CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

Dulbecco’s Modified Eagle Medium (DMEM), Antibiotics (penicillin/streptomycin), Trypsin Dimethylsulfoxide (DMSO), Propidium iodide (PI), NBT (Nitro blue Tetra Zolium), DCF-DA, Rhodamine-123 were procured from Sigma-Aldrich Chemicals Pvt Limited. Fluo-4-AM, BCIP (5-Bromo-4-chloro-3-indolyl phosphate), MTT (3-(4, 5-Dimethylthiazol-2-yl) -2, 5-Diphenyltetrazolium Bromide), Phenyl methyl sulfonyl fluoride (PMSF), Protease /Phosphatase inhibitor cocktail, RIPA buffer were obtained from Cell signaling technologies. Trypanblue, diethylether. ethanol, SDS (Sodium dodecyl sulfate), APS (Ammoniumpersulphate), Mgcl₂, TEMED tetramethylethylenediamine), and TRIS (tris(hydroxymethyl)aminomethane)) were obtained from HIMEDIA. The antibodies of Anti-α Sarcomeric Actin, pAkt, p38 MAPK and FITC tagged secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies of Akt, pAkt, p38, pp38 MAPK, BAD, pBAD, XIAP and cytochrome c, Wortmannin, SB203580 was obtained from cell signaling technologies. All reagents were prepared using deionized (Millipore) or glass-distilled water (d. H₂O).

2.2 CELL CULTURE AND MAINTENANCE

H9C2 cells from NCCS Pune were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) containing 10% FBS (GIBCO) with penicillin and
streptomycin antibiotics (Pen-Strep) under a fully humidified atmosphere of 5% CO\textsubscript{2} at 37\textdegree C.

2.3 PASSAGING AND CRYOPRESERVATION

H9C2 cell lines reached confluence in 4 to 5 days in T-25 flask and were passaged in order to maintain viability. Passaging was done as per protocol:

a) The culture medium was removed by decanting into an autoclaved tube inside the laminar flow hood, and cells were rinsed with the PBS

b) 1ml of Trypsin solution was added to the flask containing cells and incubated at 37\textdegree C for a few minutes. As soon as cells start detaching from the surface of the flask, the trypsin was removed and the flask was rinsed with DMEM containing 10% FBS to remove all the cells.

c) The suspensions of cells were collected in a sterile 2 ml centrifuge tube and the cells were pelleted at 1500 RPM for 5 min. The cell pellet was resuspended in fresh DMEM containing 10% FBS and an aliquot of the cells was seeded back into the flask and the remaining cell pellet was resuspended in cryopreservative medium (10% DMSO +90% FBS) in a cryovial and frozen at -80\textdegree C or in liquid nitrogen.

2.4 COUNTING CELLS AND VIABILITY EVALUATION

After being harvested, an aliquot of pelleted cells was resuspended in 10 ml of DMEM medium and 50 \textmu l of the cell suspension was taken and added to 50 \textmu l trypan blue (Sigma Aldrich) (1:1 dilution) and mixed properly.
10 µl was transferred to haemocytometer slides, over which a cover slip was placed. The cells that were round or slightly oval and bright were counted. For measuring the yield and viability, both total number of cells and the number of stained (dark) cells were counted. 5,00,000 cells were plated per 60 mm dish. Yield and viability were calculated as follows:

- **Dead cells per ml** = mean number of stained cells per square
- **Total Cells** = cells per ml X volume
- **Yield** = \(2 \times \left(\frac{\text{total number of cells in four grids}}{4}\right) \times \left(10^4 \times \text{cell suspension volume}\right)\)
- **Viability (%)** = \(\frac{\text{Total cells counted} - \text{stained cell}}{\text{Total cells counted}} \times 100\)

### 2.5 ISOLATION OF NEONATAL RAT CARDIOMYOCYTES

Our research work on Neonatal rat pups was approved by the Institutional animal ethics committee (Ref. No. CBT 2011-1). All our research work was done in Centre for Biotechnology, Anna University, Taramani Campus Chennai.

a) Anesthetization and sterilization: Neonatal (1-3 days old) Sprague-Dawley Rat pups were anesthetized with diethyl ether and decontaminated with 75% ethanol, and transferred to a Laminar flow hood.

b) Dissection was performed on ice, beating hearts were surgically removed by making a midline cut through the sternum, after perfusion with Ca\(^{2+}\) and Mg\(^{2+}\) free PBS buffer,
and placed in the fresh cold PBS buffer, free from divalent cations.

c) The ventricles were excised and transferred to fresh ice-cold PBS buffer and were minced with fine scissors into 1 – 3 mm³ pieces after washing the blood RBC’s were removed by centrifugation for two times.

d) Preconditioning (20 min): The minced tissues were transferred to a 40 ml conical flask containing trypsin solution (0.08%, 0.5 ml per rat) and a magnetic bead. Then the flask was allowed to settle on ice for 20 min, followed by shaking for 3 min interval.

**Trypsin (0.08%)**

Trypsin stock solution of 0.25% (Gibco) was diluted to 0.08% using sterile 1XPBS and allowed to mix at 4°C overnight, then the solution is filtered using a 0.22µm filter and stored at 4°C.

e) Trypsinization (10 min): Following preconditioning, the tissue was allowed to digest in the flask for 10 min at 37°C, which was subjected to constant stirring (150–200 RPM). The trypsin used for preconditioning was as such used for the first digestion; fresh trypsin was added for subsequent digestion.

f) Centrifugation (5 min): After trypsinization, cells were allowed to disperse from the digested tissue by smoothly pipeting and the cell suspension was settled on ice for 30 secs. The supernatant was transferred to a 15 ml centrifuge tube. Trypsin activity was stopped by adding a mixture of trypsin
inhibitor and cold culture medium supplemented with 10% FBS (1:1, v/v). The cells were pelleted by spinning at 1000 RPM for 5 min, and were resuspended in 2 ml of warm culture medium (DMEM).

g) Repeating trypsinization: The remaining tissue in the conical flask from step 5 was continuously digested by adding 5–10 ml fresh pre-warmed trypsin solution containing DNase I (0.05 mg /ml). This protocol protocol was repeated twice or thrice depending on the left out of undigested tissue.

h) Cell harvest (10 min): Cell suspension from all dissociated steps was pooled in one centrifuge tube and settled for 5 min. The suspension was then transferred to a fresh centrifuge tube excluding the precipitates to the bottom. Cells were then harvested by centrifugation at 1000 RPM for 5 min. Eventually the cells were plated in a culture flask and incubated at 37°C in a humidified atmosphere (5% CO2, 95% air).

i) Purification (2 hrs): Cells harvested from step (g) were incubated for 2 hrs to allow the non-cardiomyocytes to attach on the surface of the culture plate. The majority of cardiomyocytes remained in culture medium itself. The cardiomyocytes in the cell suspension were collected by centrifugation and plated at a density of 2 X 10^5 cells/ml. BrdU (0.1 mM/l, Sigma Aldrich) was then added to the culture medium for 48 hrs to prevent proliferation of non cardiomyocytes that might be present in the culture.
j) Cultivation: The cells were cultured at 5% CO₂, 95% humidity, and was kept undisturbed during the initial 24 hrs. Then the medium was substituted with fresh media without BrdU for every 48 hrs (Fu et al 2005). Later on three days, beatings NCM were video graph using Nikon Camera at 20X and 40X.0X.

### 2.6 GELATINISATION OF CULTURE PLATES

Cardiomyocytes require adherence support and were cultured in gelatinised culture plates. The culture vessel was filled with 0.1% gelatin.

#### 0.1% Gelatin preparation

0.1% Gelatin was prepared by mixing 0.1g of gelatin from bovine skin (Sigma Aldrich) in 100 ml triple d.H₂O, the gelatin was sterilized by autoclaving at 121°C for 15 mins, just within 30 mins after adding to d.H₂O. The sterilized gelatin was allowed to stick out at room temperature for at least 24 hours before coating.

Then it is left to incubate at 37°C for at least 10 minutes. Then the plates were washed with PBS free from divalent cations. And then the plates were dried by keeping the lid open in the laminar flow hood. The culture vessels were always maintained sterile as the same will be used for seeding the cells.

### 2.7 CHARACTERIZATION OF NEONATAL RAT CARDIomyocytes AND H9C2 CELLS

Before characterization of NCM and H9C2 cells were fixed by using 4% paraformaldehyde. The paraformaldehyde (Sigma Aldrich) is prepared by using the following protocol. 4g of paraformaldehyde powder
were added to 80 ml of PBS and allowed to stir on hot plate for about 60°C by slowly adding NaOH to adjust the pH of about 7.4 and stirred until the powder gets dissolved completely. Once it is dissolved, then the volume was made up to 100 ml and was allowed to cool at room temperature. It was sterile filtered and stored at 2 - 8°C up to one month.

2.7.1 Hematoxylin & Eosin Staining Method

NCM was stained with Hematoxylin and Eosin (H&E) (Sigma Aldich) to visualize the morphology of NCM clearly. The media were removed from 60 mm culture dish and NCM were washed using 1X PBS. Appropriate volume of 4% paraformaldehyde was added to the sample and spread properly and kept for 30 mins. After 30 mins paraformaldehyde was removed and discarded. Then the hematoxylin was added and retained for 8 mins. The dye was taken out and the sample was washed with 1X PBS and then again washed with 95% ethanol. Then Eosin was added to the hematoxylin stained NCM and kept for 1 min. Then H&E stained NCM were washed with 1X PBS and with 95% ethanol. It was allowed to dry for 5 – 10 mins and pictures were captured under phase contrast Nikon microscope.

2.7.2 Immuno Staining Method

Subsequently on a gentle wash in 1XPBS, NCM were incubated for 10 mins at 37°C with 4% (v/v) paraformaldehyde. Then paraformaldehyde is removed carefully and fixed NCM were washed 3 times with 1XPBS (5 minutes per wash). Then it is permeabilized with 0.1% Triton X-100 for 5 - 10 mins at room temperature. The cover slip was gently washed in 1XPBS. NCM were incubated at 37°C for about 3 - 4 hrs with Monoclonal Anti-α-Sarcomeric Actinin (A7811) (Sigma Aldrich) at a dilution of 1:200 in 1X PBS at 37°C. NCM Samples were washed and then incubated with biotinylated
anti-mouse secondary antibodies at a dilution of 1:500 dilutions (Santa Cruz Biotechnology) for 45 mins at 37°C. After washing with 1X PBS, samples were again incubated for 15 mins at 37°C with avidin-conjugated 1:500 FITC. NCM samples were washed with 1X PBS, and place the coverslip over the glass slide and images were captured using confocal microscopy (Carl Zessis, Zen 2010) with excitation 490 nm & emission 525 nm.

2.8 TREATMENT OF H9C2 CELLS AND NEONATAL RAT CARDIOMYOCYTES

H9C2 cells and NCM were cultured to 70% - 80% confluency and were synchronized in serum-free DMEM + 0.1% Bovine Serum Albumin overnight. For our experiments, Normoxic control group or groups induced to H/R after treatment with or without two applications of EPO (10 U/ml, 15 U/ml and 20 U/ml). The first application of EPO was accomplished 24 hrs before H/R. Second application of EPO immediately before induction of hypoxia and hypoxic conditions were induced by incubating NCM in an airtight chamber (94% N₂ -5% CO₂ and remaining 1% O₂) for 8 hrs without 10% FBS and DMEM medium without glucose (Sigma Aldrich). Reperfusion was for 16 hrs by replacing glucose containing DMEM + 10% FBS for hypoxic medium (Dhanasekaran et al 2008). For some of the experiments, cells were treated with 20 U/ml of EPO alone in normoxic condition. In some experiments 10 µM SB203580 (SB), p38 MAPK inhibitor and 1 µM Wortmannin (WT), PI3K/AKT inhibitor were added 30 mins prior to each application of EPO. Control cells were maintained in DMEM+10% FBS throughout the duration of the experiments.

2.9 MTT ASSAY

H9C2 cells and NCM were cultured in 0.1% gelatin coated 96 well plates for about 70 - 80% confluency. NCM were pretreated with different
concentration (10 U/ml, 15 U/ml and 20 U/ml of EPO) and incubated for 24 hrs and followed the treatment as described earlier. The MTT [3-(4, 5-dimethylthiazole-2-yl) -2, 5- diphenyltetrazolium bromide] were added to each well and incubated at 37°C in a CO₂ incubator for 4 hrs (Lingadurai et al 2011). Soluble yellow color MTT is reduced by mitochondrial succinyl dehydrogenase into insoluble purple formazan. The insoluble formazan product was dissolved in 50 µl DMSO and incubated for 10 mins, mixed well and read absorbance at 540 nm (Dhanasekaran et al 2008). Cell viability was determined using the formula.

\[
\% \text{ Cell Viability} = \frac{\text{Sample OD} - \text{Blank OD}}{\text{Control OD} - \text{Blank OD}}
\]

2.10 ACRIDINE ORANGE (AO) AND ETHIDIUM BROMIDE (ETBR) DOUBLE STAINING METHOD

Followed by the pretreatment H9C2 cells and NCM was washed with 1XPBS and myocytes were collected by centrifugation. After that the pellet were resuspended with 100 µl of 1XPBS, 10 µl of the cell suspension were put on a glass slide and then mixed with 1 µl of Ao (1 mg/ml, Sigma Aldrich) and 1 µl of EtBr (10 µg/ml, Sigma Aldrich). Coverslip is placed over the glass slide and immediately viewed under a confocal laser scanning microscope at a wavelength of excitation 502 nm and emission 526 nm for Ao and for EtBr excitation 510 nm and emission 595 nm (Lingadurai et al 2011). The images were quantified by using image J software.

2.11 DETECTION OF MITOCHONDRIAL MEMBRANE POTENTIAL AND ROS

After pretreatment H9C2 cells and NCM cultured on 0.1% gelatine coated cover slips were washed with 1XPBS and incubated with Rhodamine-
123 (5 µg/ml, Invitrogen) and DCFH-DA (100 µM, Invitrogen) reagent for 30 mins in dark at 37°C. Samples were washed thrice with 1XPBS. The fluorescence was measured using a confocal laser scanning microscope using 514 nm and 570 nm for Rhodamine-123 and DCF-DA respectively (Chung et al 2008; Scaduto Jr & Grotyohann 1999). Before that confocal laser scanning microscope adjusts to the laser power (2%), detector gain, and resolution (512x512) then it was focused on 40X oil immersion (Joshi & Bakowska 2011). The same parameter is used for all sets of experiments. The images were quantified by using image J software.

2.12 **CALCIUM LIVE CELL IMAGING**

H9C2 cells were grown to 80% confluency in six-well plates and subjected to 8 hrs hypoxia and 15 mins reperfusion or normoxia with or without pretreatment with EPO as described earlier. Cells with or without pretreatment of EPO were incubated with 4 µM of cell permeant Fluo-4 AM in the dark before induction of hypoxia (Fluo-4 acetoxymethyl ester; Molecular Probes). After 8 hrs of hypoxia cells were visualized under confocal microscope and live cell images were taken in the time series mode of about 0.2 secs for 15 mins (Lemasters et al 1998). Hypoxia medium was replaced with fresh DMEM + 10% FBS to reoxygenate those cells and live cell images were taken about 15 mins of reperfusion. Control cells were incubated with 4 µM of permeant Fluo-4-AM for 15 mins and live cell images were taken (excitation at 485 nm, emission at 520 nm). The images were quantified by using image J software.

2.13 **CELL LYSATE PREPARATION**

H9C2 cells and NCM were cultured on 0.1% gelatin coated cover slips 35 mm dishes and were treated under 8 hrs hypoxia and 30 min reperfusion with or without EPO pretreatment. Cells were kept on ice and
washed thrice with cold 1XPBS. Cells were removed by scraping the dishes
with a Teflon coated cell scraper and centrifuged for 5 minutes at 1200 x g.
Cell pellets were then lysed by RIPA buffer

50 mM Tris (pH 8.0)

150 mM NaCl

0.5% SDS

1% Nonidet P40

0.5% sodium deoxycholate

1 mM EDTA

1X protease and phosphatase inhibitor cocktail (Cell Signaling
technologies) were added prior to cell lysis.

400 μl of 1x RIPA buffer/10 cm dish were added. Cells were
incubated on ice for 5 minutes and the cells were scraped and centrifuged for
10 mins at 14,000 x g at 4°C in refrigerated centrifuge. Discarded the pellet
and supernatant was quantified by using the Bradford assay for Western blot.
The lysate were used to estimate protein content with the Bradford Assay
Reagent.

2.13.1 Estimation of Protein using Bradford’s Method

Protein estimations were performed according to the method of

a) 20 mg of Coomassie brilliant blue G-250 (Sigma Aldrich) was
dissolved in 10 ml of methanol followed by the addition of 20
ml of orthophosphoric acid. The final volume was made up to 200 ml using d. H₂O and filtered. This solution appears as pale-straw and stored in the dark.

b) The standard BSA was added to the eppendorf tubes at concentrations of 10 µg, 20 µg, 40 µg, 60 µg, 80 µg and 100 µg in 0.15 M NaCl. 5 µl of the test samples were added to each well and the volume was made up to 100 µl with 0.15 M NaCl. 1 ml of Bradford’s reagent was added to the samples and the standard and incubated for 5 mins in the dark. NaCl served as blank.

c) The optical density was read at 595 nm. The readings from protein standards were used to plot a standard graph and to estimate the amount of protein in the test samples.

2.13.2 SDS Polyacrylamide Gel Electrophoresis

Equal measures of protein (20 - 50 µg) from each sample were electrophoresed on a 12% SDS-polyacrylamide gel. Buffers, reagents and gel composition for SDS-PAGE were as follows:

**SDS – PAGE gel Composition**

Separating gel buffer – Tris 1.5 M (pH 8.8)

Stacking gel buffer – Tris 1.0 M (pH 6.8)

**12% Separating Gel (10ml):**

D. H₂O – 3.3 ml

30% Acrylamide solution - 4 ml
1.5 M Tris buffer (pH 8.8) – 2.5 ml

10% SDS solution – 100 µl

14% APS – 100 µl

TEMED – 5 µl

**5% Stacking Gel: 5ml**

d.H₂O – 3.3 ml

30% Acrylamide solution -0.83 ml

1. M Tris buffer (pH 6.8) – 0.63 ml

10% SDS solution – 50 µl

14% APS – 50 µl

TEMED – 5 µl

Samples were mixed with SDS gel loading buffer and the composition is as follows

**SDS gel-loading buffer (5X):**

Glycerol – 2 ml

1M Tris (pH 6.8) – 2.25 ml

β Mercapto ethanol – 1 ml

D. H₂O – 9 ml
SDS – 1 g

Pinch of Bromophenol Blue was added

After addition, samples were heated to 95°C for 5 minutes and placed on ice. 20 - 40 µl corresponding to 20-50 µg of protein was loaded onto the gel. Electrophoresis was carried out at 50 mA per gel for approximately 45 mins at room temperature in 1XRGB buffer. The composition of 10XRGB buffer is as follows. From 10 X RGB buffers 1X buffer was prepared.

10X RGB Buffer (Running Gel Buffer)

Tris – 30.8 g

Glycine – 144 g

Make up to 1 liter adjusted pH to 8.3 and 1% SDS was added.

2.13.3 Western Blotting and Detection of Target Proteins

Polyacrylamide gels were transferred from the SDS PAGE apparatus and placed on top of a piece of nitrocellulose membrane which had been cut to size. Gel and membrane were sandwiched between Whatman filter paper and placed on the semi dry transfer apparatus. 5 ml of Transfer buffer (Tris - 0.303 g, Glycine - 1.441g, Methanol – 20 ml, make up to 100 ml) were added to the surface of Gel and membrane sandwich and allowed for electroblotting. The electroblotting was carried out for 1 hr at 10 mA. Once the electroblotting was done nitrocellulose membrane was separated from the gel and rinsed with 1XTBST for 1 min. The membrane was blocked by incubating with blocking buffer for 1 hr at room temperature and the composition of blocking buffer was as follows
**Blocking buffer (5% non fat dry milk solution):**

Milk powder – 1.25 g

1X TBS – 25 ml

The membrane was washed with 1X TBS-T for 3 minutes at room temperature the membrane was probed with specific primary antibody (1:1000 dilutions) with the primary antibody dilution buffer and the composition is as follows

**Primary antibody dilution buffer**

5% BSA in 1X TBS

0.1% Tween -20 and

20% Sodium Azide

The membrane was incubated for overnight at 4°C with constant agitation. The membrane was washed thrice with 1XTBST for 5 mins each and incubated with polyclonal Anti-Rabbit IgG conjugated to Alkaline Phosphatase in 1:10000 in TBS-T for 1 hr at room temperature with constant agitation. The membrane was washed thrice with 1X TBS-T at room temperature for 3 times. The protein band of interest was visualized by the calorimetric detection by using working Alkaline Phosphatase substrate (Sigma Aldrich) (ALP) solution. The composition of substrate and substrate buffer as follows.

**ALP substrate buffer**

12.11g Tris (0.1M),
0.5844g Nacl (100mM)

1.015mg MgCl₂ (5mM)

Dissolved in 100ml d. H₂O

**Substrate stock solution**

NBT – 10 mg/ml in d.H₂O

BCIP – 50 mg/ml in d. H₂O

**Working solution**

33 µl of 50 mg/ml BCIP,

330 µl of 10 mg/ml NBT in 10 ml substrate buffer

Once the band is developed then the reaction was stopped by using d.H₂O. The membrane was washed with d. H₂O twice and allowed to dry completely. Then the image is captured in gel documentation instrument (Bio-Rad) using calorimetric detection. The bands were quantified using Image J Software.

### 2.14 DETECTION OF CYTOCHROME C RELEASE INTO CYTOSOL

The release of mitochondrial cytochrome c into the cytosol was measured according to the protocol described previously (Jin et al 2005). NCM was washed briefly with 1XPBS and homogenized in 0.25 M sucrose, 20 mm Tris HCl, and 5 mm EDTA, pH 7. The homogenates were centrifuged at 800 g, discarded the pellet and the supernatant was again centrifuged at 8,000 g for 10 min. The pellet contains the mitochondrial fraction and the
The supernatant contains a soluble cytosol-enriched fraction. The supernatant was used to measure cytochrome c released into the cytosol and the pellet was resuspended for Western blot analysis using anti rabbit cytochrome c as described previously in Western blot analysis.

2.15 DETECTION OF CASPASE-3 ENZYMATIC ACTIVITY

H9C2 cells and NCM were cultured in 60 mm cell culture dishes for about 70 - 80% confluency. After induction of H/R with or without pretreatment with EPO, samples were washed with 1XPBS and lysed with cell lysis buffer. The enzymatic activity of the caspase-3 is measured by following the protocol given in the caspase-3 Colorimetric assay kit (R&D systems). After that, the cell lysate was incubated for 10 min on ice and centrifuged at 10,000 x g for 1 min. The supernatant was transferred to a fresh tube and held on ice. Then 50 µl of the supernatant was added to 50 µl of 2X Reaction buffer containing DTT in 96 well plates. At the end, reaction mixtures were incubated with 5 µl of DEVD-pNA (caspase-3 colorimetric substrate) for 1-2 hrs. The caspase activities were quantified by using a spectrofluorometer at a wavelength of 405 nm.

2.16 STATISTICS

Statistical data were analyzed using ANOVA followed by TUKEYs tests in Graph Pad Prism. For all the experiments, data are presented as means ± SEM from five experiments for MTT and three experiments for other assays. Values for P < 0.05 were considered as statistically significant.