CHAPTER 3
MATERIALS AND METHODS
3.1. ANIMALS

Adult male Sprague-Dawley (SD) rats weighing 280±20g were procured from National Animal Laboratory Centre (NALC) of Central Drug Research Institute (CDRI), Lucknow, INDIA and used throughout in the study. All procedures were conducted in strict accordance with care and use of laboratory animals after necessary approval by Institutional Animal Ethical Committee (IAEC) vide no. 34/06/S/Pharma/IAEC dt. 19.4.2006 and 92/07/Pharmaco/IAEC dt.16.8.2007. Rats were allowed food and water *ad libitum* in 12 h light-dark cycle before the experiment.

3.2. MATERIAL USED

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<td>Rabbit anti goat HRP conjugated</td>
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### 3.3. METHODOLOGIES

#### 3.3.1. Induction of diabetes

Diabetes was induced in overnight fasted rats by intraperitoneal (i.p.) administration of streptozotocin (STZ) at a dose of 50 mg/kg body weight, freshly prepared in 0.1M citrate buffer (pH 4.5). Streptozotocin was selected to induce experimental diabetes because of its greater selectivity of β-cells for STZ, the lower mortality and longer half life (15 min) of STZ in the body.

Diabetes was confirmed 72 h after STZ administration with Accu-Chek glucometer (Nicolas Piramal, Mumbai). All animals with plasma glucose level >16mM were considered diabetic and included in the study. Age matched rats were injected
with an equal volume of citrate buffer, which served as nondiabetic normoglycemic control and exhibited blood glucose level \( \leq 5 \text{ mM} \). Before the induction of ischemia, the animals were fasted again overnight with the free access to drinking water.

### 3.3.2. Surgical procedure for induction of cerebral ischemia in diabetic rats

Focal cerebral ischemia was induced by occlusion of the middle cerebral artery (MCA) using a modification of the intraluminal technique (Longa et al., 1989; Belayev et al., 1999; Manhas, 2006). The animals were anesthetized with chloral hydrate (300 mg/kg i.p., Sigma-Aldrich). During the whole surgical period, the body temperature of the animals was maintained constant at 36.5±0.5°C by the use of a thermoregulated dissecting heating surgical table. Briefly, the left common carotid artery (CCA) was exposed through the midline incision in the neck region. The CCA was further cleared to expose external carotid artery (ECA) and internal carotid artery (ICA). A 3-0 nylon suture (Ethicon, Johnsons & Johnsons Ltd. Mumbai) with rounded tip was introduced into the internal carotid artery through a small nick in the ECA and then gently advanced 20-22 mm from the CCA bifurcation till the resistance was felt, which blocked the origin of MCA. The suture was pulled back after 0.5, 1 and 2 h to reestablish the CBF and rats of different groups allowed reperfusion for 24h for primary evaluation, and 1 h of ischemia followed by 3, 6, 12 and 24 h of reperfusion for detailed investigation. In sham operated rats all surgical procedures except the occlusion of the MCA were performed. Upon regaining right reflex, the animals were returned to cages with free access to food and water.

### 3.3.3. Melatonin treatment

Rats, subjected to 1/24 h of I/R, were administered melatonin (5 mg/kg i.p.) at onset and 6 h post reperfusion (Pei et al., 2003). Melatonin (Sigma) was dissolved in 1 mL of normal saline containing less than 5% of dimethyl sulfoxide (DMSO, Sigma). Control group of animals received equal amount of vehicle alone.

### 3.3.4. Assessment of neurobehavioral deficit

Animals were examined for neurological deficit (ND) after cerebral ischemia/reperfusion to ascertain the successful induction of ischemia and subsequent decrease in motor response due to cerebral damage as a result of I/R injury (Longa et al., 1989). A neurological sign includes flexion, contralateral circling, hemiparesis and non-spontaneity. ND was assessed as per ND score given in table 5 after respective time point of I/R. Rats without ND were excluded from the study. The brain samples
for histological, flow cytometry, western blot analysis and immunofluorescence were collected at the end of I/R injury.

**Table 5.** Shows grading of neurological score.

<table>
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<th>S. No.</th>
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<th>Score</th>
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<td>i</td>
<td>No neurological deficit</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td>ii</td>
<td>Flexion</td>
<td>1</td>
<td>Mild</td>
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<tr>
<td>iii</td>
<td>Circling</td>
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</tr>
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<td>iv</td>
<td>Hemi paresis</td>
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<td>v</td>
<td>Non-spontaneous movement</td>
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</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>10</td>
<td>Death</td>
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</tbody>
</table>

3.3.5. **Biochemical studies**

Oxidative stress due to overproduction of free radicals during cerebral ischemia/reperfusion may damage membranes, proteins and DNA. The lipid peroxidation of subcellular membranes and accumulation of such products during the reperfusion period can be monitored to assess the brain damage. Lipid peroxidation was estimated in terms of malondialdehyde (MDA) and glutathione (GSH), an endogenous antioxidant was estimated to depict the level of endogenous stores of antioxidant level.

3.3.5.1. **Malondialdehyde (MDA) estimation**

Malondialdehyde is a highly reactive three carbon dialdehyde produced as a byproduct of polyunsaturated fatty acid (PUFA) peroxidation and is the most often measured as an index of lipid peroxidation. The amount of MDA formed as a byproduct of lipid peroxidation was quantitated by reaction with thiobarbituric acid (TBA) in serum (Colado et al., 1997). The pink coloured MDA-TBA complex formed at low pH and high temperature was measured at 532 nm.

3.3.5.2. **Glutathione (GSH) estimation**

Glutathione was estimated by the 5, 5’dithiobis-(2-nitrobenzoic acid) (DTNB)-glutathione reductase coupled assay essentially (Anderson, 1985). The yellow coloured chromogen formed by reaction of GSH with DTNB (Sigma) was read at 412 nm.

3.3.6. **Histological studies**

3.3.6.1. **Brain infarct assessment and morphometric measurement**

2, 3, 5- triphenyltetrazolium chloride (TTC) staining was used to demarcate the brain damage from viable tissue and to calculate the infarct volume (Bederson et al., 1986, Benedek et al., 2006). TTC acts as a proton acceptor for pyridine nucleotide-
linked dehydrogenases that along with the cytochromes form an integral part of the inner mitochondrial membrane and make up the electron transport chain (ETC). The enzymes reduce the tetrazolium salt into a red, lipid-soluble formazan. The live tissue therefore stained red, while the infarct tissue remained unstained and pale. The brain slices were then fixed in 4% paraformaldehyde solution. The brains were rapidly dissected out and sectioned coronally at 2 mm intervals from the rostrum to caudal end. All slices were incubated for 20 min in a 1% solution of TTC at 37°C. The slices were scanned using a computerized image analysis system (Biovis Image Plus) and the infarction area in each section was calculated by subtracting the non-infarct area (mm²) of the ipsilateral side from the area of the contralateral side. The total volume of infarct (mm³) was calculated as: summation of average infarct area (mm²) and multiplied thickness (2 mm) of the brain slices from the same hemisphere. The corrected stroke volume compensating for edema in the ischemic hemisphere was obtained by subtracting the volume of non-ischemic volume from the ischemic hemisphere volume (Khan et al., 2000)

3.3.6.2. Cellular morphological alterations

The cellular changes were analyzed with differential haematoxylin & eosin (H&E) staining. H&E is a differential staining used to analyze the cellular morphology in thin sections. Haematoxylin is a base that preferentially colors acidic components of the cell blue. The most acidic components of cell, the nucleus (DNA and RNA) stain dark blue and the basic components of cell (cytoplasm) constituents stain pink.

Brains from both nondiabetic and diabetic animals were lightly perfusion-fixed with 4% paraformaldehyde at the end of respective time point of reperfusion and carefully dissected out. These brains were cut into thick slices and dehydrated with acetone, acetobenzene (1:1) for 30 min each and finally cleared with benzene (3x30 min). Upon complete dehydration, the brain tissue was kept in molten paraffin wax for 4 h at 65°C in Histocentre2 workstation (Shandon) for proper paraffin infiltration and made into blocks for sectioning. The brain tissue blocks were cut into 5 μm thick sections with a semi-automated microtome (Leica) and the serial sections were taken on SilanePrep slides.

Slides were cleared of paraffin with xylene and sections were rehydrated with decreasing grades of alcohol (100%, 90%, 70%, 50%, 30% and water) for 5 min each. These sections were then stained with haematoxylin for 5 min, washed under running
tap water to remove excess stain and stained with eosin (~1 min). The sections were dehydrated in acetone (5-6 quick dips), acetone:xylene (1:1) and xylene respectively and mounted with DPX. Neuronal death was evaluated under a light microscope at x400 magnification. Bright red stained acidophilic neurons with shrunken, triangular dense purple nuclei were considered dead neurons and alternatively having altered shape, size, stainability of cellular perikarya and vacuolation (Li et al., 1998). The alternative brain sections from same rat were used for immunoflorescence and TUNEL staining.

3.3.7. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay

The terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) assays is based on the specific binding of terminal deoxynuclotidyl transferase (TdT) to 3'-OH ends of fragmented DNA. Following proteolytic treatment of histological sections, TdT incorporates X-dUTP (X = biotin, DIG, or fluorescein) at sites of DNA breaks. Termini modified nucleotides allow the examination of labeled cells under light or fluorescent microscopy, flow cytometry, or immunohistochemistry (Gavrieli et al., 1992).

Paraffin embedded 5.0 μm brain sections were processed for quantification of in situ DNA fragmentation (TUNEL) using BD ApoAlert™ DNA Fragmentation Assay Kit (BD Biosciences) based on the incorporation of fluorescein dUTP at the free 3'-hydroxyl ends of fragmented DNA. Briefly, slides containing paraffin embedded section were cleared of paraffin with xylene and sequentially dehydrated with decreasing order of alcohol and finally in water. The sections were immersed in 0.85% NaCl for 5 min at room temperature (RT) and washed with PBS (2x5 min). The slides were then immersed in 4% paraformaldehyde/PBS at RT for 15 min and washed with PBS. The all sections including negative controls were then treated with TdT incubation buffer. The reaction was terminated by immersing the slides in 2X SSC for 15 min at RT and washed with PBS. For nuclear morphology, the sections were counterstained with Hoechst 33342 (Molecular Probes) for 20 min at RT and washed with PBS for excess staining. The slides were mounted in 90% glycerol containing anti-fade reagent and analyzed for DNA fragmentation with fluorescence microscope (Leica DM5000 B, Leica Microsystem). The three different areas comprising cortex, striatum and hippocampus were scanned for DNA fragmentation and cell with fragmented DNA
were calculated after averaging the number of cells in minimum 5 fields per sections with IM50 software (Leica Microsystem).

### 3.3.8. Preparation of single cell suspension for flow cytometry

Rats were sacrificed after 1 h of ischemia and 3, 6 and 24 h of reperfusion. Brains were quickly removed and brain regions viz., cortex and striatum were dissected out and subjected to collagenase treatment in HEPES-buffered Hanks’ (HBH) solution (pH 7.4) containing 3 mM CaCl₂ at RT. Single cell suspension obtained was centrifuged at 500 g for 5 min and resuspended in HBH for further studies.

#### 3.3.8.1. Measurement of reactive oxygen species (ROS)

Intracellular ROS was estimated by flow cytometry using the oxidation sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate (DCF-DA), which is the oxidation product of DCDHF with sensitivity for H₂O₂/NO-based radicals. DCDHF-DA is an ester that is freely membrane permeable and enters the cells. After entering the cells, DCDHF-DA loses its diacetate group and becomes DCDHF by esterase action and can be oxidized to highly fluorescent DCF. Isolated 10⁶ cells were incubated with 75 mM of DCF at RT for 1 h in dark and subjected to flow cytometric estimation for signal recording using the 530 nm (Yamawaki et al., 2004).

#### 3.3.8.2. Measurement of mitochondrial potential with JC-1

5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide or JC-1 was used to estimate mitochondrial potential (ΔΨm). JC-1 is a lipophilic cationic dye that exhibits potential-dependent accumulation in negatively charged mitochondria (Reers et al., 1991). At low concentrations (low ΔΨm), JC-1 exists mainly in a monomeric form, which emits green fluorescence. JC-1 at high concentration (high ΔΨ) forms aggregates called “J” complexes, which emit red fluorescence at 590 nm. Thus, a reduction in the ratio of red to green fluorescence indicates a fall in ΔΨm. Potassium ionophore, valinomycin (10 mM) was loaded as a reference standard to induce depolarization by dissipation of the mitochondrial potassium gradient. Cells were loaded with 5 M of JC-1 for 20 min at 37°C and then immediately applied to the flow cytometer for signal recording using the 530 nm (FL-1 channel) and 585 nm (FL-2 channel) bandpass filters simultaneously.
3.3.8.3. *Estimation of apoptotic cell population using Annexin V-FITC Kit*

Annexin V-FITC was used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis. It relies on the property of cells to lose membrane asymmetry in the early phases of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing PS to the external environment. Annexin V is a Ca\(^{2+}\)-dependent phospholipid-binding protein that has a high affinity for PS, and is useful for identifying apoptotic cells with exposed PS. Propidium Iodide (PI) is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. The cells that stain positive for Annexin V-FITC and negative for PI are undergoing apoptosis. Cells that stain positive for both Annexin V-FITC and PI are either in the end stage of apoptosis, are undergoing necrosis, or are already dead. Cells that stain negative for both Annexin V-FITC and PI are alive and not undergoing measurable apoptosis.

The rat brains of sham, 1/3 and 1/24 h of I/R stress were quickly removed under chilled condition and brain parts viz. cortex and striatum dissected out and a single cell suspension (SCS) was prepared using collagenase enzyme treatment containing 3 mM CaCl\(_2\). The single cells obtained were washed twice with cold HBH and then resuspended in 1X binding buffer at a concentration of 1 x 10\(^6\) cells/ml. Transferred 500 \(\mu\)l of the solution in tube and added 6.5 \(\mu\)l of Annexin V-FITC and 6.5 \(\mu\)l of PI. Then cells were gently vortexed and incubated for 45 min at RT in the dark. After incubation, cells were immediately applied to the flow cytometer for signal recording at log FL1 (X-axis; FITC) excitation (488 nm) and emission (515 nm).

3.3.9. *Western blot analysis*

The rat brains were quickly removed after I/R injury in ice-cold condition. Brain parts viz. cortex and striatum were dissected out and homogenized with Teflon homogenizer in 5 volume of buffer (250 mM sucrose, 200 mM HEPES-KOH, 10 mM KCl, 1.5 mM MgCl\(_2\), 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF and protease inhibitors). The nuclear, mitochondrial and cytosolic fractions were extracted by a series of centrifugations (Zhang *et al.*, 2002). Briefly, the homogenate was centrifuged at 800 g for 10 min to obtain a crude nuclear pellet (P1) and supernatant (S1). The S1 containing mitochondria and cytosol were separated from the
nuclear pellet (P1). S1 was centrifuged at 8,000 g for 20 min to obtain the mitochondrial pellet (P2) and supernatant (S2). P2 containing mitochondrial fraction was suspended in buffer and S2 was obtained as cytosolic fraction. Nuclear pellets were resuspended in buffer, incubated on ice for 15 min, and centrifuged at 16,000 g at 4°C for 20 min. Protein concentrations were determined using Lowry’s method (Lowry et al., 1951). For western blot analysis proteins were separated using 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, an equal amount of protein (50 μg) was applied to each lane. Following electrophoresis, proteins were transferred onto nitrocellulose membrane, Hybond ECL (GE Healthcare). The membrane was incubated with primary antibodies against HSP70, HSP60, cytochrome c, Bax and Bcl-2, cleaved caspase-3, AIF, PARP, (1:800) for 3 h at RT. The membrane was washed and incubated with horseradish peroxidase (HRP) conjugated secondary antibodies for 1 h at RT. The blots were then developed using the enhanced chemiluminescence (ECL) method (GE Healthcare).

3.3.10. Immunocytochemistry

Paraffin embedded 5.0 m brain sections of 0.5/24 and 1/24 h of I/R were incubated with 5% serum to block nonspecific binding sites. The sections were incubated overnight with primary antibodies for cleaved caspase-3 (1:100, polyclonal antibody; Cell Signaling Technology), AIF (1:100, goat polyclonal antibody; Santa Cruz, Cell Signaling Technology), PARP (1:100, rabbit polyclonal antibody; Santa Cruz) and NeuN (1:500, Chemicon). The sections were washed and then incubated with Alexa Fluor-conjugated secondary antibody (Molecular Probes). Sections were counterstained with Hoechst 33342, mounted and analyzed under fluorescence microscope (Leica DM5000 B, Leica Microsystems)

3.3.11. RNA isolation and quality assessment

Brain parts viz. cortex and striatum were snap-frozen in liquid nitrogen. Approximately 100 mg of frozen brain tissue was crushed manually in liquid nitrogen and immediately homogenized in 1 ml, TRI reagent (Sigma). The homogenized tissue was incubated for 10 min at RT followed by the addition of 200 μl chloroform (Sigma) per ml of TRI reagent used and mixed vigorously for 10-15 sec. The sample was allowed to stand undisturbed for 15 min at room temperature and then centrifuged at 12,500 g for 20 min at 4°C. The aqueous phase was carefully transferred to a fresh tube and RNA was precipitated with 500 μl isopropyl alcohol (Merck). Further
centrifugation at 12,000 g for 15 min at 4 C and washing (twice) with 1 ml, 75% ethanol was done to recover pure RNA pallet. Finally, the RNA pallet was air dried and resuspended in molecular grade water (Sigma). The quality of RNA was assessed electrophoretically on denaturing formaldehyde agarose gel and spectrophotometrically with GeneQuant Pro. Briefly, 3-4 µl of RNA sample was mixed with 10 µl formamide (Sigma), 2 µl 5X formaldehyde gel running buffer and 3.5 µl formaldehyde. It was heated at 65°C for 15 min and immediately placed on ice. After 5 min, 2 µl formaldehyde gel loading dye was added followed by 0.5 µl EtBr (10 mg/ml stock). The contents were mixed properly and loaded on to gel for electrophoretic separation. RNA samples with approximately 2:1 ratio of 28S: 18S rRNA and 260/280 values >1.7 were used for gene expression studies.

3.3.11.1. Primer designing

Primers used for gene expression studies were designed using online free primer designing software and procured from Sigma. The nucleotide sequences of selected genes were downloaded from reference sequence (RefSeq) and served as input data files. Default settings of primer length ranged between 17 and 24 nucleotide having Tm values ranging between 55-60°C and product length ranging between 300-600 bp (Table 6). Primer sequences were checked for their self complementary properties and avoided if they did not fulfill default parameters.

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<td>1</td>
<td>Caspase-3</td>
<td>FP: 5' TGG ACA TGA CGA CAG GGT GCT A 3'</td>
<td>64°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RP: 5' TGC CAG GGC CCT TCA TTT CTA 3'</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>AIF</td>
<td>FP: 5' CCG TTC GGA GAG TGA GAC AGA GT 3'</td>
<td>63.5°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RP: 5' CTT TTA TCC ACC CAT TCC CTG TCA 3'</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PARP</td>
<td>FP: 5' ACG CTT GGC CTG GTG GAC A 3'</td>
<td>65.5°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RP: 5' TGA GGG TGT AGA AGC GAT TGG AGA 3'</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>HSP70</td>
<td>FP: 5' TAT TCA TCC ACT CCA TCG CCT CAT 3'</td>
<td>64°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RP: 5' ACG CTT GGC CTG GTG GAC A 3'</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>β-actin</td>
<td>FP: 5' CCC TAA GGC CAA CCG TGA AAA GAT 3'</td>
<td>55.5°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RP: 5' CGA CCA GAA GCA TAC AGG GAC AAC 3'</td>
<td></td>
</tr>
</tbody>
</table>

3.3.11.2. PCR Protocol

The PCR reaction was set as per the manufacturer’s guidelines. Briefly, thawed the template RNA, primer solutions, dNTP Mix, 5X QIAGEN OneStep RT-PCR Buffer, and RNase-free water, and placed on ice. The master mix for the reaction was prepared according to the table 7.
Thawed the template RNA, primer solutions, dNTP Mix, 5X QIAGEN OneStep RT-PCR Buffer, and RNase-free water, and place them on ice. It is important to mix the solutions completely before use to avoid localized differences in salt concentration. Mixed the master mix thoroughly and dispensed the appropriate volumes into PCR tubes. Mixed gently by pipetting the master mix up and down a few times and then added 2 μg/reaction RNA template to the individual PCR tubes. The RT-PCR reaction was set in the thermal cycler according to the program outlined in table 8.

**Table 7**: Reaction components for one-step RT-PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Master mix</td>
<td></td>
</tr>
<tr>
<td>RNase-free water</td>
<td>13 μl</td>
</tr>
<tr>
<td>5x QIAGEN OneStep RT-PCR Buffer</td>
<td>5.0 μl</td>
</tr>
<tr>
<td>dNTP Mix (containing 10 mM of each dNTP)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>QIAGEN OneStep RT-PCR Enzyme Mix</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Template RNA</td>
<td>1.0 μl (2.0 μg/μl)</td>
</tr>
<tr>
<td>Total volume</td>
<td>25.0 μl</td>
</tr>
</tbody>
</table>

**Table 8**: Thermal cycler conditions

<table>
<thead>
<tr>
<th>Reverse transcription</th>
<th>30 min</th>
<th>50°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial PCR activation step</td>
<td>15 min</td>
<td>95°C</td>
</tr>
</tbody>
</table>

**3-step cycling**

| Denaturation                                   | 1 min  | 94°C |
| Annealing                                      | 0.5 min| 50–68°C |
| Extension                                      | 1 min  | 72°C |

| Number of cycles                               | 25–40  |     |
| Final extension                                | 10 min | 72°C |

The relative expression changes following amplification were determined using gel documentation system.

**3.4. Statistical Analysis**

Significant differences between diabetic and normoglycemic I/R groups were made using ANOVA followed by Newman-Keuls multiple comparison test. The comparison between identical time points of I/R was made using Student’s t-test and represented as mean±SEM. */p<0.05 and ***/p<0.001 were considered statistically significant and highly significant respectively.