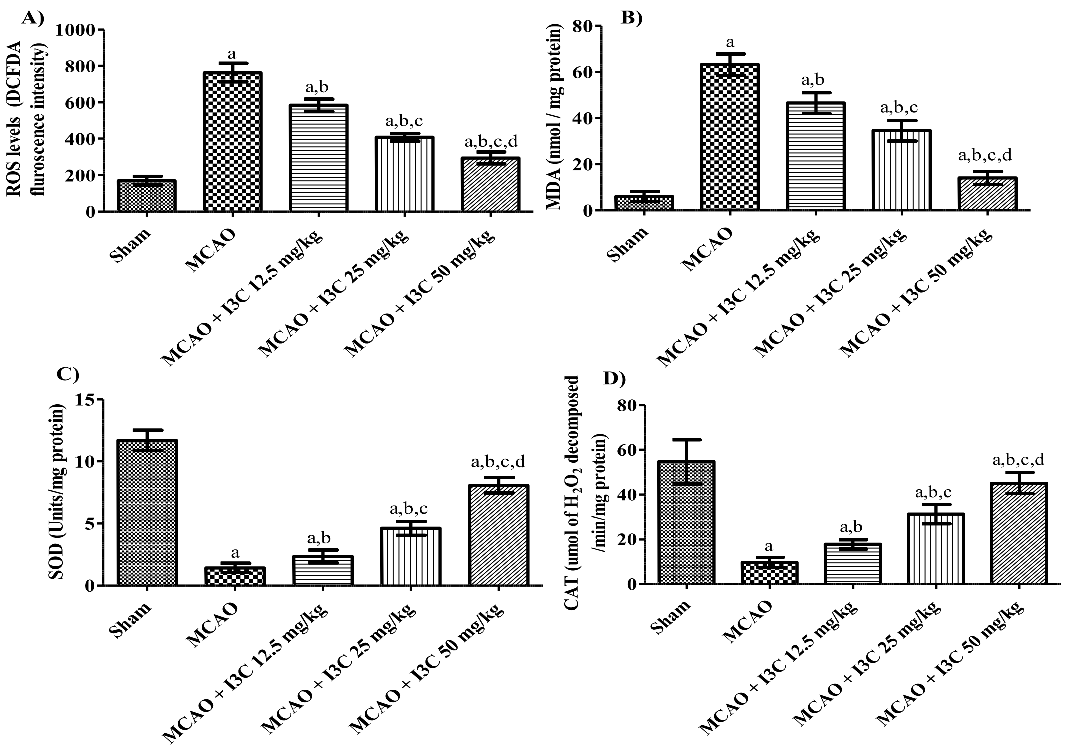

Figure 5.8. The effect of I3C treatment on oxidative stress in MCAO rats. Figures 5.8A, 5.8B, 5.8C, and 5.8D represent the levels of ROS, MDA, SOD, and CAT levels. $^{a}p<0.001$ vs. Sham, $^{b}p<0.001$ vs. MCAO, $^{c}p<0.001$ vs. MCAO + I3C 12.5 mg/kg, and $^{d}p<0.001$ vs. MCAO + I3C 25mg/kg. All values are expressed as Mean ± SD (n = 4). One-way ANOVA followed by Tukeys post hoc test.

5.2.7 I3C attenuated inflammation factors

The alterations in inflammatory markers in MCAO rats are depicted in figure 5.9. One-way ANOVA identified the significant differences in TNF-α [$F(4, 19) = 156.4$, $P < 0.0001$], [$F(4, 19) = 51.84$, $P < 0.001$], and IL-10 [$F(4, 19) = 337.7$, $P < 0.001$] among the groups.

MCAO rats significantly elevated the inflammatory markers (TNF-α and IL-6) and decreased the anti-inflammatory marker (IL-10) compared to sham animals. I3C dose-dependently decreased the inflammatory markers and elevated the anti-inflammatory markers in MCAO rats than in untreated MCAO rats and sham rats. These results suggest that I3C ameliorated the inflammation associated with stroke.

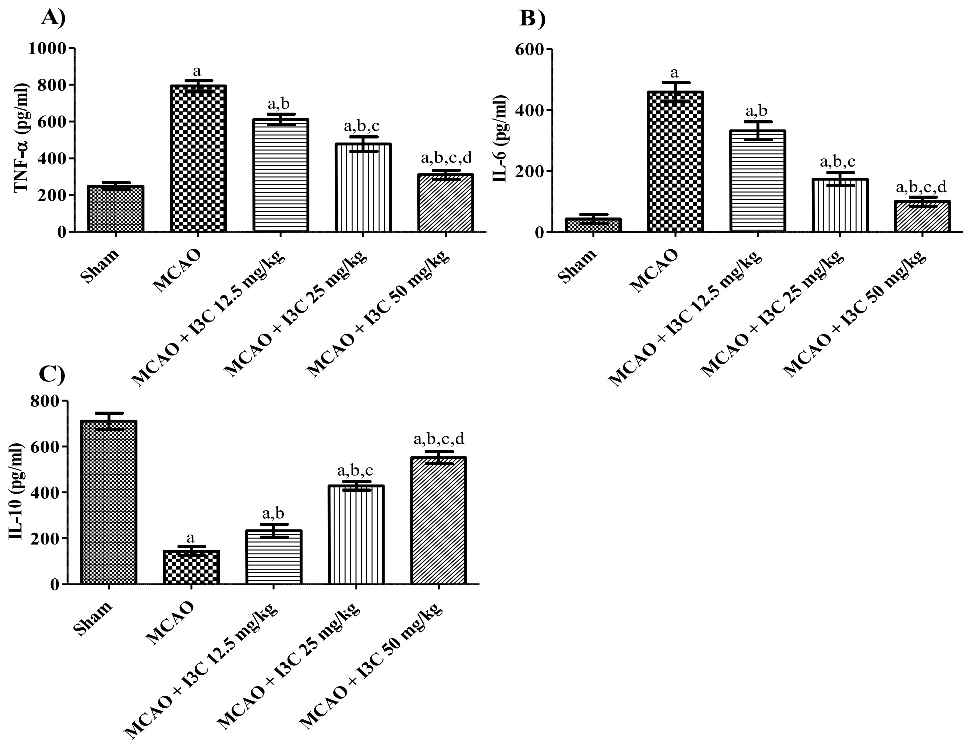

Figure 5.9. The effect of I3C treatment on inflammation factors. Figures 5.9A, 5.9B, and 5.9C represent the TNF-α, IL-6, and IL-10 levels. [a]p<0.0001 vs. Sham, [b]p<0.001 vs. MCAO, [c]p<0.001 vs. MCAO + I3C 12.5 mg/kg, and [d]p<0.001 vs. MCAO + I3C 25mg/kg. All values are expressed as Mean ± SD (n = 4). One-way ANOVA followed by Tukeys post hoc test.

5.2.8 I3C increased the protein levels of p-AMPK and PGC-1α

Changes in protein levels of p-AMPK and PGC-1α are depicted in figure 5.10. One-way ANOVA revealed significant differences in protein levels of p-AMPK [F (4, 19) = 195.6, P <

0.001] and PGC-1α [F (4, 19) = 288.1, P < 0.001] among groups. MCAO rats significantly decreased protein levels of p-AMPK and increased the PGC-1α than sham animals. I3C treatment significantly increased p-AMPK and PGC-1α levels in MCAO rats than in untreated MCAO rats and sham animals dose-dependently.

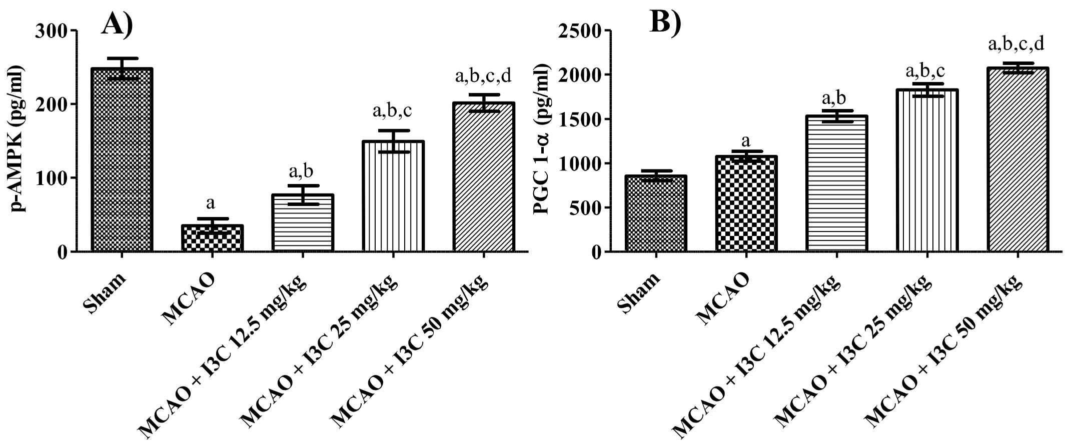

Figure 5.10. The effect of I3C treatment on protein levels of p-AMPK and PGC-1α in MCAO rats. Figures 5.10A and 5.10B represent the changes in p-AMPK and PGC-1α levels. ^{a}p<0.001 vs. Sham, ^{b}p<0.001 vs. MCAO, ^{c}p<0.001 vs. MCAO + I3C 12.5 mg/kg, and ^{d}p<0.001 vs. MCAO + I3C 25mg/kg. All values are expressed as Mean ± SD (n = 4). One-way ANOVA followed by Tukeys post hoc test.

5.2.9 I3C increased mRNA levels of mitochondrial biogenesis markers

Figure 5.11 represents the alterations in mitochondrial biogenesis markers in MCAO rats. One-way ANOVA revealed significant differences in mRNA levels of PGC-1α [F (4, 19) = 81.18, P < 0.001], NRF1 [F (4, 19) = 94.24, P < 0.001], NRF2 [F (4, 19) = 56.21, P < 0.001], and tFAM [F (4, 19) = 119.8, P < 0.001] among groups. MCAO rats significantly had higher levels of PGC-1α, NRF1, tFAM, and lower levels of NRF2 than sham animals. I3C treatment significantly increased mRNA levels of PGC-1α, NRF1, NRF2, and tFAM in MCAO rats than in untreated MCAO rats and sham animals dose-dependently. These results indicate that I3C improved mitochondrial biogenesis in MCAO rats.

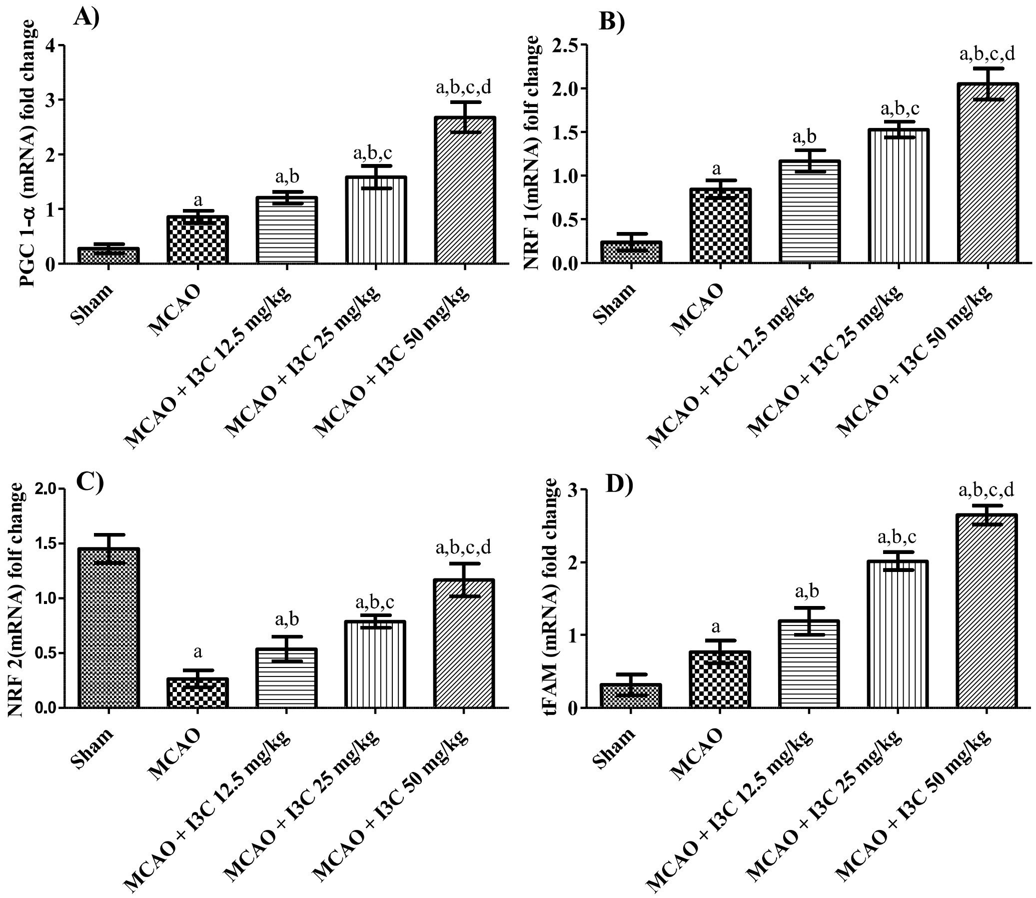

Figure 5.11. The effect of I3C treatment on mRNA levels of PGC-1α, NRF1, NRF2, and tFAM in MCAO rats. *Figures 5.11A, 5.11B, 5.11C, and 5.11D represent the mRNA expression of PGC-1α, NRF 1, NRF2, and tFAM levels.* $^{a}p<0.001$ *vs. Sham,* $^{b}p<0.001$ *vs. MCAO,* $^{c}p<0.001$ *vs. MCAO + I3C 12.5 mg/kg, and* $^{d}p<0.001$ *vs. MCAO + I3C 25mg/kg. All values are expressed as Mean ± SD (n = 4). One-way ANOVA followed by Tukeys post hoc test.*

5.2.10 I3C altered apoptosis

The alteration in apoptosis markers after treatment with I3C in MCAO rats is illustrated in figure 5.12. One-way ANOVA identified the significant differences in cytochrome C [F (4, 19) = 441.9, P < 0.001], caspase 9 [F (4, 19) = 133.2, P < 0.001], and caspase 3 [F (4, 19) = 130.5, P < 0.001] among the groups. Post hoc analysis revealed that MCAO rats significantly increased the levels of cytochrome C, caspase 9, and caspase 3 compared to sham animals. I3C dose-dependently reduced these enzyme levels in MCAO rats than in untreated MCAO rats and sham animals. These findings indicate that I3C protected the brain from apoptosis.

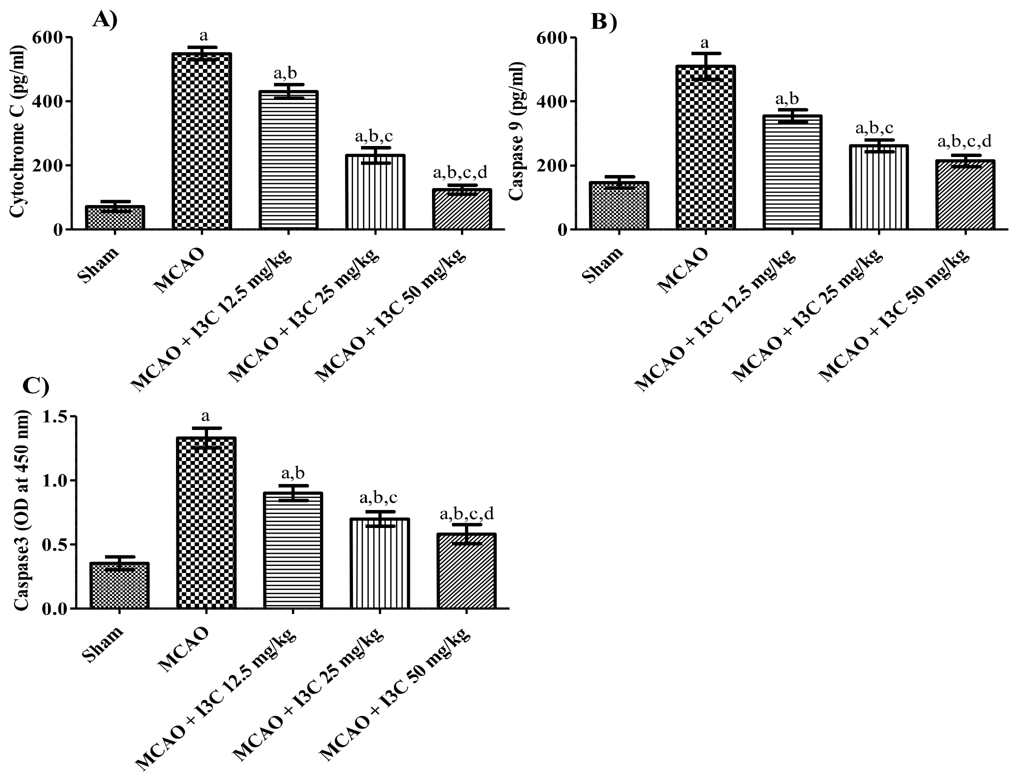

Figure 5.12. The effect of I3C treatment on apoptosis markers in MCAO rats. Figures 5.12A, 5.12B, and 5.12C represent the levels of expression of cytochrome-C, caspase-9, and caspase-3, respectively. ^{a}*p<0.001 vs. Sham,* ^{b}*p<0.001 vs. MCAO,* ^{c}*p<0.001 vs. MCAO + I3C*

12.5 mg/kg, and $^{d}p<0.001$ *vs. MCAO + I3C 25mg/kg. All values are expressed as Mean ± SD (n = 4). One-way ANOVA followed by Tukey post hoc test.*

5.2.11 I3C improved cortical architecture

Figure 5.13 represents the changes in histopathological abnormalities with I3C treatment in MCAO. One-way ANOVA revealed significant differences in healthy neuron ratio among the groups [$F (4, 19) = 66.62$, $P < 0.001$. Post hoc analysis revealed that MCAO rats significantly had a lower healthy neuron ratio than sham-operated animals. I3C dose-dependently increased the healthy neuron ratio in MCAO rats than in untreated MCAO rats and sham animals. These findings indicate that I3C restored the structural abnormalities in MCAO rats.

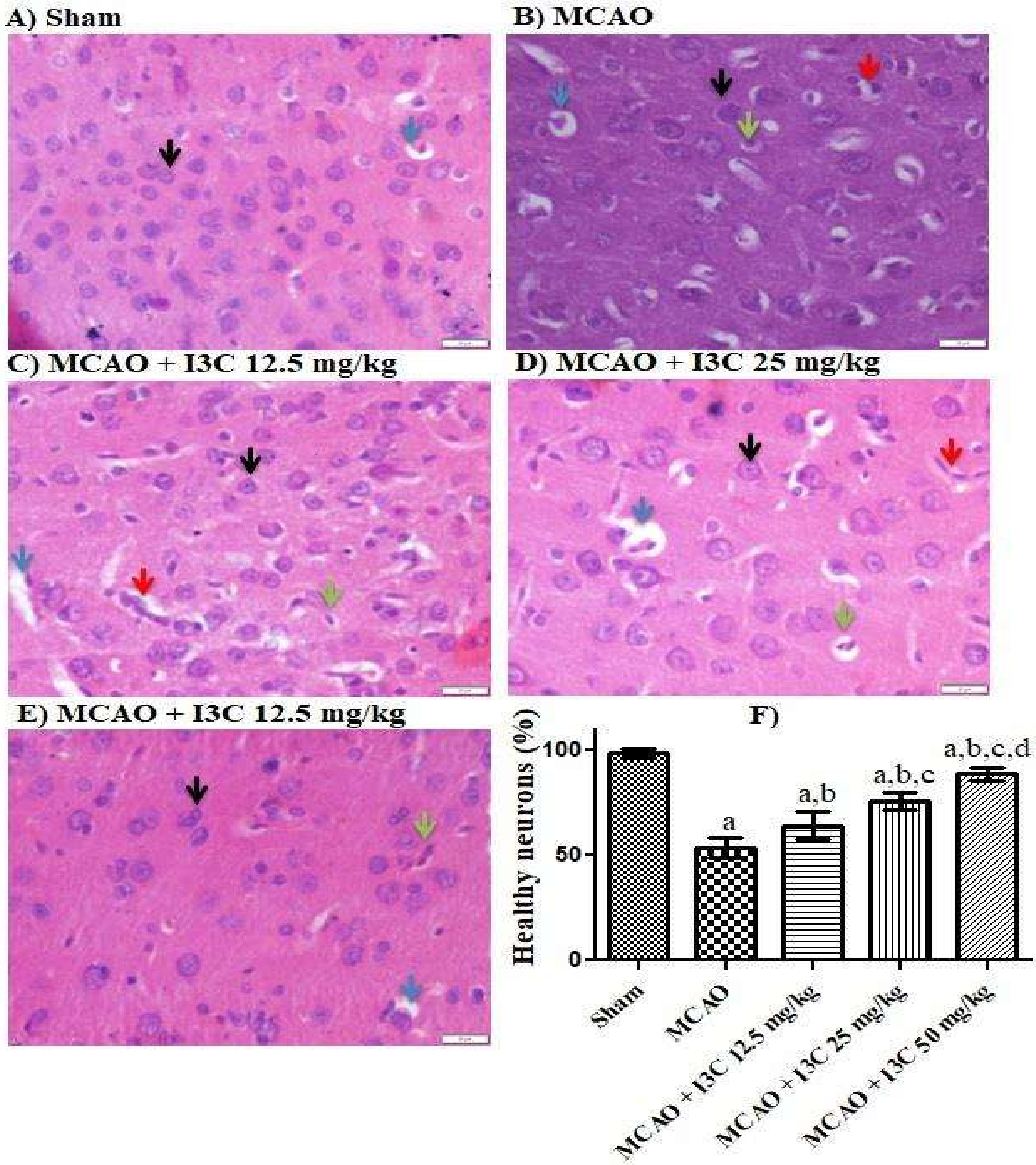

Figure 5.13. The effect of I3C on histological changes after MCAO. Figure 5.13A, 5.13B, 5.13C, 5.13D, and 5.13E represents the H and E staining images of sham, MCAO, MCAO + I3C 12.5, MCAO + I3C 25 mg/kg, and MCAO + I3C 50 mg/kg, respectively. The black arrow represents the normal cells with an intact nucleus. The red arrow represents nuclear fragmentation. The sky blue arrow indicates the vacuoles, and the green arrow indicates the nuclear shrinkage. [a]p<0.001 vs. Sham, [b]p<0.001 vs. MCAO, [c]p<0.001 vs. MCAO + I3C 12.5

mg/kg, and $^{d}p<0.001$ *vs. MCAO + I3C 25mg/kg. All values are expressed as Mean ± SD (n = 4). One-way ANOVA followed by Tukeys post hoc test.*

5.3 Discussion

The present study showed the neuroprotective mechanisms mediated by I3C against focal cerebral ischemic injury in rats. I3C treatment significantly improved neurological function by alleviating infarction, BBB leakage, and brain water content. This neuroprotection could be due to restoring mitochondrial function by enhancing mitochondrial biogenesis by activating the AMPK/PGC-1α pathway and alleviating oxidative stress, inflammation, and apoptosis. Comprehensively, I3C ameliorated mitochondrial dysfunction, thereby protecting the neuronal cells from ischemic injury.

Stroke treatment academic-industrial research (STAIR) recommended that neuroprotective agents essentially lessen brain infarction, neurological deficits, BBB leakage, and brain water content (Belayev, Alonso, Busto, Zhao, & Ginsberg, 1996b; Fisher et al., 2009; Longa et al., 1989). Earlier reports indicated that I3C treatment ameliorated brain infarction and neurological deficits (P. Paliwal et al., 2018). Similarly, we have observed a reduction in brain infarction and neurological deficits. BBB leakage occurs after an ischemic event and causes deleterious effects on the brain by allowing water, toxicants, and other substance into the brain (Hawkins & Davis, 2005). The elevated brain edema largely influences neurological deficits and contributes to the development of intracranial pressures, which further puts the brain under detrimental conditions (Ioannides et al., 2017). I3C treatment alleviated the BBB leakage and brain water content indicates that I3C buffers the structural and functional integrity after an ischemic event, thereby maintaining brain homeostasis.

AMPK is the master regulator of cellular energy (Day, Ford, & Steinberg, 2017). Following energy decline, kinases phosphorylate the AMPK resulting in phosphorylated AMPK (p-AMPK), which is considered an active form of AMPK (Hardie, Scott, Pan, & Hudson, 2003). Further, pAMPK shuts the energy-consuming process and simultaneously accelerates ATP generating process (Day et al., 2017). Cerebral ischemia causes energy deprivation, making the brain more susceptible to energy deficits and hampers brain function by inducing mitochondrial dysfunction (Golpich et al., 2017; Yang et al., 2020), resulting in increased energy demand (Guan et al., 2018). We found that I3C treatment significantly increased pAMPK levels in MCAO rats indicating the activation of pAMPK. To meet the energy demand, pAMPK up-regulates the PGC-1α, thereby upregulating the MB (J. Gao, Qian, & Wang, 2020; C. Ma et al., 2019). We found that I3C significantly increases mRNA and protein levels of PGC-1α in ischemic animals, suggesting the activation of mitochondrial biogenesis. Further, I3C upregulated the mRNA expressions of PGC-1α downstream regulators of MB, including NRF 1 and tFAM, indicating the initiation of MB. Indeed, activation of AMPK or PGC-1α ameliorates inflammation and oxidative stress (C. Ma et al., 2019; Palomer et al., 2009).

It has been observed that there is a distinct elevation of oxidative stress, inflammation, mitochondrial function, and structural abnormalities of the brain remain impaired after a rise in mitochondrial number in cerebral ischemia (Mehta et al., 2012; Novitzky et al., 2016; Xie, Li, Fan, Qi, & Li, 2014). Similarly, we have observed a significant increase in oxidative stress, inflammation, and mitochondrial dysfunction in MCAO rats. Further, I3C treatment ameliorated oxidative stress, inflammation, and mitochondrial dysfunction in MCAO rats, which is reflected in improved neurological function. Besides, pAMPK directly regulates inflammation by decreasing pro-inflammatory cytokines such as TNF-α and IL-6 (C. Ma et al., 2019; Wang, Zhou, Dong, Ma, & He, 2018). Moreover, the downregulation of TNF-α can activate the PGC1-α (Palomer et al., 2009; Qi et al., 2015). I3C treatment reduced the inflammatory cytokines TNF-α, and IL-6, and elevated the anti-inflammatory cytokines IL-10 in MCAO rats. Therefore, these findings confirm that I3C ameliorated the inflammation in MCAO rats partially or fully due to activation of AMPK or PGC-1α. Further AMPK and PGC-1α activation have been reported to promote the antioxidant enzyme levels through upregulating the NRF2 (Felszeghy et al., 2019; Yu et al., 2020). We found that mRNA levels of NRF2 were decreased in MCAO rats and I3C treatment significantly elevated the NRF2 mRNA levels indicating the activation of antioxidant signaling mechanisms. In support, we found that I3C treatment significantly increased the antioxidant enzymes, such as SOD and CAT, with a simultaneous decrease in ROS and MDA levels. Therefore, these findings confirmed that I3C activates mitochondrial biogenesis and mitigates oxidative stress and inflammation by activating the AMPK/ PGC-1α pathway in ischemic rats.

Few studies reported that, though MB slightly increased after reperfusion, neurological functions, motor functions, and cortical architecture remained dysfunctional (Dong et al., 2019; Xie et al., 2014). Similarly, we have observed neurological deficits, brain infarction, and cortical architecture impairment in MCAO rats with the elevation of PGC-1α. I3C treatment significantly ameliorated these impairments with significant elevation of pAMPK/PGC-1α and its downstream proteins. I3C treatment significantly elevated the antioxidant enzymes such as SOD and CAT, suggesting that I3C acts as an antioxidant by activating the PGC-1α. In connection with this pathway's activation, we have observed a significant reduction of neurological deficits, brain infarction, BBB leakage, and brain water content, suggesting that I3C exhibited neuroprotection by activating the AMPK/PGC1-α pathway. Therefore, I3C can be used as a neuroprotective for managing stroke. Previous reports suggest that I3C reported activating the AMPK/PGC-1α pathway to inhibit adipocyte differentiation in obesity disorder (H.-S. Choi et al., 2014; Y Choi et al., 2013). Similarly, the present study also reported that the I3C-mediated activation of AMPK/PGC-1α is beneficial for mitigating oxidative stress and neuroinflammation by enhancing mitochondrial biogenesis.

Mitochondrial ETC complex enzymes are involved in ATP generation through oxidative phosphorylation (Hatefi, 1985). Numerous studies reported mitochondrial ETC enzyme dysfunction aggravated cerebral injury (L. Yang, Youngblood, Wu, & Zhang, 2020). Therefore, restoring mitochondrial ETC enzymes is critical for neuronal survival (Ames III, 2000; Franklin, 2011). AMPK activates the ATP synthesizing machinery (Day et al., 2017). I3C treatment significantly enhanced the ETC enzyme activities, indicating that I3C maintains energy homeostasis, thereby promoting cell survival due to AMPK activation. MMP is maintained by mitochondrial ETC complex enzymes (complexes I, III, and IV), and diseased state MMP facilitates the removal of abnormal mitochondria. Moreover, disrupted MMP in ischemic conditions facilitates MPTP opening, which recruits water and other toxicants into the mitochondria, leading to mitochondrial swelling (Zorova et al., 2018). Therefore, maintaining MMP and MPTP functions are crucial for cell survival (J.-L. Yang et al., 2018). I3C treatment restored the MMP and inhibited MPTP opening, indicating the normalization of mitochondrial membrane morphology and permitting essential compounds entry into mitochondria. Cytochrome C enzyme (cyt C) is released into the cytosol when MMP and MPTP are compromised in ischemic conditions. The presence of cyt C in cytosol initiates apoptosis by activating caspase 9 and caspase 3 (Borutaite, Toleikis, & Brown, 2013; Chowdhury et al., 2008; De La Lastra & Villegas, 2007; Utkina-Sosunova et al., 2013). I3C ameliorated the cyt C, caspase 9, and caspase 3 is an indication of reduction in mitochondrial-mediated apoptosis in ischemic brains. Supportive to these findings, our histological (H and E) findings indicated that I3C reestablished the cortical architecture by ameliorating vacuoles, nuclear fragmentation, and nuclear shrinkage.

As per earlier findings, I3C acts peripherally and inhibits platelet aggregation and thrombus generation (P. Paliwal et al., 2018). In the present study, I3C acts centrally and protects brain function by stimulating mitochondrial biogenesis. Overall, I3C could be used as an antiplatelet and antithrombotic agent or neuroprotectant in the treatment of stroke.

5.4 Summary

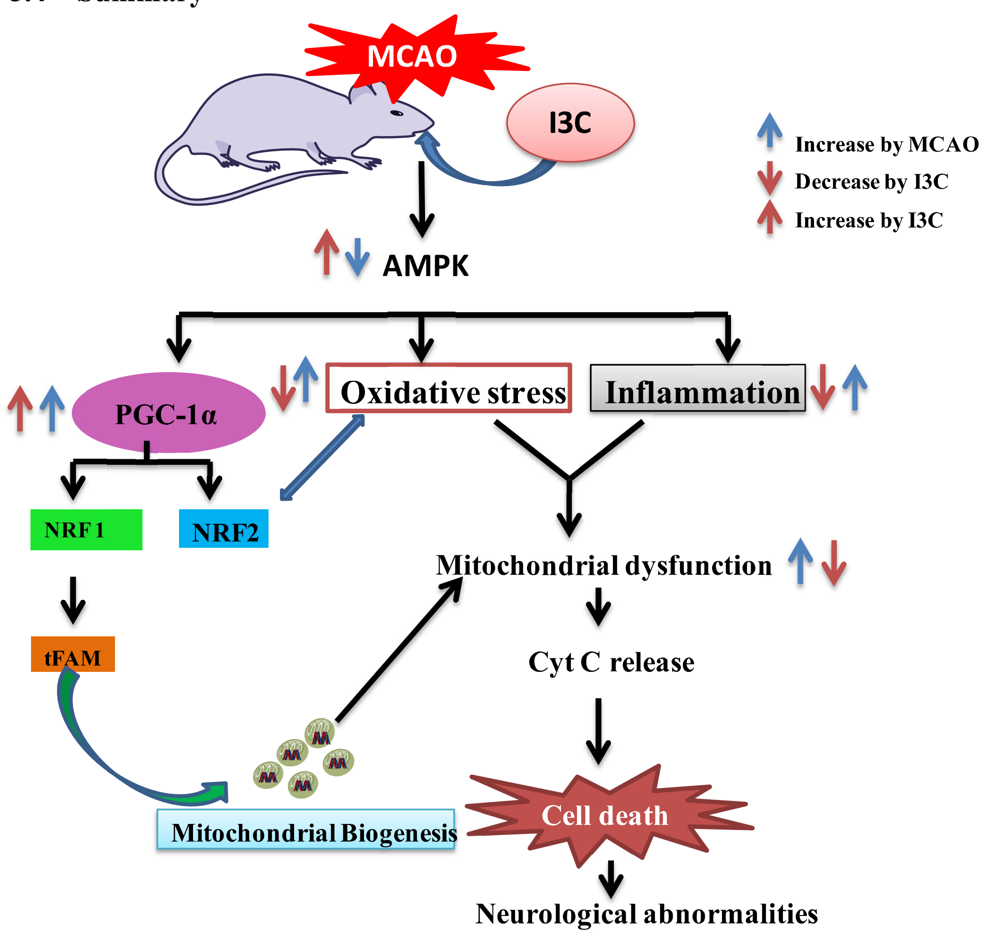

Figure 5.14. The mode of action of I3C against MCAO injury-induced pathological abnormalities.

- I3C treatment ameliorated the neurological dysfunction in cerebral ischemia.

- I3C preserved the mitochondrial function by improving the ETC enzyme activities, MMP and decreased the MPTP opening.
- Repeated I3C treatment significantly alleviated oxidative stress and inflammation in MCAO rats.
- I3C treatment ameliorated apoptosis and restored cortical architecture in MCAO rats.
- I3C stimulated mitochondrial biogenesis by activating the AMPK/PGC-1α in MCAO rats.
- In conclusion, I3C improved the mitochondrial function in cerebral ischemia by activating AMPK/PGC-1α pathways, protecting the neurons from ischemic injury. Therefore, I3C can be a promising candidate for the management of stroke.