CHAPTER 4

RESULTS
The results obtained at various stages of the present study have been presented here in a sequential manner to accomplish the objectives and highlight the aims. The study has been conducted on following outlines, which includes: 1. Assessment of mode of cellular damage in the progression of ischemic brain damage in diabetes. 2. Further, to delineate the nuclear changes and DNA fragmentation following cerebral ischemia and reperfusion in diabetics. 3. Moreover, efforts were made to analyze the expression and localization of various cell survival/death regulatory proteins to elucidate the involvement of mitochondria in the progression of the cellular damage via the release of mitochondrial proteins and to explore the mitochondria mediated end point(s) in diabetic stroke. 5. Besides, studies were also undertaken to assess the role of oxidative stress using melatonin.

The cerebral ischemia induced by MCAo at various time points (0.5, 1 and 2 h) following reperfusion of 24 h in normoglycemic and diabetic rats was examined to differentiate the relative cerebral damage. It was observed that I/R injury led varying degree of brain damage and severity of which was assessed by several parameters that includes neurological deficit (ND), oxidative stress markers of lipid peroxidation: malondialdehyde, intracellular antioxidant (GSH) status and besides monitoring size of cerebral infarct. Further, at microscopic level the cellular and nuclear changes were analyzed with H&E and TUNEL staining.

Once the preliminary evidence was accumulated, then a detailed investigation was undertaken to reveal the cellular and molecular mechanisms of ischemic brain damage at 1 h of ischemia followed by reperfusion time points of 3, 6, 12 and 24 h. The I/R led brain damage was assessed by ND, MDA, GSH, TTC, H&E and TUNEL staining, ROS and mitochondrial potential, These changes were further assessed at transcription and translation level by analyzing the expression and translocation of HSP70, HSP60, Bax, Bcl-2, cytochrome c, caspase-3 AIF and PARP.
4.1. NEUROBEHAVIORAL ASSESSMENT

The neurological deficit (ND) scored in all the experimental rats at the end of reperfusion following varying degree of ischemia, showed varying degrees of flexion, circling, hemi-paresis and non-spontaneity exhibiting impaired sensori-motor ability. For normoglycemic rats, this deficit was mild and showed contralateral forelimb impairment, loss of postural reflex, showed problems in balance and grip strength on the side of the occlusion. In contrast, diabetic rats showed a more pronounced deficit.

The ND test scale used provides a general indication of neurological differences between the two groups studied presently. The I/R injury of varying degree produced ND in increasing order in normoglycemic subjects with 0.5/24 h of I/R, the average score was 3.25 and with 1/24 h it was 5.91, which further enhanced to a score of 7.37 with 2/24 h of I/R. In the diabetic group, the ND score was conspicuously further increased as compared to normoglycemic rats. The average ND score was about 4.62, 7.64 and 8.62 at 0.5/24, 1/24 and 2/24 h post I/R injury respectively (Figure 7A). Further, diabetic 2/24 h I/R group showed neurological score above 8 with very high mortality rate (>90 %). The neurological deficit was significantly higher in diabetic rats at each time point of I/R as compared to normoglycemic ones and this reflects the damage in both cortical and subcortical areas of the brain.

Additionally, when ischemia time point was fixed for 1 h and varied reperfusion time for 3, 6, 12 and 24 h, it was observed that the varying degree of I/R injury produced increasing order of neurobehavioral alterations (Figure 8A). The ND increased with increasing I/R with an average score of 3.37, 4.70, 4.80 and 5.91 respectively in normoglycemic subjects at 1/3, 1/6, 1/12 and 1/24 h. The increased ND score was more conspicuous in diabetic group with an average score of 5.16, 5.83, 6.75 and 7.64±0.21 at respective time points. In general, the ND was significantly (P<0.001) higher in diabetics as compared to normoglycemic rats at respective time points of I/R.

4.2. BIOCHEMICAL STUDIES

4.2.1. Malondialdehyde (MDA)

The MDA level estimated in blood serum of normoglycemic and diabetic rats following ischemia/reperfusion are shown in figure 7B. MDA level gradually increased with increasing I/R injury in normoglycemic animals. In contrast, MDA increased further in diabetic group at each time point of I/R except 2/24 h, where MDA level was comparable to respective time point in normoglycemic rats. The increase was
significantly higher at 0.5/24 \((P<0.01)\) and 1/24 h \((P<0.05)\) of I/R in diabetic as compared to normoglycemic animals at respective time point of I/R injury. The MDA level reached maximum at 1/24 h of I/R in diabetics, whereas the level reached maximum at 2/24 h of I/R in normoglycemic group.

Similarly, the MDA level gradually increased with increasing (1/3, 1/6, 1/12 and 1/24 h) I/R time points and remained elevated up to 24 h of reperfusion in normoglycemic animals (Figure 8B). In contrast, MDA increased further in diabetic group and increase was significant at 1/3 \((P<0.01)\), 1/6 \((P<0.05)\) and 1/24 h \((P<0.01)\) at respective time point of I/R as compared to normoglycemic rats. The rise in MDA level in serum of both I/R groups depicts the impact of oxidative stress on lipid peroxidation and subsequent ischemic brain damage.

The rise in MDA level in serum of both I/R groups depicts the oxidative stress induced lipid peroxidation and relates to the brain damage.

4.2.2. Glutathione (GSH)

Glutathione is a central component in the antioxidant defense mechanism, acting both directly to detoxify reactive oxygen species and as a substrate for various peroxidases. The I/R induced stress depletes the glutathione level in the brain and showed overall decrease. The GSH level decreased significantly at each time point of I/R injury in normoglycemic as well as in diabetic ischemic rats as compared to respective sham control. Moreover, when comparison was made between normoglycemic and diabetic I/R groups, the GSH level decreased significantly at 0.5/24 \((P<0.01)\) and 1/24 h \((P<0.05)\) of I/R injury. The decrease in GSH level was maximum at 2/24 h I/R in both the groups. The stress induced synthesis of glutathione does not replenish the depleted stores (Figure 7C)

Similar trend was also observed at 1/3, 1/6, 1/12 and 1/24 h of I/R injury and level further decreased in diabetic rats at respective time point of I/R injury. The decrease was significantly lower at 1/3 and 1/24 h \((P<0.01)\) of I/R in diabetics as compared to normoglycemic animals (Figure 8C).

4.3. HISTOLOGICAL STUDIES

4.3.1. Infarct volume and morphometric analysis with TTC

The brain stained with TTC revealed clear distinction between viable as deep red in colour and damaged tissue as unstained white mass. The cerebral damage progressively increased with increasing I/R injury in both normoglycemic and diabetic
groups as compared to respective sham control, which showed normal tissue structure. The cerebral damage was nonconspicuous but showed characteristics of developing infarction at 0.5/24 h of I/R in normoglycemic rats. In contrast a small infarct was clearly visible in diabetic rat brain at same time point of I/R. Moreover, the infarct size became significantly greater \((P<0.01)\) in diabetics after 1/24 and 2/24 h of I/R as compared to normoglycemic animals (Figure 9-10). The infarction virtually extended to the entire affected brain side and encompassed the subcortical and the cortical area at 2/24 h of I/R injury, thereby having high impact on mortality rate at this time point.

Similar trend was also observed at 1/3, 1/6, 1/12 and 1/24 h of I/R injury, and the brain tissue showed small infarct in diabetic rat brain at 1/3 h of I/R, whereas the infarct was not conspicuous in brain of normoglycemic animals. Moreover, the infarction became larger after increasing time point of I/R in the brain of diabetic rats as compared to normoglycemic animals (Figure 11).

Interestingly, the infarct volume correlated well with the ND score at each time point and depicts the severity of ischemic stress in diabetics.

### 4.3.2. Cellular morphological alterations with haematoxylin & eosin (H&E)

The cerebral I/R injury was revealed cellular level with H&E staining. The I/R injury induced changes were more pronounced in diabetics in comparison to normoglycemic animals (Figure 12). It was observed that striatal region was greatly affected following I/R injury followed by cortex but hippocampus was not affected by ischemic insult and showed normal morphology.

The cellular damage at 0.5/24 h appeared immature in normoglycemic subjects, whereas, the cell damage matured in diabetics at respective time point of I/R and progressively increased upon extending the I/R injury with nearly complete damage in striatal area at 1/24 h of I/R in diabetics. A very similar pattern was also observed in the cortex. Further, hippocampal region showed no cellular alterations at 0.5/24 h of I/R but some changes were observed at 1/24 h of I/R. The ischemic injured cells exhibited features of both necrosis and apoptosis. Necrotic cells were characterized by nuclear pyknosis, karyolysis, cytoplasmic eosinophilia (red neurons) and nuclei lacking cellular structures (ghost neurons). Whereas, apoptotic cells were characterized by more than two vesicle shaped protuberances, called the apoptotic bodies, on the cell surface roughly spherical or ovoid in shape.
It was observed that besides necrotic changes, the apoptotic features were also increased with the increased ischemic insult. The cellular damage indicated by shrunken dark cell morphology was greater at each time point of I/R in diabetics as compared to normoglycemic animals. Further, cell death in striatum although was more of necrotic nature, few apoptotic cells were also visible at 0.5/24 h of I/R in diabetic rats and, which increased further at 1/24 h. Apoptotic cell density was higher in cortex of diabetic as compared to normoglycemic subjects at 1/24 h of I/R injury. Further, a large number of cavitations observed indicate the loss of cells, which were higher in diabetics at 1/24 of I/R in cortical and striatal area.

These results suggested that cerebral damage progression besides necrosis was also mediated in part by apoptotic mechanism in diabetics following I/R injury.

4.3.3. Nuclear changes and DNA fragmentation with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay

The TUNEL assay was used to demarcate I/R stress induced alterations at nuclear level and to demonstrate nucleosomal DNA fragmentation in situ. The TUNEL staining have distinguished cells with normal physiology and demarcated cells with fragmented DNA undergoing through various stages of apoptotic cell damage such as a condensed nuclear appearance and margination of nuclear structure into vesicle like appearance called apoptotic bodies. These cell characteristics were further confirmed by colabelling sections with Hoechst dye (Figure 13-16).

It was observed that DNA fragmentation represented by TUNEL positive cells increased with increasing I/R injury. However, the increase was further enhanced in diabetic subjects. TUNEL positive cells were greater in striatum followed by cortex and hippocampus at respective time point of I/R injury. The TUNEL positive cells were absent or negligible in sham, hippocampal and cortical areas of 0.5/24 h I/R group. A quantitative evaluation of TUNEL positive cells is shown in figure13-15. TUNEL positive cells significantly \((P<0.001)\) increased in diabetic rats when compared to normoglycemic at 0.5/24 and 1/24 h of I/R in striatum. Similarly, significant increase in TUNEL positive cells was also evident at 1/24h of I/R in cortex and hippocampus of diabetic animals as compared to normoglycemic ones, indicating the severity of I/R injury in diabetes. The striatum seems to be the most affected region following I/R injury, followed by cortex. Further significant increase in the frequency
of TUNEL positive cells along with apoptotic features in diabetes compared to controls indicate a very significant contribution of apoptosis in diabetes.

**Figure 7:**

A- Neurological deficit in normoglycemic versus diabetic rats subjected to 0.5/24, 1/24 and 2/24 h of I/R stress. The significance ***$P<0.001$, *$P<0.05$ (n=≥5 each) is between normoglycemic and diabetic groups at respective time points. The data is represented as mean±SEM and analyzed by one-way ANOVA followed by Newman-Keuls test.

B- The serum MDA in normoglycemic versus diabetic rats at different time points of I/R injury. The MDA level significantly increased (**$P<0.01$, *$P<0.05$) in diabetics as compared to normoglycemic rats at respective time point.

C- The effect of I/R injury of different time points on GSH level in normoglycemic and diabetic rats. The GSH level significantly decreased (ϕ$P<0.01$, ϕ$P<0.05$) in diabetics as compared to normoglycemic rats at respective time points of I/R injury (n=≥5 each).
**RESULTS**

A- Neurological deficit in normoglycemic versus diabetic rats subjected to 1/3, 1/6, 1/12 and 1/24 h I/R stress. The significance ***P<0.001 (n=≥5 each) is between normoglycemic and diabetic groups at respective time points. The data is represented as mean±SEM and analyzed by one-way ANOVA followed by Newman-Keuls test.

B- The serum MDA in normoglycemic versus diabetic rats at different time points of I/R injury. The MDA level significantly increased (**P<0.01, *P<0.05) in diabetics as compared to normoglycemic rats at respective time point (n=≥ 5 each).

C- The effect of I/R injury of different time points on GSH level in normoglycemic and diabetic rats. The GSH level significantly decreased (ϕϕP<0.01, ϕP<0.05) in diabetics as compared to normoglycemic rats at respective time points of I/R injury (n=≥5 each).
The effect of I/R injury of 0.5/24, 1/24 and 2/24 h time points on brain damage. TTC stained brain slices clearly shows the progression of ischemic brain damage at varying time point of I/R injury.

The effect of I/R injury of different time points on infarct volume in normoglycemic and diabetic rats. The infarct significantly increased in diabetics as compared to normoglycemic rats at respective time point. The significance **P<0.01, *P<0.05 (n=≥4 each) shown as between normoglycemic and diabetic groups at respective time point. The data is represented as mean±SEM and determined by one-way ANOVA followed by Newman-Keuls test.

TTC stained brain slices showing clear distinction between the normal and infarcted tissue at 1/3, 1/6, 1/12 and 1/24 h of I/R injury.

Figure 9: The effect of I/R injury of 0.5/24, 1/24 and 2/24 h time points on brain damage. TTC stained brain slices clearly shows the progression of ischemic brain damage at varying time point of I/R injury.

Figure 10: The effect of I/R injury of different time points on infarct volume in normoglycemic and diabetic rats. The infarct significantly increased in diabetics as compared to normoglycemic rats at respective time point. The significance **P<0.01, *P<0.05 (n=≥4 each) shown as between normoglycemic and diabetic groups at respective time point. The data is represented as mean±SEM and determined by one-way ANOVA followed by Newman-Keuls test.

Figure 11: TTC stained brain slices showing clear distinction between the normal and infarcted tissue at 1/3, 1/6, 1/12 and 1/24 h of I/R injury.
Figure 12: H&E stained representative photomicrographs showing the brain damage in cortex, striatum and hippocampal brain areas in normoglycemic and diabetic rats following 1/24 h of I/R injury. Arrow=Shrunken neurons, Arrowhead=A apoptotic cells (inset). NM=Normoglycemic MCAo and, DM=Diabetic MCAo, Bar=50 μm.
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The effect of I/R injury of 0.5/24 and 1/24 h on DNA fragmentation in cortex of normoglycemic and diabetic rats. TUNEL positive cells (green) and Hoechst staining (blue) displayed distinct features of apoptosis in cortical area (Arrow=Apoptotic cells, shown in Inset). The TUNEL positive brain cells significantly (***P<0.001) increased in diabetics as compared to normoglycemic rats at 1/24 h (n≥3 each). NM=Normoglycemic MCAo, DM=Diabetic MCAo, Bar=50 μm.

Figure 13. The effect of I/R injury of 0.5/24 and 1/24 h on DNA fragmentation in cortex of normoglycemic and diabetic rats. TUNEL positive cells (green) and Hoechst staining (blue) displayed distinct features of apoptosis in cortical area (Arrow=Apoptotic cells, shown in Inset). The TUNEL positive brain cells significantly (***P<0.001) increased in diabetics as compared to normoglycemic rats at 1/24 h (n≥3 each). NM=Normoglycemic MCAo, DM=Diabetic MCAo, Bar=50 μm.
**RESULTS**

**Figure 14.** The effect of I/R injury of 0.5/24 and 1/24 h on DNA fragmentation in striatum of normoglycemic and diabetic rats. TUNEL positive cells (green) and Hoechst staining (blue) displayed distinct features of apoptosis (Arrow=Apoptotic cells, shown in Inset). The TUNEL positive brain cells significantly (**P<0.001**) increased in diabetics as compared to normoglycemic rats at 1/24 h (n=≥3 each). NM=Normoglycemic MCAo, DM=Diabetic MCAo, Bar=50 μm.
Results

The effect of I/R injury of 0.5/24 and 1/24 h on DNA fragmentation in hippocampus of normoglycemic and diabetic rats. TUNEL positive cells (green) and Hoechst staining (blue) displayed distinct features of apoptosis in striatal area (Arrow=Apoptotic cells, shown in Inset). The TUNEL positive brain cells significantly (**P<0.001) increased in diabetics as compared to normoglycemic rats at respective time point (n=≥3 each). NM=Normoglycemic MCAo, DM=Diabetic MCAo, Bar=50 μm.

Figure 15. The effect of I/R injury of 0.5/24 and 1/24 h on DNA fragmentation in hippocampus of normoglycemic and diabetic rats. TUNEL positive cells (green) and Hoechst staining (blue) displayed distinct features of apoptosis in striatal area (Arrow=Apoptotic cells, shown in Inset). The TUNEL positive brain cells significantly (**P<0.001) increased in diabetics as compared to normoglycemic rats at respective time point (n=≥3 each). NM=Normoglycemic MCAo, DM=Diabetic MCAo, Bar=50 μm.
Detailed screening of various changes at nuclear level demarcated with TUNEL (green) and Hoechst (blue) staining in cortical and striatal area of normoglycemic and diabetic groups following 1/24 h of I/R injury. TUNEL positive cells colabelled with Hoechst (blue) displayed distinct features of apoptosis (Arrow=Apoptotic cells, shown in Inset). Features of caspase-dependant and independent peripheral chromatin condensation at 1/24 h of I/R in diabetics. NM=Normoglycemic MCAo, DM=Diabetic MCAo, Bar=50 μm.
4.4. FACs STUDIES

4.4.1. Apoptotic changes at early and late I/R time point using Annexin V-FITC apoptosis detection kit

It was observed that besides necrotic changes, cells also appeared to be damaged by apoptotic mechanism. The cellular morphological features, indicative of apoptosis, characterized by H&E and TUNEL were further confirmed at early (1/3 h) and late (1/24 h) I/R time points by using Annexin V-FITC apoptosis detection kit. It had quantitatively determined the percentage of cells within the population that are actively undergoing apoptosis and relies on the property of cells to lose membrane asymmetry in early phases of apoptosis by exposing phosphatidylserine (PS) to the external environment upon flipping form inner leaflet of the plasma membrane to outer leaflet. Annexin V with affinity binds to the exposed PS and indicates the early apoptotic changes in the cell.

It was found that cells appear to lose its symmetry as early as 1/3 h of I/R in the affected cortical and striatal area of both normoglycemic and diabetic rats as indicated by Annexin V-FITC positive cells (Figure 17). The brain cells undergoing apoptotic changes in the cortex of normoglycemic animals increased further at 1/24 h of I/R. In contrast, the increase in brain cells undergoing apoptotic changes were enhanced further by diabetes and number of these cells were significantly ($P<0.01$) higher at 1/3 and 1/24 h of I/R in diabetic rats as compared to normoglycemic ones at respective time point of I/R.

Similarly, the brain cells undergoing early apoptotic changes were also detected in the striatal area of normoglycemic and diabetic animals. The number of these cells, following early increase at 1/3 h of I/R, showed marked decrease at 1/24 h of I/R. Similar trend was also showed by the brain cells of diabetic group. However, cells with early apoptotic changes were greater in diabetic rats and significantly ($P<0.01$) higher at 1/3 h of I/R injury as compared to normoglycemic animals (Figure 18).
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Figure 17: Representative flow cytogram of an Annexin V binding (abscissa, FL1) versus propidium iodide uptake (ordinate, FL2) in the affected cortical area of the normoglycemic and diabetic rat brain cells at 1/3 and 1/24 h of I/R injury. The numbers in the left upper quadrant, right upper quadrant, left lower quadrant and right lower quadrant represent the percentage of damaged (Annexin V-/PI+), necrotic (Annexin V+/PI+), live (Annexin V-/PI-), and apoptotic cells (Annexin V+/PI-), respectively. NM=Normoglycemic MCAo, DM=Diabetic MCAo.

Relative percentage of Annexin V positive cells in cortical area. The number of these cells increased significantly after I/R injury in diabetics as compared to normoglycemic rats.
**Figure 18:** Representative flow cytogram of an Annexin V binding (abscissa, FL1) versus propidium iodide uptake (ordinate, FL2) in the affected striatal area of the normoglycemic and diabetic rat brain cells at 1/3 and 1/24 h of I/R injury. The numbers in the left upper quadrant, right upper quadrant, left lower quadrant and right lower quadrant represent the percentage of damaged (Annexin V-/PI+), necrotic (Annexin V+/PI+), live (Annexin V-/PI-), and apoptotic cells (Annexin V+/PI-), respectively. NM=Normoglycemic MCAo, DM=Diabetic MCAo.

Relative percentage of Annexin V positive cells in striatal area. The number of these cells increased significantly after I/R injury in diabetics as compared to normoglycemic rats.
4.4.2. Cerebral ischemia/reperfusion induced ROS generation

We examined cellular ROS levels in cortex and striatum of brain following 1/3, 1/6 and 1/24 h of I/R injury by flow cytometry using oxidation sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate (DCF-DA). The time course experiments revealed the marked elevation of ROS level following restoration of cerebral blood flow at 1/3 of reperfusion and showed gradual decline upon extending the reperfusion time to 24 h in cortical and striatal area of the brain of normoglycemic animals. In contrast, the ROS level further increased in diabetics and the elevated ROS level persisted for 1/24 h of reperfusion in cortex. In the striatal area, the ROS level decreased sharply at 1/6 h of reperfusion and showed increased tendency thereafter. The increase in ROS level was significant at 1/6 ($P<0.05$) and 1/24 h ($P<0.01$) of I/R in cortex and 1/3 and 1/24 h ($P<0.01$) of I/R in striatum of diabetics as compared to normoglycemic animals respectively (Figure 19). The rapid rise in the ROS level following withdrawal of the intraluminal filament seems to be due to the instantaneous reperfusion, which resulted in damage to striatal area as early as 1/3 h of I/R in diabetic animals.

![Figure 19: ROS generation in cortex and striatum of normoglycemic and diabetic rats at different time points of I/R injury (n=4 each). Results were analyzed with ANOVA followed by Newman-Keuls test. *$P<0.05$ and **$P<0.01$ between normoglycemic and diabetic groups at respective time point of I/R.](image-url)
4.4.3. Alteration in mitochondrial membrane potential (ΔΨm)

Cerebral I/R injury at different time points affected the ΔΨm of the cells. The JC-1 red-to-green ratio of control was normalized and converted to 1.0. The increase and decrease from this value reflects the alteration in ΔΨm (Figure 20). At early time points of I/R, the ΔΨm gradually had a tendency to increase, and as the reperfusion time further increased, the ΔΨm exhibited decreasing trend. In cortex, the ΔΨm increased from 3 h (1.12±0.12) to 6 h (1.38±0.10) post-reperfusion and thereafter decreased at 24 h (0.90±0.11) in normoglycemic rats. In diabetic animals, the ΔΨm increased further and fell close to normoglycemic animals at 24 h post-reperfusion. However, in striatal region, the ΔΨm increase (1.95±0.29) peaked at 3 h in diabetic as comparison to normoglycemic animals, which showed ΔΨm increase (1.82±0.21) at 6 h post-reperfusion. The ΔΨm decreased after 6 h of reperfusion in both the groups. The increase in ΔΨm during early reoxygenation appears to be due to hyperpolarization, while upon extending the reperfusion time; the ΔΨm remained below 1.0, thereby showing the depolarizing effect.

![Diagram](image)

**Figure 20:** The graph indicates the JC-1 fluorescence change of ΔΨm in cortical and striatal area measured using flow cytometer. Results showed hyperpolarization, followed by depolarization of the ΔΨm in normoglycemic and diabetic rats. *P<0.05 and **P<0.01 vs. respective time point of I/R (n=4 per time point)
4.5. MOLECULAR STUDIES

4.5.1. Effect of cerebral ischemia on stress responsive and cell death regulatory proteins, HSP70, HSP60, Bax, Bel-2, cytochrome c, caspase-3, AIF, and PARP

Based on the results obtained using histological (morphological alteration) and DNA fragmentation analysis, we analyzed the expression pattern of various proteins involved in stress response and cell death regulation following cerebral I/R injury. The regulatory proteins of interest viz HSP70, HSP60, Bax, Bcl-2, cytochrome c, caspase-3, AIF, and PARP were analyzed in ischemic brain tissue. Further, the quantification of these proteins was done to ascertain the significance of these proteins at different time points of I/R injury in diabetics.

4.5.1.1. Expression of stress marker HSP70 and HSP60

**HSP70:** Western blot analysis revealed an increase in expression of HSP70 in the striatal region of normoglycemic animals with increasing I/R time points, peaked at 12 h of reperfusion with slight decrease thereafter (Figure 21). However, HSP70 protein expression displayed exactly a reverse trend in diabetics at each time points of I/R as compared to normoglycemic rats with a tendency of recovery at 24 h of reperfusion. The expression profile of HSP70 was virtually similar in cortex of normoglycemic animals, however, there was slight decrease initially in diabetics but it increased later being significantly higher ($P<0.05$) at 24 h of reperfusion as compared to normoglycemic rats. Further, HSP70 induction revealed that its expression persists in mild stress, whereas in severe stress, its expression reduces markedly. Thus, HSP70 serve as a marker of stress underlying the severity of ischemic stress in normoglycemic and diabetic rats.

**HSP60:** HSP60 is a resident of the mitochondrial matrix, constitutively expressed and it facilitates the folding of newly translated mitochondrial proteins transported into the mitochondrial matrix. In the present study, western analyses of mitochondrial fraction of normoglycemic and diabetic rat brains subjected to ischemia and reperfusion of different time points revealed that HSP60 contents in normoglycemic ischemia significantly ($P<0.01$) increased in the cortex at 3 h of recovery, while diabetes caused HSP60 to increase in sham-operated diabetic controls as well as each time point of I/R injury (Figure 22). However, HSP60 did not change significantly in diabetic group as compared to normoglycemic animals. The significant reduction in HSP60 in striatum of normoglycemic and diabetic rats clearly indicates the I/R induced mitochondrial stress.
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**Figure 21**: HSP70 expression in the cortex and striatum of the normoglycemic and diabetic rats. HSP70 protein expression in the cytosolic fraction reduced in diabetics in striatum and cortex at early hours of I/R and recovered later in cortex as compared to normoglycemic rats at respective time points of I/R injury. Semiquantitative changes of HSP70 are expressed as mean±SEM (n=4 per time point). fP<0.05, fP<0.01 and fP<0.001 decrease vs. respective time of I/R. SN=Sham Normoglycemic, SD=Sham Diabetic, NM=Normoglycemic MCAo and, DM=Diabetic MCAo.

**Figure 22**: HSP60 expression in the cortex and striatum of the normoglycemic and diabetic rats. Semiquantitative changes of HSP60 are expressed as mean±SEM (n=4 per time point). */P<0.05 increase vs. respective time of I/R. SN=Sham Normoglycemic, SD=Sham Diabetic, NM=Normoglycemic MCAo and, DM=Diabetic MCAo.
4.5.1.2. Expression pattern of Bcl-2, Bax translocation and cytochrome c release

The cellular and DNA fragmentation analysis has revealed that cerebral ischemia activates several cell death signaling pathways and one such response is the activation of apoptotic cell signaling. Bcl-2 (antiapoptotic) and its related protein Bax (proapoptotic) are essential for the regulation of apoptotic cell death, through formation of pore in the mitochondrial membrane and release of apoptotic mitochondrial proteins such as cytochrome c.

**Bcl-2:** In the present study, western analysis revealed that Bcl-2 expression in the cortical area increased gradually up to 1/12 h of I/R and reduced thereafter in normoglycemic rats (Figure 23). In contrast Bcl-2 expression exhibited biphasic pattern in the cortex of diabetic group. Following an initial significant ($P<0.05$) increase at 1/3 h, the Bcl-2 expression reduced at 1/6 and the expression significantly was declined at 1/12h of I/R in diabetic rats as compared to normoglycemic ones. But its expression elevated significantly ($P<0.01$) at 1/24 h of I/R in diabetics as compared to normoglycemic subjects.

Similarly, the expression profile of Bcl-2 in the striatum revealed that Bcl-2 in normoglycemic rats, elevated at 1/3 h, reduced but exhibited recovery at 1/24 of I/R. The expression profile was very similar to that of cortex in diabetic group. Interestingly, Bcl-2 expression in striatum of diabetic rats decreased considerably and the expression remained below the basal level (sham value). The decrease was significant at 1/3 h ($P<0.01$) and 1/24 h ($P<0.001$) of I/R injury in diabetics as compared to normoglycemic animals at respective time point.

**Bax:** The Bax protein translocated from cytosol to mitochondria, where it seems to be involved in mitochondrial membrane permeabilization. The level of Bax, after initial increase at 1/3 h, reduced at 1/6 h and increased gradually thereafter up to 1/24 h of I/R in mitochondrial fraction of cortex in normoglycemic rats. Similar trend is seen in diabetic group at each time points of I/R. However, Bax level remained elevated at each time points of I/R in diabetic animals as compared to normoglycemic rats. The quantitative analysis revealed that Bax expression was significantly higher in diabetic at 1/3 and 1/6 h ($P<0.05$) as compared to normoglycemic rats at respective time point of I/R.

In contrast, the Bax expression in striatum of diabetic group decreased following reperfusion (1/3h) from the control value but increased gradually thereafter and the
increase was significant ($P<0.05$) at 1/12 h of I/R as compared to normoglycemic rats (Figure 24).

**Figure 23:** Bcl-2 expression in cortex and striatum of the normoglycemic and diabetic rats. Bcl-2 in the mitochondrial fraction was higher in cortex in diabetics as compared to normoglycemic rats, whereas Bcl-2 level reduced markedly in striatum with increasing I/R stress. Semiquantitative changes of Bcl-2 are expressed as mean±SEM (n=4 per time point). *$P<0.05$ and **$P<0.01$=increase and $P<0.05$=decrease vs. respective time of I/R. SN=Sham Normoglycemic, SD=Sham Diabetic, NM=Normoglycemic MCAO and, DM=Diabetic MCAO.

**Figure 24:** Western blots analysis of Bax in the mitochondrial fraction from the cortex and striatum of the normoglycemic and diabetic rats. Bax was higher in cortex in diabetics at each time point of I/R as compared to normoglycemic rats whereas Bax level reduced initially in striatum, but increased with increasing I/R. Semiquantitative changes of Bax are expressed as mean±SEM (n=4 per time point). *$P<0.05$ vs. respective time of I/R. SN=Sham Normoglycemic, SD=Sham Diabetic, NM=Normoglycemic MCAO and, DM=Diabetic MCAO.
Cytochrome c: The mitochondrial intermembrane space (IMS) contains a heterogeneous class of proteins whose release promotes cell death. The first molecule belonging to this class of proapoptotic proteins that has been identified at the molecular level is cytochrome c. Release of cytochrome c from mitochondria into the cytoplasm is an important step in the activation of apoptotic cell death mechanism. In the present study, the mitochondrial release of cytochrome c was detected using western analysis in cytosolic fraction (Figure 25). The results revealed a biphasic pattern, an increase at early and late time point of I/R) in cortex of normoglycemic rats. Similar pattern was also exhibited in diabetic group, although the release was higher and significantly ($P<0.01$) greater at 1/12 and 1/24 h of I/R in diabetics as compared to normoglycemic rats. Semiquantitation of immunoblots showed that the percentage change of relative density was significantly higher in diabetic than in nondiabetic animals.

The there was mild release of cytochrome c in the striatum of normoglycemic animals, which significantly ($P<0.01$) increased in diabetics at 1/3, 1/6 and 1/24 h of I/R as compared to normoglycemic animals at respective time points.

**Figure 25:** Western blot analysis of cytochrome c in the cytosolic fraction from the cortex and striatum following different time points of I/R injury. Cytochrome c level is elevated slightly in the striatum and at cortex. The release was markedly enhanced by diabetes. Semiquantitative changes of cytochrome c in the cortex and striatum. Data are mean±SEM (n=4 each time point). *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs. respective time point of NM. SN=Sham Normoglycemic, SD=Sham Diabetic, Cyto C=Cytochrome c, NM=Normoglycemic MCAo and, DM=Diabetic MCAo.
4.5.1.3. Expression of caspase-3, AIF translocation and PARP cleavage

Caspase-3: The results of the present study have revealed various changes at cellular and nuclear level that directly indicate the activation of executioner caspase-3. Further, the activation of caspase-3 is dependent on cytochrome c, which is released from mitochondria to the cytosol. On the basis of the above results, we investigated the activation of caspase-3 in cerebral ischemia under diabetic condition using monoclonal antibody specific for the cleaved (active) product p17 of caspase-3 (Figure 26), since it is synthesized as a 32 kDa inactive proenzyme and its activation that is cleaved during activation into a large subunit of 19 or 17 kDa, and a small subunit of 12 kDa.

The results showed that the normoglycemic sham animals displayed very low level of active caspase-3 expression. Further, as the I/R time point increased, the caspase-3 become more and more activated in normoglycemic rats and significantly increased at each time point of I/R as compared to sham control rats in the cortex. However, caspase-3 activation got further increased in diabetic rats at each time point of I/R as compared to normoglycemic rats. Densitometric semiquantitation of immunoblots revealed that the percentage change of relative density of active caspase-3 was significantly higher in diabetics at 1/3 ($P<0.01$), 1/6 and 1/12 h ($P<0.001$) of I/R as compared normoglycemic rats. The caspase-3 activation was maximum at 1/24 h of I/R in both normoglycemic and diabetic groups.

Caspase-3 expression in the striatal area showed similar trend as that of cortex, although the level of increase was lower in the striatum. Interestingly, the caspase-3 activation increase was more in diabetics except 1/6 and 1/24 h of I/R as compared to normoglycemic rats at respective time point. Semiquantitation of immunoblots revealed that the expression was significantly higher at 1/3 ($P<0.05$) and 1/24 h ($P<0.001$) of I/R in diabetic as compared to normoglycemic rats at respective time point.

Thus, the processing of caspase-3 to an active form detected presently in ischemic rat brain early during reperfusion indicates the initiation of apoptotic cell death mechanism, which seems to be involved in ischemic damage progression in normoglycemic and diabetics rats.

AIF: The present study has revealed the compaction of nuclear material at the peripheral region of the nucleus by TUNEL and Hoechst staining. Such condensation of nuclear material is subject to caspase-3 independent cell death program. One of such event is suggested to be mediated by AIF, which is a mitochondrial protein and
involved in caspase-independent cell death following its translocation to nucleus. Presently, we reported for the first time that AIF was processed and translocated from mitochondria to cytosol and nucleus in the both cortical and striatal area of the brain following I/R injury (Figure 27). AIF increased gradually up to 1/6 h and declined thereafter in the nuclear fraction of the cortex of normoglycemic rats. A very similar trend was also observed in the cortex of diabetic rats, however, AIF presence was higher at each time point and significantly ($P<0.001$) greater at 1/3 and 1/6 h of I/R as compared to normoglycemic rats.

The striatal area however, showed marked elevation of AIF in the nuclear fraction and AIF increased gradually with increase in reperfusion time in normoglycemic rats. However, the AIF significantly further increased in the diabetic samples at 1/3 ($P<0.01$) and 1/6 h ($P<0.05$) of I/R as compared to normoglycemic rats. AIF declined after 6 h post reperfusion and remained comparable at 1/12 and 1/24 h of I/R.

Thus, the significant increase of AIF in diabetics as early as 3 h post reperfusion indicates the essential role of AIF in cell death mechanisms leading to enhanced ischemic cerebral damage in diabetes.

**PARP:** PARP is involved in the regulation of chromatin structure, DNA integrity and repair, gene transcription, cell cycle, and chromosomal segregation during physiological conditions. Its activation following the presence of low level of DNA damage may promote cell survival. However, in the presence of widespread DNA damage, the excessive activation of these enzymes has been linked to cell death. In the present study we have found that cerebral ischemia leads to massive DNA damage as revealed by TUNEL positive staining. Therefore, we analyzed the expression of PARP at different time points of I/R injury in normoglycemic and diabetic rats (Figure 28).

Consistent with the AIF translocation and activation of caspase-3, the PARP expression also increased in diabetic animals following I/R injury. However, PARP expression remained nearly similar to the sham (control) value in cortex of normoglycemic rats, but PARP expression increased in diabetic rats as compared to normoglycemic ones at respective time points of I/R and expression remained elevated and significantly higher at 1/3 ($P<0.001$), 1/6 and 1/24 h ($P<0.05$) respectively in diabetics as compared to normoglycemic rats.
The PARP expression in striatum after initial increase at 1/3 and 1/6 h of I/R, however, showed slight decrease. Similar trend was also observed in diabetic rats, although PARP expression was higher at each time point of I/R except 1/24 h. Further, the significant elevation of PARP at 1/3 ($P<0.01$) and 1/6 h ($P<0.05$) of I/R in diabetics is consistent with the rise of AIF in nuclear fraction.

In addition to the involvement of PARP in the translocation of AIF form mitochondria to nucleus, PARP, being a direct substrate to caspase-3, is also the sensor of caspase-3 activation. Therefore, the cleavage of PARP studied indicates that PARP was cleaved in diabetic animals as early as 3 h of reperfusion. The cleaved PARP was located in the nuclear fraction of both cortex and striatum of normoglycemic and diabetic rats. However, cleaved product of PARP was significantly higher in diabetics as compared to the normoglycemic rats (Figure 28).

Therefore, the significant increase of PARP at early time point of I/R in diabetics is consistent with the translocation of AIF at respective time point of I/R, although, its increased cleavage with increasing I/R time points indicates the activation of caspase-3.

![Figure 28](image-url)

**Figure 28:** Caspase-3 (active) expression in the cortex and striatum of normoglycemic and diabetic rats after different time points of I/R injury. Caspase-3 gets activated as early as 1/3 h of I/R and the expression was significantly higher in diabetics as compared to normoglycemic rats. Data are represented as mean±SEM (n=4 each time points). *$P<0.05$, **$P<0.01$ and ***$P<0.001$ increase, and ϕϕ$P<0.01$ decrease vs. respective time point of NM SN=Sham Normoglycemic, SD=Sham Diabetic, NM=Normoglycemic MCAo and, DM=Diabetic MCAo.
FIGURE 27: AIF presence in the nuclear region in cortical and striatal area of normoglycemic and diabetic rats following different time points of I/R injury. Data are represented as mean±SEM (n=4 each time points). *P<0.05, **P<0.01 and ***P<0.001 increase, and §P<0.01 decrease vs. respective time point of NM. SN=Sham Normoglycemic, SD=Sham Diabetic, NM=Normoglycemic MCAo and, DM=Diabetic MCAo.
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PARP expression in cortical and striatal area at varying degrees of I/R injury. PARP level is elevated mildly after 3 and 6 h of reperfusion in both the striatum and cortex. The increase was further enhanced in diabetics relative to normoglycemic rats. Additionally, PARP was cleaved in both the groups but the level of cleaved product was higher in affected region of the brain in diabetics. Semiquantitative analysis of PARP in cortex and striatum are represented as mean±SEM (n=4 each time points). *P<0.05 and ***P<0.001 vs. respective time point of I/R of NM. SN=Sham Normoglycemic, SD=Sham Diabetic, NM=Normoglycemic MCAo and, DM=Diabetic MCAo.

Figure 28: PARP expression in cortical and striatal area at varying degrees of I/R injury. PARP level is elevated mildly after 3 and 6 h of reperfusion in both the striatum and cortex. The increase was further enhanced in diabetics relative to normoglycemic rats. Additionally, PARP was cleaved in both the groups but the level of cleaved product was higher in affected region of the brain in diabetics. Semiquantitative analysis of PARP in cortex and striatum are represented as mean±SEM (n=4 each time points). *P<0.05 and ***P<0.001 vs. respective time point of I/R of NM. SN=Sham Normoglycemic, SD=Sham Diabetic, NM=Normoglycemic MCAo and, DM=Diabetic MCAo.
4.5.2. mRNA expression of HSP70, caspase-3, AIF and PARP

**HSP70 mRNA:** HSP70 at transcriptional level in the cortical and striatal area of normoglycemic and diabetics did not alter significantly. However, HSP70 mRNA significantly ($P<0.001$) increased at 1/6 h of I/R in cortical area of diabetic rats as compared to normoglycemic ones (Figure 29), indicating the translation inhibition at respective time point at protein level due to increased stress in diabetes. Conversely, the mRNA expression of HSP70 in striatum at 1/6 h in diabetics decreased significantly ($P<0.01$) as compared to the normoglycemic rats, which is consistent with the protein expression at respective time point of I/R. These results, therefore, highlight the transcriptional and translational behavior of HSP70 under mild and severe stress caused by I/R.

![Figure 29](image)

**Figure 29:** Relative mRNA expression of HSP70 from the cortical and striatal tissues following varying degree of I/R injury. Semiquantitative changes of HSP70 mRNA are expressed as mean±SEM (n=4 each time points). *$P<0.001$ increase and $\ddot{\phi}\ddot{\phi}P<0.01$ decrease vs. respective time point of NM. SN=Sham Normoglycemic, SD=Sham Diabetic, NM=Normoglycemic MCAo and, DM=Diabetic MCAo.
Caspase-3 mRNA: In normoglycemic animals, the caspase-3 mRNA showed gradual increase in cortex of normoglycemic at each time points of I/R injury. In contrast, caspase-3 mRNA increase enhanced in diabetic rats as compared to normoglycemic ones at respective time points of I/R, which is well correlated with the protein expression in the same region of the brain.

Moreover, the caspase-3 mRNA in striatum of normoglycemic animals exhibiting an increase from sham value did not change further. However, caspase-3 expression showed maximum increase in striatal area at 3 h of I/R in diabetics as compared to normoglycemic animals and the trend declined thereafter (Figure 30).

![Graph](image)

**Figure 30:** mRNA expression of caspase-3 from the cortical and striatal tissues following varying degree of I/R injury. Semiquantitative changes of caspase-3 mRNA. Data are mean±SEM (n=4 each time points). *P<0.05, **P<0.01 and ***P<0.001 increase and φP<0.05 decrease vs. respective time point of NM. SN=Sham Normoglycemic, SD=Sham Diabetic, NM=Normoglycemic MCAo and, DM=Diabetic MCAo.
AIF mRNA: The mRNA expression of AIF in cortical area of normoglycemic rats did not change markedly from sham control to 1/24 h of I/R. In contrast, the expression increased significantly ($P<0.001$) in sham of diabetic group as compared to normoglycemic sham. However, the AIF mRNA expression did not change significantly in diabetic rats following I/R injury as compared to normoglycemic ones except significant ($P<0.001$) reduction at 1/6 h in diabetics.

Moreover, the mRNA expression of AIF studied in striatal area, showed significant elevation in normoglycemic rats following I/R injury as compared to respective sham control. Further, the expression remained stable up to 1/24 h of I/R injury. In contrast, the mRNA expression of AIF in striatum of diabetic animals showed increase in sham as well as 1/3 h of I/R as compared to respective normoglycemic group. However, as the I/R time point increased further from 1/3 h of I/R, the AIF mRNA in diabetic animals decreased significantly ($P<0.001$) as compared to normoglycemic ones at respective time point of I/R injury (Figure 31).

![Figure 31](image)

**Figure 31:** mRNA expression of AIF from the cortical and striatal tissues following varying degree of I/R injury. Semiquantitative changes of AIF mRNA are represented as mean±SEM (n=4 each time points). ***$P<0.001$ increase and $\phi\phi\phi P<0.001$ decrease vs. respective time point of NM. SN=Sham Normoglycemic, SD=Sham Diabetic, NM=Normoglycemic MCAo and, DM=Diabetic MCAo.
PARP mRNA: The mRNA expression of PARP in cortical area of the brain of normoglycemic rats was similar to the mRNA expression of AIF in the respective brain region and did not change considerably from sham control. However, PARP mRNA reduced significantly ($P<0.001$) in diabetic sham as well as 1/3 and 1/6 h of I/R as compared to the respective group of normoglycemic animals. Similar trend was also found in striatal area, where the PARP mRNA decreased significantly ($P<0.001$) at 1/6 and 1/24 h of I/R as compared to normoglycemic rats (Figure 32).

**Figure 32**: mRNA expression of PARP in the cortical and striatal area of the brain following varying degree of I/R injury. Semiquantitative changes of analyzed PARP mRNA are represented as mean±SEM (n=4 each time points). $\phi\phi\phi P<0.001$ decrease vs. respective time point of NM. SN=Sham Normoglycemic, SD=Sham Diabetic, NM=Normoglycemic MCAo and, DM=Diabetic MCAo.
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4.6. IMMUNOLOCALIZATION OF CASPASE-3, AIF AND PARP

Using immunohistochemical staining for active caspase-3, AIF and PARP, we detected the cells with active caspase-3, AIF and PARP at 24 h post-reperfusion (Figure 33-36). Further, the presence of these proteins in different cell types and various cell compartments are crucial in determining their role in cellular survival/damage following I/R injury. Therefore, sections stained with neuronal marker NeuN depict the localization of these proteins in the neurons and nonneuronal cells. Further, staining with Hoechst has demarcated the localization of these proteins in nuclear compartment following I/R injury.

Caspase-3: Immunolocalization of active caspase-3 was detected in the brains of normoglycemic and diabetic rats following 1/24 h of I/R injury. These results are in agreement with the western analysis and mRNA level of caspase-3 in cortical and subcortical area. It was observed that the caspase-3 localization was mostly confined to neurons, when stained with NeuN, although some non-neuronal cells also exhibited caspase-3 positive staining in normoglycemic and diabetic rats. Additionally, the number of caspase-3 positive neurons in both cortex and striatum was higher in diabetic animals. Further, the staining was localized at nuclear site in diabetic rats, when stained with Hoechst. The caspase-3 positive cells displayed well developed morphological signs of apoptosis with multiple apoptotic bodies, which were scattered throughout the ischemic zone and were considerably enhanced in diabetics (Figure 33-34).

AIF: It was observed that AIF showed well distinguished pattern of localization following I/R injury. In the control rats, AIF staining appeared punctated indicating mitochondrial localization, whereas diffused pattern of AIF signifies its release and translocation from mitochondria to cytosolic and nuclear compartment. The number of cells with diffused pattern was greater in diabetics as compared to normoglycemic rats at 1/24 h of I/R injury (Figure 35)

Further, colabelling of caspase-3 with AIF, showed distinct features of their localization and indicates the concomitant activation of cell death pathway dependent on caspase-3 and AIF (Figure 33).

PARP: In the present study, PARP was coimmunolocalized with AIF to ascertain its importance in ischemic brain damage (Figure 36). The triple staining of PARP, AIF and Hoechst clearly demarcates the presence of these proteins in the nuclear compartment of the cell following I/R injury. It was observed that AIF typically coincide in nuclear region of the cell having intense PARP staining, which was more in
the cortical area of diabetics after 1/24 h of I/R injury. Further, reduced PARP staining in severely affected striatal area is well in correlation to the results of western analysis. However, increased AIF staining in these areas indicate the early activation of PARP and subsequent translocation of AIF to the nuclear site.

Figure 33: Active caspase-3 (red fluorescence, a&g) and AIF (green fluorescence, b&h) in rat brain after focal ischemia of 1/24 h, performed on 5 μm thick brain sections. The sections were counterstained with Hoechst (blue fluorescence, c&i) to visualize nuclear changes. Active caspase-3 and AIF showed more cytoplasmic (diffused) localization in normoglycemic rats. Whereas, they have nuclear localization with scattered cells at this time point in diabetic rats when stained with Hoechst. Note the changes in cell structure. Apoptotic changes seem to be initialized in normoglycemic, whereas, such changes are well developed in diabetics with colocalization of caspases-3 and AIF. Multiple 400x images were captured using Leica fluorescent microscope and composite images (d,e,f,j,k&l) were created by IM50 software (Leica Microsystem), NM=Normoglycemic MCAo, DM=Diabetic MCAo, Bar=50 μm
A: Active caspase-3 (red fluorescence, a&e) colocalization with neuronal marker NeuN (green fluorescence, b&f) in cortex of normoglycemic and diabetic rat brain after focal ischemia of 1/24 h, performed on 5 μm thick brain sections. The sections were counterstained with Hoechst (blue fluorescence, c&g) to visualize nuclear changes. Note the caspase-3 localization is more in neurons as well as nonneuronal cells in diabetics as compared to normoglycemic animals.

B: Active caspase-3 (red fluorescence, i&m) colocalization with neuronal marker NeuN (green fluorescence, j&n) in striatum of normoglycemic and diabetic rat brain after focal ischemia of 1/24 h, performed on 5 μm thick brain sections. The sections were counterstained with Hoechst (blue fluorescence, k&o) to visualize nuclear changes. Note the caspase-3 reduced in striatum at 1/24 h as compared to cortex. Multiple 400x images were captured using Leica fluorescent microscope and composite images (d&h, i&p) were created by IM50 software (Leica Microsystem), NM=Normoglycemic MCAo, DM=Diabetic MCAo, Bar=50 μm.
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A: AIF (red fluorescence, a&e) colocalization with neuronal marker NeuN (green fluorescence, b&f) in cortex of normoglycemic and diabetic rat brain after focal ischemia of 1/24 h, performed on 5 μm thick brain sections. The sections were counterstained with Hoechst (blue fluorescence, c&g) to visualize nuclear changes. Note the localization of AIF is more in neurons of diabetics as compared to normoglycemic animals.

B: AIF (red fluorescence, i&m) colocalization with neuronal marker NeuN (green fluorescence, j&n) in striatum of normoglycemic and diabetic rat at 1/24 h of I/R injury. The sections were counterstained with Hoechst (blue fluorescence, k&o) to visualize nuclear changes. AIF nuclear translocation increased in striatum at 1/24 h of diabetic as compared to normoglycemic rats. Multiple 400x images were captured using Leica fluorescent microscope and composite images (d&h, i&p) were created by IM50 software (Leica Microsystem). NM=Normoglycemic MCAo, DM=Diabetic MCAo, Bar=50 μm.
PARP+Hoechst  AIF+Hoechst  Merged

Picture 36: PARP (red fluorescence, a,d,g&j) and AIF (green fluorescence, b,e,h&k) immunolabelling in cortical and striatal region of the brain after focal ischemia of 1/24 h, performed on 5 μm thick brain sections. The sections were counterstained with Hoechst (blue fluorescence) to visualize nuclear localization. PARP and AIF showed nuclear localization at this time point. Multiple 400x images were captured using Leica fluorescent microscope and composite images were created by IM50 software (Leica Microsystem), NM=Normoglycemic MCAo, DM=Diabetic MCAo, Bar=50 μm.
4.7. STUDY WITH ANTIOXIDANT MELATONIN: to unravel the role of oxidative stress in enhancing ischemic brain damage in diabetes

In the present study it was observed that I/R at different time points lead to the brain damage, the severity of which was greater in diabetic rats as compared to normoglycemic ones. One of the reasons for this increased brain damage in diabetes appeared to be excessive oxidative stress due to the generation of ROS, which leads to lipid peroxidation, depletion of GSH, mitochondrial dysfunction. This activates the intracellular cell death signaling either apoptosis or necrosis that seems to further enhance the brain damage in diabetics. Therefore, we investigated the role of oxidative stress in aggravating the ischemic brain damage in diabetes, using melatonin, which is well known for its neuroprotective effect mainly due to free radical scavenging and antioxidant properties.

4.7.1. Effect of melatonin treatment on ROS generation and mitochondrial potential

The ROS generation and mitochondrial dysfunction appear to be key event in progression of cerebral ischemic damage. Therefore, being a potent free radical scavenger and antioxidant, we investigated the effect of melatonin on ROS generation and mitochondrial potential. It was found that melatonin reduced the ROS level in both cortical and striatal area following 1/24 h of I/R injury as compared to respective groups (Figure 37). Further, lowering of ROS level following melatonin treatment was significantly more in cortical ($P<0.001$) and striatal area ($P<0.05$) of diabetic group as compared to vehicle treated ones.

![Figure 37](image-url) Effect of melatonin treatment on ROS generation in cortex and striatum of normoglycemic and diabetic rats at 1/24 h of I/R injury ($n = 4$ each time points). Results were analyzed by t-test and one-way ANOVA followed by Newman-Keuls test, and represented as mean±SEM. $\phi P<0.05$ and $\phi\phi P<0.01$ decrease vs. respective time point of I/R. NM=Normoglycemic MCAo, DM=Diabetic MCAo.
Moreover, the reduction in ROS level seems to have positive effect on mitochondrial functioning as melatonin treatment normalized the ΔΨm in both cortical and subcortical areas of normoglycemic and diabetic rats following 1/24 h of I/R injury as compared to vehicle treated controls (Figure 38).

4.7.2. Effect of melatonin treatment on DNA fragmentation

The DNA damage due oxidative stress appears to be one key event in progression of cerebral damage following I/R injury. Therefore, the effect of ROS generation was investigated on DNA damage at 1/24 h of I/R. It was observed that melatonin treatment reduced TUNEL positive cells in the ischemia affected territory of the brain. Further, quantitative analysis revealed that the reduction in TUNEL positive cells and hence DNA fragmentation was significantly (P<0.001) improved after melatonin treatment in the striatal area (Figure 39).
Figure 39. The effect melatonin treatment on DNA fragmentation at 1/24 h of I/R injury in striatal area of normoglycemic and diabetic rats. Melatonin treatment significantly (P<0.001) reduced TUNEL positive cells (green) in both normoglycemic and diabetic rats. Hoechst staining (blue) was used to demarcate total number of cells (n≥3 each). Results were analyzed by t-test and one-way ANOVA followed by Newman-Keuls test, and represented as mean±SEM, NM=Normoglycemic MCAo, DM=Diabetic MCAo, Bar=50 µm.

4.7.3. Effect of melatonin on neurological outcome and brain damage

The melatonin administration at the onset and 6 h post reperfusion significantly (P<0.001) reduced the neurological score from 5.91±0.27 to 3.00±0.28 and 7.64±0.21 to 4.07±0.46 in both normoglycemic and diabetic rats respectively as compared to vehicle treated groups, when analyzed after 24 h of reperfusion (Figure 40). Further, the ND score was not significantly different when comparison was made between melatonin treated normoglycemic and diabetic groups. The improvement ND was clearly reflected by significant (P<0.001) reduction in infarct volume from 31.25±2.05 mm³ to 4.06±2.65 and 87.50±14.72 mm³ to 14.83±5.55 mm³ in both normoglycemic and diabetic rats respectively as compared to vehicle treated groups (Figure 41).
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TTC stained brain slices from vehicle and melatonin treated normoglycemic and diabetic rats showing clear reduction in infarct area at 1/24 h of I/R. Morphometric analysis revealed that the infarct volume significantly \((P<0.001)\) reduced following melatonin treatment in both groups. Data represents mean\(\pm\)SEM for each group \((n=\geq4)\) by \(t\) test and one-way ANOVA followed by Newman-Keuls test. NM=Normoglycemic MCAo, DM=Diabetic MCAo.

**Figure 41:** TTC stained brain slices from vehicle and melatonin treated normoglycemic and diabetic rats showing clear reduction in infarct area at 1/24 h of I/R. Morphometric analysis revealed that the infarct volume significantly \((P<0.001)\) reduced following melatonin treatment in both groups. Data represents mean\(\pm\)SEM for each group \((n=\geq4)\) by \(t\) test and one-way ANOVA followed by Newman-Keuls test. NM=Normoglycemic MCAo, DM=Diabetic MCAo.