4.1. Ethical approval

The experimental protocols were performed in agreement with the principles of the Guide for the Care and Use of Laboratory Animals, published by the National Institute of Health (NIH publication no. 86–23, revised 1996), and all animal experiments were approved by the Institutional Animal Ethical Committee of Institute of Medical Sciences (IMS), Banaras Hindu University (BHU), Varanasi, India (**No. Dean/2015/CAEC/1423**). Some part of the study (experiments of objective-III) was approved by the Institutional Animal Care and Use Committee of Hong Kong University.

4.2. Drugs, chemicals and antibodies

The list of drugs, chemicals and antibodies used in this study were represented in **Table 4.1**. All other remaining drugs/chemicals/reagents used were of analytical grade.

Table 4.1: Drugs, chemicals and antibodies

S.No.	Drugs/Chemicals/Antibodies	Procured from
1	Pterostilbene	Sami Labs, Bangalore, India
2	Corn Starch	Research Diet, USA
3	High Fructose Diet	Research Diet, USA
4	Rat insulin ELISA kit	Thermo Fisher Scientific, USA
5	TNF-α and IL-6, IL-1β ELISA Kits	Thermo Fisher Scientific, USA
6	Lactate dehydrogenase assay kit	Cayman Chemicals, USA
7	Creatine kinase (CK)-MB kit	Cayman Chemicals, USA
8	Aspartate aminotransferase (AST) kit	Cayman Chemicals, USA
9	Free 8-isoprostane immunoassay kits	Cayman Chemical, Ann Arbor, USA
10	Terminal deoxynucleotidyl nick-end labelling (TUNEL) assay kit	Roche Applied Science, Indianapolis, IN,USA
11	Triglyceride kit, HDL-C kit	Siemens, India
12	Serum Cholesterol Kit	Siemens, India
13	Uric acid kit	Abcam, India
14	Bradford protein assay kit	Bio-Rad, CA, USA
15	Bovine Serum Albumin (BSA)	Sigma-Aldrich, St. Louis, MO, USA

16	Reduced Glutathione (GSH)	SD Fine Chem Ltd, Mumbai, India
17	Thiobarbituric acid (TBA)	SD Fine Chem Ltd, Mumbai, India
18	Pyrogallol Tris hydrochloride	SD Fine Chem Ltd, Mumbai, India
19	Disodium EDTA	SD Fine Chem Ltd, Mumbai, India
20	Hydrogen Peroxide	SD Fine Chem Ltd, Mumbai, India
21	Griess Reagent	SD Fine Chem Ltd, Mumbai, India
22	AMPK, p-AMPK (Thr 172)	Cell Signaling Technology, USA
23	Nrf2	Cell Signaling Technology, USA
24	HO-1	Cell Signaling Technology, USA
25	PGC-1α	Cell Signaling Technology, USA
26	NF-κB	Cell Signaling Technology, USA
27	GAPDH	Cell Signaling Technology, USA
28	Complex III	Invitrogen, USA
29	Complex V	Invitrogen, USA
30	Bax	Cell Signaling Technology, USA
31	Bcl2	Cell Signaling Technology, USA
32	Caspase 3, cleaved caspase 3	Cell Signaling Technology, USA
33	β-actin	Cell Signaling Technology, USA
34	TRIzol Reagent	Ambion, USA
35	5X Prime Script RT master mix	Takara Clontech, USA
36	SYBR Premix Ex Taq II	Takara Bio, USA
37	DEPC-treated water	Ambion, USA
38	RIPA buffer	Thermo Fisher Scientific, UK
39	Phosphate buffered saline (PBS)	Fisher Scientific, UK
40	β-cyclodextrin	Sigma-Aldrich, MO, USA
41	Compound C	Sigma-Aldrich, MO, USA
42	Streptozotocin	Sigma-Aldrich, MO, USA
43	1% 2, 3, 5-triphenyltetrazolium chloride	Sigma-Aldrich, MO, USA
44	Hematoxylin & Eosin	Sigma-Aldrich, MO, USA
45	Dihydroethidium (DHE)	Sigma-Aldrich, MO, USA
46	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT)	Sigma-Aldrich, MO, USA

4.3. Equipment and Software

The list of equipments and software used in this study were enlisted in **Table 4.2**.

Table 4.2: List of equipments and software

S.No	Equipment/Software	Purchased from
1	Noninvasive tail-cuff apparatus	Narco Bio-System, Houston, TX
2	Glucose meter	One Touch Select, UK
3	Automated blood analyzer	Bayer Corp. USA
4	Microplate reader	Bio-Rad Laboratories, CA, USA
5	Fluorescence microscope	Olympus IX51
6	Electrophoretic transfer system	Bio-Rad, CA, USA
7	Polytron homogenizer	Kinematica, Switzerland
8	Microplate reader	BioTek Instruments, USA
9	Nanodrop instrument	Thermo Scientific, USA
10	RT-PCR StepOnePlus Instrument	Applied Biosystem, USA
11	Centrifuge	Thermo Scientific, USA
12	Nitrocellulose membranes	Millipore, USA
13	Clarity Western ECL blotting substrates	Bio-Rad, USA
14	Micropipettes	Eppendorf, UK
15	Microtome	Leica, Bensheim, Germany
16	Rat ventilator	Kent Scientific, USA
17	Perfusion apparatus	AD Instruments, China
18	GraphPad Prism 5.0	GraphPad Software, Inc., USA
19	ImageJ software	downloaded from NIH website

4.4. Animal Husbandry

Three weeks old, adult male Sprague-Dawley rats (220 ± 60 g) were procured from the Central Laboratory Animal House of IMS, BHU, Varanasi, India. Animals were housed in polypropylene cages (40 × 33 ×17 cm), two per cage, under standard environmental conditions (humidity, 50 ± 10%; temperature, 22 ± 3°C; 12-h light/dark cycle, 7 a.m.-7 p.m.), with free access to water and diet. All efforts were taken to reduce animal pain and to utilize the least number of animals essential to generate reliable data.

4.5. Experimental Groups

4.5.1. Experimental groups for objective-I

After two weeks of adaptation period, animals with a body weight of 330 ± 30 gm were randomly segregated into five groups (n=10).

Group-I	Control (C)	Rats received 65% corn starch (Research Diet, USA) and vehicle (10% β-cyclodextrin) for eight weeks.
Group-II	Diabetic (D)	Rats received 65% fructose diet (Research Diet, USA) for an interval of eight weeks for the induction of an established model of T2DM (Padiya et al., 2011). Vehicle (10% β-cyclodextrin) was given orally for eight weeks.
Group-III	D+PT20	Rats received 65% fructose diet along with pterostilbene (PT) 20 mg/kg/day for eight weeks. PT was dissolved in 10% β-cyclodextrin and given orally to rats.
Group-IV	D+PT40	Rats received 65% fructose diet along with PT 40 mg/kg/day for eight weeks. PT was dissolved in 10% β-cyclodextrin and given orally to rats.
Group-V	D+M	Rats received 65% fructose diet along with metformin (M) 100 mg/kg/day for eight weeks (Wessels et al., 2014). Metformin was dissolved in 0.9% saline and given orally to rats.

In experimental studies, PT was administered as a prophylactic modality for various diseases; Gomez-Zorita et al. (2015) found that PT administered as a dietary supplement for six weeks to obesogenic rats ameliorated insulin resistance and glycemic control through stimulation of Akt pathway (Gomez-Zorita et al., 2015). Lv et al. (2015) reported that PT administration five min before reperfusion decreased cardiac infarct size by inhibiting neutrophil infiltration and the rise of serum TNF-α levels in normal rats (Lv et al., 2015). Thus, prophylactic administration of PT and metformin along with fructose induction were chosen for the amelioration of T2DM development.

The oral dose range of 20 and 40 mg/kg (approximately equivalent to one and two times the human equivalent dose) was chosen based on previous efficacy studies in rats (Pari & Satheesh, 2006; Zhang et al., 2016a) and in a recent clinical trial where 250 mg of PT demonstrated an anti-hypertensive effect (Riche et al., 2014b). Presume that the average body surface area of an adult human is 1.8 m^2, the dose of PT validated in the clinical trial was 138.9 mg/m^2. Furthermore, it seems safe to consider it non-toxic since the oral administration of 250 mg daily for 6-8 weeks to humans (Riche et al., 2013) and an ultra-high chronic dose of PT (3 g/kg, 28 days) (Ruiz et al., 2009) did not cause any notable side effects. Therefore, clinically appropriate doses of 20 and 40 mg/kg in rats (equivalent to 120 and 240 mg/m^2) were selected to investigate the impact of dose escalation on diabetes and cardiac complications in diabetic rats.

4.5.2. Experimental groups for objective-II

After two weeks of adaptation, the animals were randomly divided into six groups (n = 8/group):

Group-I	C	Rats received 65% corn starch (Research Diet, USA) and vehicle (10% β-cyclodextrin) for eight weeks.
Group-II	C+PT20	Rats received 65% corn starch along with pterostilbene (PT) 20 mg/kg/day for eight weeks. PT was dissolved in 10% β-cyclodextrin and given orally to rats. The PT dose was chosen based on our earlier experiments (Kosuru & Singh, 2017).
Group-III	D	Rats received 65% high fructose diet (Research Diet, USA) for eight weeks for the induction of an established model of T2DM (Padiya et al., 2011). Vehicle (10% β-cyclodextrin) was given orally for eight weeks.
Group-IV	D+PT20	Rats received 65% high fructose diet along with PT 20 mg/kg/day for eight weeks. PT was dissolved in 10% β-cyclodextrin and given orally to rats.

To validate the PT mechanistic pathway (for western blot experiments)		
Group-V	D+CC	Rats received 65% high fructose diet with AMPK inhibitor, i.e. Compound C (CC; Sigma-Aldrich, MO, USA) 20 mg/kg/day, intraperitoneally.
Group-VI	D+PT20+CC	Rats received 65% high fructose diet with an oral dose of 20 mg/kg/day of PT along with an intraperitoneal dose of 20 mg/kg/day of CC.

4.5.3. Experimental groups for objective-III

4.5.3.1. *In vivo* experiments

After eight weeks, the diabetic animals were randomly segregated into four groups (n=8/group):

Group-I	D sham	Streptozotocin-induced diabetic rats received vehicle (10% β-cyclodextrin) for four weeks. Rats underwent surgery without coronary artery ligation.
Group-II	D+IR	Streptozotocin-induced diabetic rats received vehicle (10% β-cyclodextrin) for four weeks. After 4 weeks treatment of vehicle, coronary artery ligation (30 minutes ischemia/2 hours reperfusion model) was employed (Liu et al., 2015).
Group-III	D+IR+PT20	Streptozotocin-induced diabetic rats received pterostilbene (PT) 20 mg/kg/day for four weeks. After 4 weeks treatment of PT, coronary artery ligation (30 minutes ischemia/2 hours reperfusion model) was employed.
Group-IV	D+IR+PT40	Streptozotocin-induced diabetic rats received PT 40 mg/kg/day for four weeks. After 4 weeks treatment of PT, coronary artery ligation (30 minutes ischemia/2 hours reperfusion model) was employed.

4.5.3.2. *In vitro* experiments

Adult rat primary cardiomyocytes were challenged with hypoxia/reoxygenation (HR) under low glucose (LG) and high glucose (HG) conditions. The rat primary cardiomyocytes were randomly divided into the following groups:

Under low glucose condition		
Group-I	LG	Primary rat cardiomyocytes were incubated with 5 mM D-glucose concentration in culture medium for 48 hours. Later vehicle (DMSO) was added to the culture medium and incubated for 4 hours.
Group-II	LG+ HR	Primary rat cardiomyocytes were incubated with 5 mM D-glucose concentration in culture medium for 48 hours. Later DMSO was added to the culture medium, incubated for 1 hour and challenged with 45 minutes hypoxia and 2 hours reoxygenation. Hypoxia was established by equilibrating a humidified chamber (37°C) containing cardiomyocytes with a gas mixture of 5% CO_2, 95% N_2 and 0.1% O_2 with the help of gas transfusion apparatus. Reoxygenation was established by transferring cardiomyocytes to a CO_2 incubator (5% CO_2, 95% O_2).
Group-III	LG+HR+PT	Primary rat cardiomyocytes were incubated with 5 mM D-glucose concentration in culture medium for 48 hours. Later, PT (0.5 μM) was added to the culture medium, incubated for 1 hour and challenged with 45 minutes hypoxia and 2 hours reoxygenation.
Group-IV	LG+HR+CC	Primary rat cardiomyocytes were incubated with 5 mM D-glucose concentration in culture medium for 48 hours. Later, CC (5 μM) was added to the culture medium, incubated for 1 hour and challenged with 45 minutes hypoxia and 2 hours reoxygenation.
Group-V	LG+HR+PT+CC	Primary rat cardiomyocytes were incubated with 5 mM

		D-glucose concentration in culture medium for 48 hours. Later, PT (0.5 µM) and CC (5 µM) were added to the culture medium, incubated for 1 hr and challenged with 45 minutes hypoxia and 2 hr reoxygenation.
Under high glucose condition		
Group-I	HG	Primary rat cardiomyocytes were incubated with 30 mM D-glucose concentration in culture medium for 48 hours. HG, 30 mM in culture medium was employed to mimic *in-vivo* diabetes since 30 mM glucose concentration represents the reported peak levels of blood glucose achieved in non-controlled diabetes (Worthley et al., 2007). Later DMSO was added to the culture medium and incubated for 4 hr.
Group-II	HG+HR	Primary rat cardiomyocytes were incubated with 30 mM D-glucose concentration in culture medium for 48 hours. Later DMSO was added to the culture medium, incubated for 1 hour and challenged with 45 minutes hypoxia and 2 hours reoxygenation.
Group-III	HG+HR+PT	Primary rat cardiomyocytes were incubated with 30 mM D-glucose concentration in culture medium for 48 hours. Later, PT (0.5 µM) was added to the culture medium, incubated for 1 hour and challenged with 45 minutes hypoxia and 2 hours reoxygenation.
Group-IV	HG+HR+CC	Primary rat cardiomyocytes were incubated with 30 mM D-glucose concentration in culture medium for 48 hours. Later, CC (5 µM) was added to the culture medium, incubated for 1 hour and challenged with 45 minutes hypoxia and 2 hours reoxygenation.
Group-V	HG+HR+PT+CC	Cardiomyocytes were incubated with 30 mM D-glucose concentration in culture medium for 48 hr. PT and CC were incubated for 1 hr and challenged with 45 min hypoxia and 2 hr reoxygenation.

4.6. Experimental Design

4.6.1. Study design for objective-I

The experimental design for objective-I experiments is depicted in the following diagram.

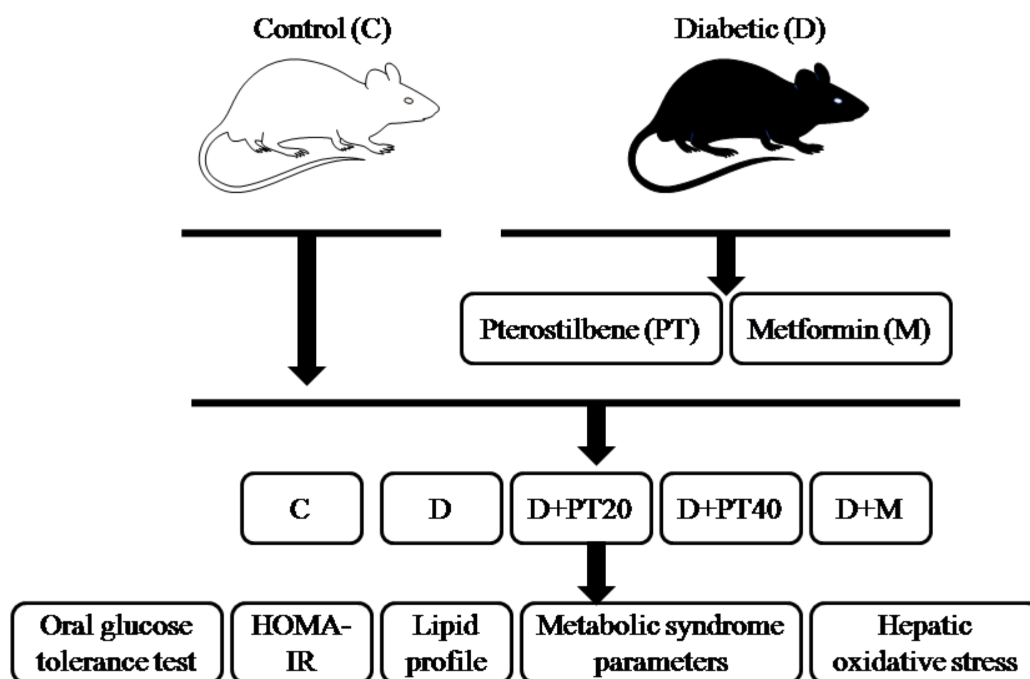

Figure 4.1: Study design for objective-I

4.6.2. Study design for objective-II

The experimental design for objective-II experiments is depicted in the following diagram.

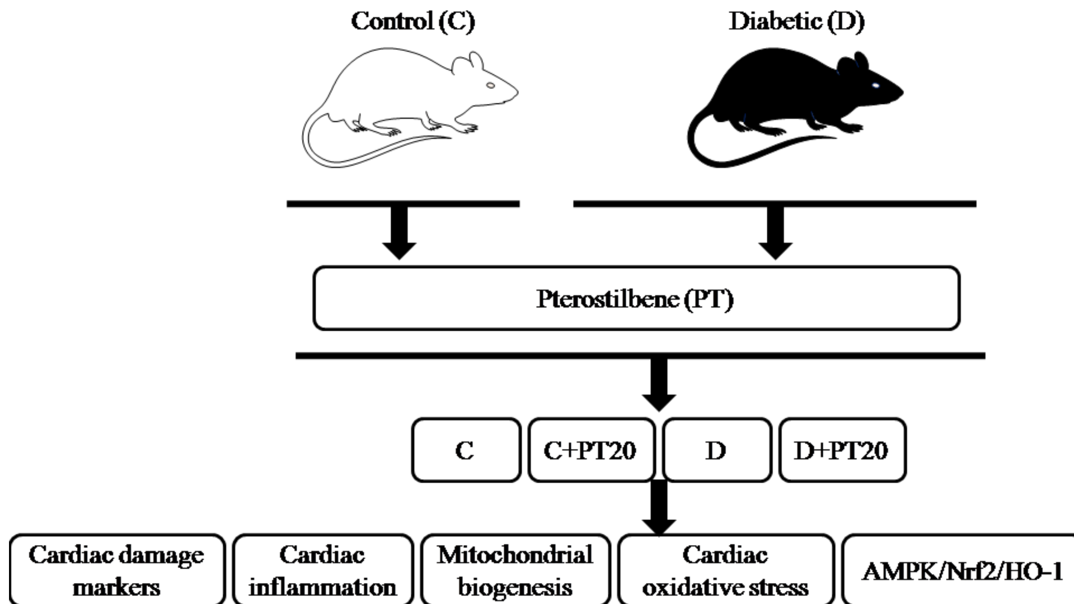

Figure 4.2: Study design for objective-II.

4.6.3. Study design for objective-III

The experimental design for objective-III experiments is depicted in the following diagram.

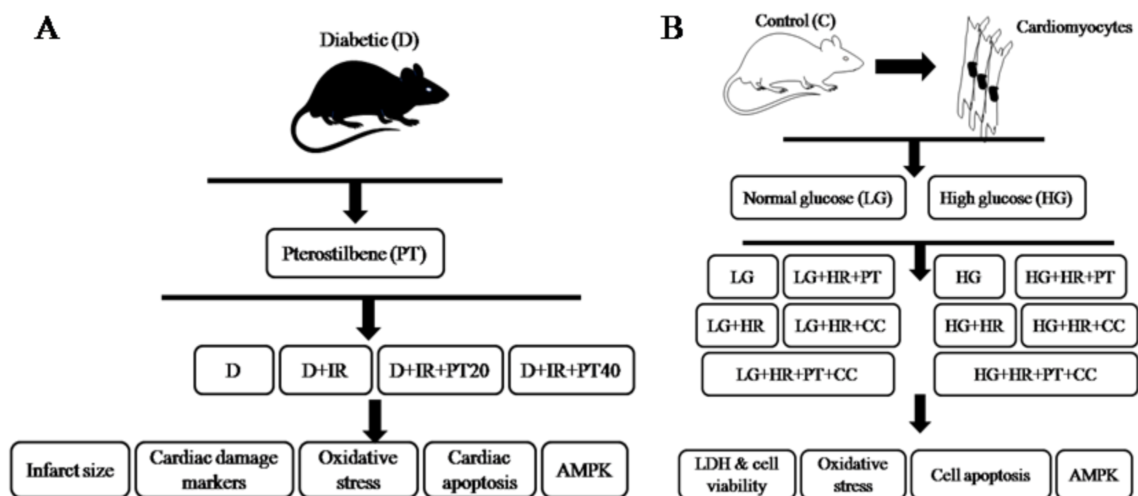

Figure 4.3: Study design for objective-III. A. *In vivo* study using diabetic rats. B. *In vitro* study in cardiomyocytes isolated from control rats.

4.7. Methods

4.7.1. Diabetes induction by high fructose diet (for experiments related to Objective-I and Objective-II)

One kg of high fructose diet contains following ingredients: Casein 200 gm; DL-methionine 3 gm; Fructose 650 gm; Cellulose (BW200) 50 gm; Corn oil 50 gm; Mineral mix (S10001) 35 gm; Vitamin mix (V10001) 10 gm; Choline bitartrate 2 gm; FD&C Red dye#40 0.2 gm. Rats were fed with 65% high fructose diet (protein 20 gm%; carbohydrate 66 gm%; fat 5 gm%) for eight weeks for the induction of an established model of T2DM (Padiya et al., 2011). Furthermore, high fructose intake has been accountable for the progression of diabetes based on its putative adverse effects on lipids (Cozma et al., 2012). Only those animals were having fasting blood glucose level ≥11.5 mM were considered as diabetic and considered for the *in vivo* experiments.

4.7.2. Diabetes induction by intravenous streptozotocin injection (for experiments related to Objective-III)

Previous reports suggest that low pH solution is essential to maintain the stability of streptozotocin, once reconstituted (Tesch & Allen, 2007). 0.1 M Sodium citrate buffer (pH=4.5; **Table 4.3**) should only be prepared immediately before injection as drug degrades after 15-20 min in the buffer.

a. **Preparation of 0.1 M citric acid buffer:** 1.929 g of citric acid is dissolved in 100 ml double distilled water.

b. **Preparation of 0.1M sodium citrate buffer**: 2.94 g of sodium citrate is added to 100 ml double distilled water.

Table 4.3: Composition of streptozotocin buffer (for 50 ml)

Component	Volume
0.1 M citric acid (FW = 192.12) buffer	28 ml
0.1 M sodium citrate (FW = 294.1)	22 ml

Diabetogenic streptozotocin is commonly administered through intraperitoneal and intravenous routes in rodents (Tay et al., 2005). Intraperitoneal route has been regarded as the rapid and regular mode of administration, particularly for induction of diabetes through multiple doses of streptozotocin. However, accidental administration of streptozotocin into the bowel or sub-dermal space may lead to decreased diabetogenic effect or enhanced moribundity. Additionally, it has been demonstrated that intravenous administration of streptozotocin (65 mg/kg i.v.) generates a more stable and reproducible model of type 1 diabetes than intraperitoneal administration (Furman, 2015; Tay et al., 2005). Therefore, intravenous delivery of streptozotocin was employed for our *in vivo* studies.

4.7.3. Myocardial ischemia-reperfusion by coronary artery ligation model (Objective-III)

Procedure:

A. **Pre-surgical Preparation:** The operating table, heating pad, needles, and sutures were disinfected with 70% alcohol before surgery. All surgical tools (scissors, forceps, needle holder, chest retractor and curved forceps) were sterilized with hot glass dry bead sterilizer before and in between surgery.

B. **Anaesthesia:** Anaesthetized the rats with 80 mg/kg pentobarbital, i.p.; verify the correct level of anaesthesia through toe-pinch reflex throughout the procedure.

C. Shaved the fur from chest and neck areas using an electric shaver and disinfected the shaved regions by scrubbing with betadine and alcohol for three times.

D. Rats were placed in a supine position on the thermostatic pad and continuously monitored body temperature of the animal at 37±1°C using a rectal temperature probe (1 mm tip).

E. **Endotracheal Intubation:** Made a 5mm mid-neck incision and exposed the trachea by retracting the muscle tissue just above the trachea. Inserted the polyethylene endotracheal tube (PE 90) by making a small cut on the trachea.

F. Mechanical ventilation is achieved by connecting intubation tube to rat ventilator (Respiration rate: 80 breaths/min; tidal volume of 1.2 ml per 100 g body weight).

G. A sterile drape was placed over the rat neck to minimize potential contamination.

H. **Transient Left Anterior Descending (LAD) Artery Ligation**

i. The chest was opened at the left 4-5th intercostals space by making a 15 mm incision. Carefully cut the tissues so that risk of bleeding can be minimized.

ii. Left ventricle was exposed by carefully spreading the ribs using chest retractor by taking maximal care not to damage the lung.

iii. Pericardium was opened and LAD artery was identified and ligated (Slipknot) using a 6-0 suture for a 30-min ischemic period. Ischemia was confirmed by blanching of cardiac tissue distal to suture (discolouration of heart surface).

iv. Myocardial reperfusion was induced by releasing the slipknot and allowed for 2 hours. Reperfusion was confirmed by restoration of red colour to cardiac tissue.

v. In sham control rats, the procedure was identical except LAD artery ligation.

4.7.4. Histological Evaluation

Procedure:

A. Following completion of myocardial IR protocol, LAD was ligated by retying slipknot.

B. **Preparation of 5% Evans blue dye:** Dissolved 5 g of Evans blue powder in 100 ml of water.

C. Injected 1 ml of 5% Evan's Blue (in phosphate-buffered saline) through the right jugular vein and allowed to perfuse for 2 minutes.

D. **TTC staining:** Hearts were rapidly excised, and infarct size was measured using 1% 2, 3, 5-triphenyltetrazolium chloride (TTC) staining as described (Xue et al., 2016).

i. **Preparation of 1% TTC (2 ml/heart):** Dissolved 100 mg of TTC in 10 ml of 1X PBS

ii. **Preparation of 10% formalin:** Dissolved 1 ml of 40% formaldehyde (commercial product) in 9 ml of DDH_2O [because 40% formaldehyde should be considered as 100%].

iii. Pre-freezed the heart sample at -80° C and sliced the heart into even slices using the mould and blades and placed them in the 6 well plates. Care should be taken that slices should not overlap each other.

iv. Added 2 ml of 1% TTC in each well and incubated for 30 min at 37°C. Later, discarded the TTC and fixed the cells with 10% formaldehyde solution in 1× PBS and observed for 2 hours or overnight.

v. Then, placed the fixed heart slices on the glass slide and snapshots were taken using the scanning device. Proceeded to measure the infarct size using Image J software.

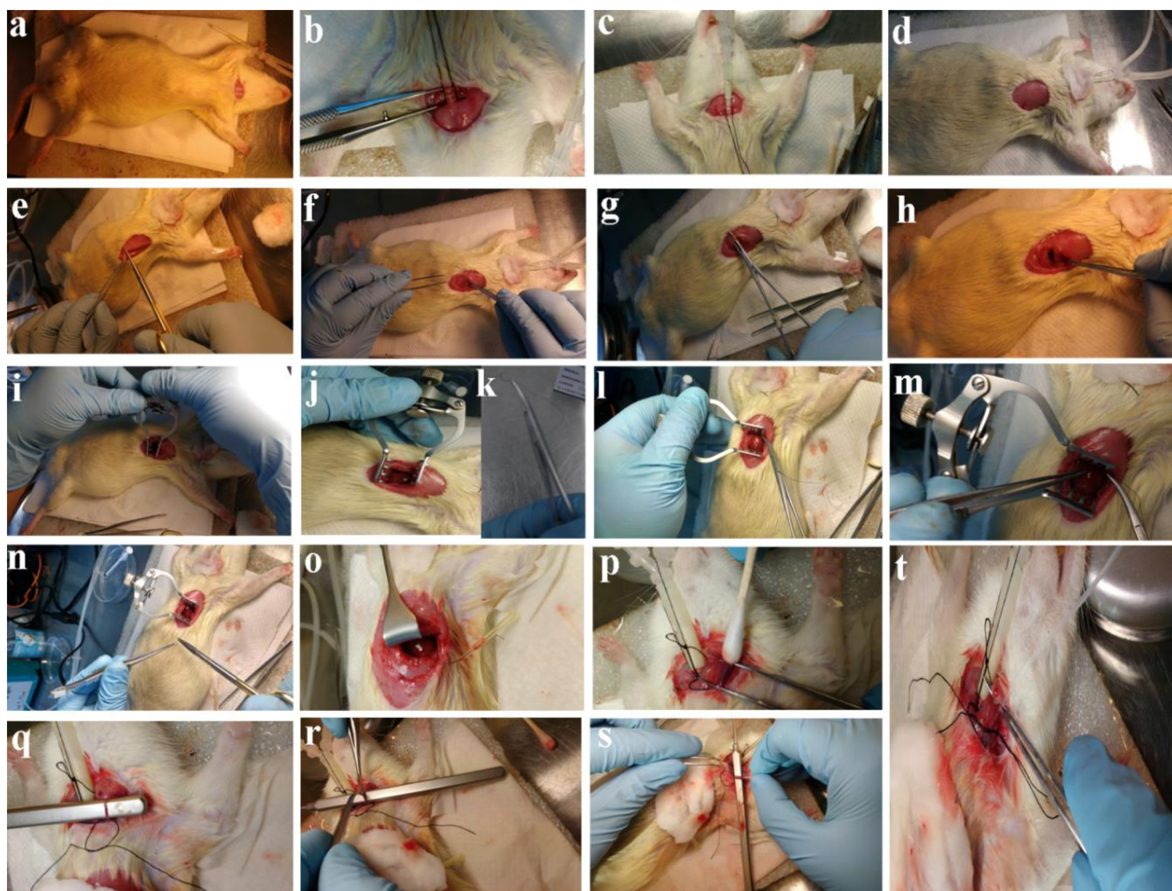

Figure 4.4: Myocardial IR injury by LAD artery ligation.

a) small incision on neck b) exposure of trachea c) endotracheal intubation d) connect to mechanical ventilation e) 15 mm incision on chest f-h) chest opening at 4-5th intercostals space by removing tissues i-j) exteriorization of heart using chest retractor k) curved needle l-m) identify and insert 6-0 suture under LAD artery n) ligate the LAD artery by slipknot to create ischemia o) release the slipknot to allow reperfusion p-q) visualize the left jugular vein r-t) cut the jugular vein and insert the polyethylene tube for injecting Evans blue dye.

vi. Brick red part indicates live tissue (blue colour by Evans blue staining); Pale coloured part indicates area at risk; White coloured part indicates infarcted.

vii. Relative infarct size to area at risk = (infarct size/area at risk)*100

4.7.5. Primary Adult Rat Cardiomyocytes Isolation and Hypoxia-Reoxygenation (HR)

4.7.5.1. Primary rat cardiomyocytes isolation

Primary cultures of adult rat cardiomyocytes were isolated from four weeks old Sprague-Dawley rats and cultured as previously described (Lei et al., 2013).

Procedure: The following steps were involved in cardiomyocytes isolation.

A. **Preparation of required solutions and Matrigel-coated dishes for myocyte isolation**

i. **Perfusion buffer (1× solution):** Prepared 1× perfusion buffer before the day of the experiment (**Table 4.4**) and stored in refrigerator at 4°C.

ii. **Digestion buffer:** Prepared digestion buffer on the day of the experiment (**Table 4.5**) and maintained at 37°C.

iii. **Stopping buffer:** Prepared stopping buffer on the day of the experiment (**Table 4.6**) and maintained at 37°C.

iv. **Planting medium:** Prepared planting medium on the day of the experiment (**Table 4.7**) and maintained at 37°C.

v. **Culture medium:** Prepared culture medium on the day of the experiment (**Table 4.8**) and maintained at 37°C.

vi. **Matrigel (Corning) matrix:** Added 5 ml of the commercial product to 45 ml of M199 medium and stored at -20°C.

vii. **Bovine serum albumin (BSA; final concentration 50 mg/ml):** For preparation of 40 ml BSA solution, dissolved 2 g (i.e. 50X40 = 2000 mg) of BSA in 40 ml of M199 medium.

viii. **BDM (mol.wt:101.10; final concentration 500 mM):** For 50 ml BDM solution, dissolved 2.5225 g of BDM in 50 ml M199 medium.

ix. **ITS stock solution (100×):** Made acidified water by adding acetic acid to reduce the pH approximately 2. Dissolved the contents of the ITS vial in 5 ml sterile, acidified water and swirl to dissolve. Then added 45 ml of sterile water.

To make 1× concentration: Added 0.5 ml of 100× ITS to 50 ml of culture medium.

x. Coated the dishes or plate with Matrigel matrix, and incubated at 37°C for >1 hour (just before the dissecting the rat heart).

Table 4.4: Perfusion Buffer Composition

Compound	Molecular weight (g.mol)	Final concentration (mM)	1× Buffer (g/L)	10× Buffer (g/L)
NaCl	58.4	120.4	7.03	70.3
KCl	74.6	14.7	1.1	11
KH_2PO_4	136.1	0.6	0.082	0.82
Na_2HPO_4	142	0.6	0.085	0.85
$MgSO_4.7H_2O$	246.5	1.2	0.30	3
Na-HEPES	1M	10	10 mL (2.6029 g)	100 mL
$NaHCO_3$	84	4.6	0.39	3.9
Taurine	125.1	30	3.75	37.5
BDM	101.1	10	1	10
Glucose	180.2	5.5	1	10

Table 4.5: Myocyte digestion buffer composition

Compound	Final concentration	Amount/Quantity
Perfusion buffer	-	50 ml
Collagenase type 2	2 mg/ml	100 mg

Table 4.6: Myocyte stopping buffer composition

Compound	Final concentration	Volume
Perfusion buffer	-	36 ml
Calf serum/FBS	10%	4 ml
$CaCl_2$ (100 mM	12.5 µM	5 µl

Table 4.7: Myocyte Planting medium (50 ml)

Compound	Final concentration	Volume
M199 medium	-	43 ml
Calf serum/FBS	10%	5 ml
BDM (500 µM)	10 µM	1 ml
PSF or P/S		0.55 ml
ITS	2 mM	0.5 ml

Table 4.8: Myocyte culture medium (50 ml)

Compound	Final concentration	Volume
M199 medium (Pink color)	-	47 ml
BSA (50 mg/ml)	0.1% (1 mg/ml)	1 ml
BDM (500 mM)	10 mM	1 ml
PSF or P/S		0.55 ml
ITS	2 mM	0.5 ml

B. **Setting up of perfusion apparatus**

i. Set the circulation water bath at 42°C.

ii. Wash the perfusion apparatus with 1M HCl for approx. 10 min.

iii. Wash the perfusion apparatus with sterile DDH_2O for approx. 10 min.

iv. Wash the perfusion apparatus with perfusion buffer for approx. 2 min.

v. Fill the perfusion system with perfusion buffer and adjust perfusion flow rate to 3-4 ml/min.

C. **Removal and cannulation of the heart**

i. Animal strain, sex and body weight were recorded.

ii. Anaesthetized the rat (300-350 g) with phenobarbital sodium (0.1 ml per 100 g) intraperitoneally (i.p.).

iii. Injected i.p. heparin (1000 U/kg; 0.1 ml diluted in PBS).

iv. Chest was cleansed with alcohol and opened chest and peritoneal cavity with small scissors and forceps to expose the heart.
v. Heart was gently lifted and cut the pulmonary vessels to identify the aorta easily.
vi. Cut the aorta at about 2 mm from its entry into the heart and immediately placed the heart in a 35-mm dish containing 2 ml of cold buffer.
vii. Extraneous tissue (thymus and lungs) was removed, if necessary, and recorded the wet weight of the heart and transferred to another 35-mm dish with cold buffer for heart cannulation.
viii. Cannulated the heart using fine-tip forceps to slide the aorta onto the cannula (21 G needle).
ix. Attached a small clip at the end of the cannula to prevent the heart from falling and tied the aorta to the cannula with 6.0 silk thread (make sure the tip of the cannula is just above the aortic valve). Total time to cannulate the heart was less than 1 min.
x. Started the perfusion immediately (3-4 ml/min).

D. **Heart perfusion and enzyme digestion**

i. Heart was perfused with perfusion buffer for 5 min (this flushes blood from the vasculature and removes extracellular calcium to stop contractions).
ii. Then, switched to digestion buffer and perfused for 20-30 min (digestion times can vary according to heart weight and animal age). Recycled the digestion buffer in a closed system. If the heart is well perfused during the enzyme digestion, the heart will become swollen and turn slightly pale, and separation of muscle fibres on the surface of the heart may become apparent. Save the digestion buffer for future use (4°C).
iii. Added cold $CaCl_2$ into the digestion buffer during the perfusion.

Initial 5 min perfused with	Perfusion buffer
Next 10 min perfused with	Digestion buffer
Later 15 min perfused with	12.5 µl $CaCl_2$ added into digestion buffer
Lastly, 20 min perfused with	25 µl $CaCl_2$
Total time of digestion:50 min	

iv. Cut the heart from the cannula just below the atria using sterile fine scissors, once digestion of the heart is completed. Placed the heart/ventricles in a 35-mm dish containing 20 ml of digestion buffer and 20 ml of stopping buffer and then minced the heart (total 40 ml mixture).

E. **Screening of cardiomyocytes**

i. Filtered 40 ml mixture using a sterilized nylon filters (200 µm) and collected the filtrate into 50 ml tube. Added 10 ml of stopping buffer to make 50 ml volume.

ii. Decantation and sedimentation process were followed instead of centrifugation to prevent the harm to the cardiomyocytes.

iii. Kept the tube at 30°, 60°, 90° angles for 3 min each respectively and then collected the precipitate and discarded the supernatant.

F. **Calcium reintroduction**

i. Resuspended the precipitate in 50 ml of perfusion buffer and introduced different concentration of $CaCl_2$ (0.1, 0.25, 0.5 µl of 1M $CaCl_2$).

ii. Kept the tube in 30°, 60°, 90° angles each time for 3 min respectively and then collected the precipitate and discarded the supernatant.

iii. Washed the pellet and repeated the process until one gets a clear supernatant and precipitate/pellet.

iv. Total time taken to complete this process was around 27 min (9 min for each concentration).

G. **Planting myocytes and culture**

i. The planting medium was equilibrated for 2-3 hours at 37°C in the water bath.
ii. Now introduced the pellet/precipitate of cardiomyocytes in the planting medium.
iii. Cell count and viability was measured using hemocytometer (cardiomyocytes should be rod-shaped). Round shaped cells were treated as non-viable.
iv. After counting cells, plated them as follow:
v. **35 mm dish:** 50K rod-shaped cells/dish – 2 ml/dish
vi. **60 mm dish:** 120K rod-shaped cells/dish – 5 ml/dish
vii. **12 well plate:** 25K rod-shaped cells/well – 1 ml/well
viii. Incubated cells in the 5% CO_2 incubation chamber for 60-90 min at 37°C. After this period, viable cells will be attached, and dead cells will be floating.
ix. Switched to cardiomyocyte culture medium for various cell studies.

Note: Weigh all the ingredients first and then make up the solutions with either perfusion buffer or M199 medium for respective solutions of the Tables (1-5). Before experimenting, take care all the necessary equipment are ready and sterilized (pipettes, tips, six-well plates and tubes, nylon filter etc.). It is always better to do the hypoxia/reoxygenation experiment next day of cardiomyocyte isolation, so that, cells will settle down properly.

4.7.5.2. Hypoxia/Reoxygenation (HR) of Cardiomyocytes

Procedure:

A. Previous culture medium was replaced with D-glucose [-] (No glucose) medium to mimic hypoxic state, i.e. no oxygen, nutrients and energy. Added 1 ml of D-glucose [-] to each well.
B. Out of 6 plates, considered three plates as control and three plates as HR groups.

C. The hypoxia chamber was connected to the gas (5% CO_2/0.1% O_2/N_2) cylinder. The flow of gas was regulated at 20 L/min for 3 minutes (care should be taken to close another outlet of the hypoxia chamber is tightly).

D. Now kept the three plates which are considered for HR in the hypoxia chamber and maintained the above-said conditions for 3 min.

E. After 3 min, locked the inlet tightly and closed the valve of the gas-cylinder to stop the flow of gas. This gas replaces the oxygen present in the hypoxia chamber with the 5% CO_2, 0.1% O_2, N_2 (mimicking the ischemic condition).

F. Now put the whole apparatus in the incubation for 45 min.

G. After 45 min, switched the medium of both the control and HR groups to culture medium again (1 ml each well). In this way, the cell lines were reoxygenated and supplied with nutrients and energy.

H. Again kept all the plates in the incubator for 2 hours. Later, the cells can be used for further tests.

4.7.5.3. Extraction of Primary Rat Cardiomyocytes from the Plates (Cell Scraping)

Procedure

A. Kept all the dishes on the ice.

B. Collected 0.5 ml of medium for LDH detection test and removed the leftover medium from all the wells.

C. Added 0.5 ml of DPBS (1× PBS) in each well.

D. Regularly scraped each well using the sterile scraper.

E. Repeated the 3 and 4 steps for three times, so that the cells were nicely suspended into the PBS.

F. Collected the PBS into centrifuge tubes and placed all the tubes on the ice.

G. Later, these samples were centrifuged at 8000 rpm at 4°C for 5 min.

H. Meanwhile, prepared 1× lysis buffer (**Table 4.9**).

I. After centrifugation, discarded the supernatant and collected the pellet. Made it dry up to the possible extent.

J. Added 100 µl of 1× lysis buffer to each pellet and re-suspended by thorough flushing using micropipette.

K. Preserved all the samples at -80°C and used for biochemical estimations.

Table 4.9: Composition of 1× lysis buffer

10× lysis buffer (commercial product)	100 µl
PI	10 µl
DD H_2O	890 µl

4.7.5.4. Estimation of Protein Concentration of Cardiomyocyte Suspension

Procedure:

A. Samples were taken out from the refrigerator (-80°C) and allowed them to melt into the liquid state (kept them on ice for 30 min). If needed, one can use a vibrator for liquefying the refrigerated samples.

B. After 30 min, centrifuged the samples in the pre-cooled (4°C) centrifuge at 13200 rpm for 15 min.

C. Collected the supernatant in the labelled centrifuge tubes and discarded the pellet.

D. In 96 well plates, added 200 µl of protein assay reagent in each well. Further, added 1µl of each sample (supernatant) and standard concentration of BSA (**Table 4.10**).

E. Checked the absorbance by Coomassie blue assay using Promega-GLOMAX (multi-detection system) and calculated the protein concentration using MS-excel.

F. Denaturing the protein sample with loading buffer: Equilibrated the sample volume according to its protein concentration. Then added 1× lysis buffer and 4× loading buffer (one-third of sample volume) to each sample. Mixed all the contents

thoroughly using a vibrator and heated the sample tubes at 100-103°C for 10 min; cooled on ice. Later stored them at -80°C and used for Western blot experiments.

Table 4.10: Standard dilution of BSA

Protein concentration (mg/ml)	BSA (1mg/ml) (µl)	DDH_2O (µl)
0	0	1000
0.125	125	875
0.25	250	750
0.5	500	500
1	1000	0
2	2000	0

4.7.6. Determination of Heart Rate and Blood Pressure

Heart rate and blood pressure were estimated in all experimental rats using a noninvasive tail-cuff method (Narco Bio-System, Houston, TX).The experimental rats were transported to a quiet environment and placed in a chamber at an ambient temperature of 37°C for 10 min, then put in an acrylic restrainer. Systolic and diastolic blood pressures of rats were measured using the tail-cuff method (Narco Bio-System, Houston, TX) (Fritz & Rinaldi, 2008). The mean of six successive recordings was calculated for each blood pressure measurement. Mean arterial pressure was derived from the following formula: Mean arterial pressure = ⅔ diastolic blood pressure + ⅓ systolic blood pressure.

4.7.7. Oral Glucose Tolerance Test (OGTT)

One day before culling, all the rats from every group were fasted overnight to perform OGTT. A glucose load of 2 g/kg was administered orally to all of the experimental rats. Blood was collected from the tip of the tail at 0, 15, 30, 45, 60, 75, 90 and 120 min time intervals to quantify the blood glucose level using glucometer (One Touch Ultra).

4.7.8. Collection of Samples and Preparation of Tissue Homogenates

The body weights of all rats were measured each week during the experimental period. After the completion treatment, all animals were weighed and euthanised. Blood was collected through the cardiac puncture into the heparinized Eppendorf tubes and centrifuged at 1, 200 *g* for 15 min to isolate plasma. The hearts and liver tissues were rapidly removed, weighed and preserved in a tube at -80°C for further biochemical evaluation. The myocardial hypertrophy index was determined by estimating the ratio of heart weight to body weight. Blood glucose levels were determined by glucose meter (One Touch Select, UK).

4.7.8.1. Preparation of cardiac and liver homogenates: Cardiac and liver tissue samples were homogenised in IP lysis buffer (Cell Signaling Technology, USA) and centrifuged at 13,000 g at 4°C for 15 min (Thermo Scientific^{TM} MicroClick 24 × 2 microtube rotor). After centrifugation, the supernatant was collected, stored at -80°C and utilized for different biochemical measurements.

4.7.8.2. Preparation of cardiomyocyte sample: Primary rat cardiomyocytes were washed with cold phosphate-buffered saline and lysed in lysis buffer on ice for 30 min. Then, whole cell lysates were centrifuged at 12,000 × g, 4°C, 15 min (ThermoScientific^{TM}MicroClick 24 × 2 microtube rotor). The supernatant was collected, stored at -80°C and utilized for different biochemical measurements.

4.7.9. Paraffin Embedding

Procedure: It involves dehydration, osmosis and embedding processes.

A. Tissue placed in 10% paraformaldehyde overnight (24-72 h).

B. Dehydration step was carried out by sequential addition of following solvents: 50% ethanol (60 min) – 70% ethanol (45 min) – 85% ethanol (45 min) – 95% ethanol (60 min) – 100% ethanol (60 min) – 100% ethanol (60 min).

C. Lucency was induced by serial mixing of following solvents: 50% ethanol + 50% xylene (10 min) – 100% xylene (10 min) – 100% xylene (5 min).

D. Osmosis was created using: paraffin 1 (40 min) – paraffin 2 (40 min).

E. Embedded of left ventricular tissue.

4.7.10. Hematoxylin & Eosin (H&E) staining

H&E staining is the most widely used staining method in the histopathological analysis of various tissues (Feldman & Wolfe, 2014).

Procedure:

A. Tissue sections were prepared at the thickness of 5-6 µm

B. Dewax and hydration: cardiac tissue sections placed in an oven for 20 min at 60°C and treated them with following solvents sequentially to deparaffinize. Placed the tissue section (slide) in xylene for 8 min – xylene for 8 min. Hydrated the tissue section by passing through decreasing concentration of alcohol and water: 100% ethanol for 8 min – 100% ethanol for 8 min – 90% ethanol for 8 min – 90% ethanol for 8 min – 70% ethanol for 5 min – 70% ethanol for 5 min – DDH_2O for 5 min – DDH_2O for 5 min.

C. Stained with Vector Hematoxylin for 5 min (check the colour under a microscope).

D. If overstain, destained with acid-ethanol (1% HCl in 95% ethanol).

E. Washed in running tap water for three times.

F. Blued in Scott's tap water, three times.

G. Washed with tap water, three times.

H. Counterstained with Eosin Y for 30-45 sec.

I. Washed with tap water, checked the colour under a microscope.

J. Dehydrated the tissue sections with increasing concentration of alcohols and clear in xylene. 70% ethanol (one time for 30 sec) - 95% ethanol (2 times, each for 30 sec) - 100% ethanol (2 times, each for 1 min) – xylene (3 times, each for 5 min).

K. Mounted with mountant and covered with a coverslip.

L. Dried overnight and observed under a microscope.

M. After H&E staining, nuclei appear blue/black; cytoplasm appears pink; muscle fibres appear deep red and fibrin as deep pink coloured.

4.8. Biochemical Estimations

4.8.1. Estimation of fasting blood glucose (FBG), fasting serum insulin (FSI) and insulin sensitivity index (ISI) and homeostasis model of insulin resistance (HOMA-IR)

The FBG levels were estimated on each week during the entire experimental period using the blood glucometer (One Touch Ultra). The FSI levels were evaluated by rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Thermo Fisher Scientific, USA), performed according to the manufacturer's protocol. The ISI was derived from the following equation: **ISI = Ln (1/FSI × FBG)**. HOMA-IR is considered as the gold standard for the estimation of insulin resistance and can be derived using the equation (Matthews et al., 1985): **HOMA-IR = [FBG (mmol/L) × FSI (mU/L)]/22.5**.

4.8.2. Estimation of glycated haemoglobin (HbA1c), hydrogen sulfide, peroxynitrite and uric acid

Serum HbA1c levels were estimated by the method described by Sudhakar and Pattabiraman (Nayak & Pattabiraman, 1981). Serum hydrogen sulfide levels were determined by the method of Cai (Cai et al., 2007). Serum peroxynitrite level as a marker of nitrosative-oxidative stress was measured according to the method described by Vanuffelen (Vanuffelen et al., 1998). Serum uric acid level was estimated by commercial diagnostic kits (Abcam, India), performed according to the manufacturer's protocol.

4.8.3. Estimation of lipid profile, cardiovascular risk indices and antiatherogenic index

Serum levels of total cholesterol (TC), triglycerides (TG) and high-density-lipoprotein-cholesterol (HDL-C) were determined using an automated blood analyzer (Bayer Corp. USA) by colourimetric method. TG (sensitivity: 10 mg/dl), TC (sensitivity: 10 mg/dl) and HDL-C (sensitivity: 10 mg/dl) commercial diagnostic kits were procured from Siemens, India. Serum low-density lipoprotein-cholesterol (LDL-C) level was derived from the equation (Friedewald et al., 1972): **LDL-C = TC – [(TG/5) + HDL-C],** while serum levels of very low-density lipoprotein-cholesterol (VLDL-C) were derived from the formula (A. Bell, 1995): **VLDL-C = TG/5**. Cardiovascular risk indices were determined using the following formulas (Ross, 1992): **cardiovascular risk index 1 = TC/HDL-C,** and **cardiovascular risk index 2 = LDL-C/HDL-C**. The antiatherogenic index (AAI) was calculated using the formula (Guido & Joseph, 1992): **AAI = HDL-C × 100/TC – HDL-C**.

4.8.4. Detection of oxidative stress and antioxidant markers

The indicators of oxidative stress and antioxidant defence in liver and cardiac homogenate were estimated, including thiobarbituric acid reactive substances (TBARS), hydrogen peroxide, peroxynitrite, total superoxide dismutase (SOD), catalase, GPx and glutathione (GSH) with commercial kits (Cayman Chemicals, USA), and performed according to the manufacturer's guidelines. Reactive oxygen species (ROS) in cardiac homogenates were estimated fluorometrically using 2, 7-dichlorofluorescein diacetate as previously described method (Maity et al., 2009).

4.8.5. Determination of proinflammatory cytokines by ELISA

The levels of interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α, in cardiac homogenate and plasma, were estimated by respective enzyme-linked immunosorbent assay (ELISA) kits (Thermo Fisher Scientific, USA) in accordance with the manufacturer's guidelines. The optical density of samples was estimated by a microplate reader (BioTek Instruments, USA).

4.8.6. Measurement of plasma creatine kinase-MB and free 8-isoprostane levels

Plasma creatine kinase-MB (CK-MB) and free 8-isoprostane are considered as cardiac damage and oxidative stress markers, respectively. After 2 hours reperfusion, blood samples (1 ml) were collected in heparinized Eppendorf tubes and plasma was separated. Plasma CK-MB (Cayman Chemical, Ann Arbor, MI, USA) & free 8-isoprostane levels were measured using enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's protocol. The values of CK-MB and free 8-isoprostane were expressed as U/L and pg/ml in plasma, respectively.

4.8.7. Determination of LDH levels in diabetic rats and primary rat cardiomyocytes

4.8.7.1. Procedure for *in vitro* experiments:

A. After completion of the HR protocol, all plates containing primary cardiomyocytes were brought out from the culture room to the workplace. Kept all the plates on the ice-box.

B. Collected 0.5 ml of medium from all the plates into centrifuge tubes, and centrifuged at 13200 rpm at 4°C for 15 min.

C. Collected supernatant and discarded the pellet. Added 0.46 µl of each sample to 96 well plate.

D. Added 0.45 µl of Cdk2 and 0.1 µl of Cdk2 to the previously added culture medium in the 96 well plates.

E. Covered the 96 well plates with aluminium foil and kept on a Rocking Shaker at a speed of 20-25 for 30 min.

F. Later, it was analysed for the cytotoxicity using MRX microplate reader. LDH release was expressed as the percentage of total cell LDH activity. All readings were measured in duplicate

4.8.7.2. Procedure for *in vivo* experiments

A. Collected blood from experimental groups and separated plasma.

B. Added 0.45 µl of Cdk2 and 0.1 µl of Cdk2 to the 96 well plates and added 0.46 µl of plasma.

C. Covered the 96 well plates with aluminium foil and kept it on a Rocking Shaker at a speed of 20-25 for 30 min.

D. Later, it was analysed for the cytotoxicity using MRX microplate reader. LDH release was expressed as the percentage of total cell LDH activity. All readings were measured in duplicate

4.8.8. Determination of cardiac cell viability

Cardiac cell viability was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay.

Procedure:

A. Coated the 96-well plate using Matrigel matrix and seeded the cardiomyocytes using planting medium inside the hood. Kept in the incubator for 1 hour.
B. Switched to the culture medium and incubated at 37°C (overnight or longer).
C. Designed and labelled the sample and control well in the plate.
D. After HR treatment, by leaning the plate, sucked away the medium with a fine tip plastic pipette carefully (don’t touch the cells).
E. **Preparation of MTT stock solution (5 mg/ml):** Dissolved 75 mg of MTT powder in 15 ml of 1× PBS (filtered solution). The stock solution is ten times concentrated, so added 10 µl of stock into 100 µl of culture medium to get a final concentration of 0.5 mg/ml.
F. Added 100 µl of MTT (0.5 mg/ml) solution into each well and covered with aluminium foil to protect from light.
G. Incubated the plate for 2-4 hours at 37°C in the incubator.
H. Centrifuged the 96 well plates for 2 min and sucked out the MTT solution with a fine tip carefully (see that purple crystals are not discarded).

I. Added 100 µl DMSO into each well and shook the plate for around 15 min at room temperature until the purple crystals dissolved to form a homogenous purple solution.

J. The absorbance of blue formazan derivative was calculated at 570 nm via microplate reader (Bio-Rad Laboratories, CA, USA) and duplicated the values.

K. Measured the cell viability by using following equation:

% cell viability = (OD sample / OD control) * 100

4.8.9. Determination of apoptotic cell death in diabetic IR hearts and primary rat cardiomyocytes

Terminal deoxynucleotidyl nick-end labelling (TUNEL) assay was employed to detect myocardial apoptotic cell death according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN, USA).

4.8.9.1. For adherent cells/*in vitro* studies:

A. Placed the sterilized glass coverslip in the culture plates.

B. Coated them as usual with the Matrigel matrix and incubated with cardiomyocyte cell suspension.

C. After HR treatment, removed culture medium; washed the wells (having coverslips with adherent cells) with 2 ml of 1× PBS, once.

D. **Fixation:** Added 2 ml of ice-cold acetone to each well and kept aside for 10 min.

E. Washed again with 2 ml of 1× PBS once.

F. **Preparation of 0.1% Triton X-100:** Added 50 µl of TritonX-100 to 50 ml of 1× PBS.

G. **Permeabilisation:** Added 1 ml of 0.1% Triton X-100 dissolved in PBS to each well and kept aside for 5 min. Washed with 1× PBS again.

H. **Light sensitive step:** Added 30 µl of TUNEL reaction mixture to each coverslip and incubated for 1 hour at 37°C in a humidified atmosphere in the dark place (black box). Later, washed with 1× PBS for three times, 5 min each.

I. Added 5 to 10 µl of DAPI mounting medium to coverslip (slide) and placed another coverslip over it, so that cells in close contact with DAPI. Incubated for 2 min at room temperature.

J. Observed under the fluorescence microscope to visualize TUNEL-apoptotic cells.

K. The percentage of apoptotic cardiomyocytes or apoptotic index was calculated by the ratio of the number of TUNEL-stained cardiomyocytes to the total number of DAPI-stained cardiomyocytes in a given field of observation.

4.8.9.2. For *in vivo* studies:

A. Deparaffinized the 5-mm thick paraffin embedded left ventricular tissue sections.

B. Washed with 1× PBS for two times.

C. Permeabilized with protease K (20 µg/ml) for 30 min at 37°C.

D. Washe with 1× PBS, two times.

E. Added 30 µl of TUNEL Reaction Mix, incubated at 37°C for 30 min

F. Washed with 1× PBS, three times.

G. Added 5 to 10 µl of DAPI mounting medium to coverslip (slide) and placed another coverslip over it, so that cells in close contact with DAPI. Incubated for 2 min at room temperature.

H. Observed under the fluorescence microscope to visualize TUNEL-apoptotic cells.

4.8.10. Determination of reactive oxygen species (ROS)

ROS production was measured based on the oxidation of dihydroethidium (DHE, a superoxide indicator) to ethidium. After HR treatment, cells were incubated with 10 µM DHE in a six-well plate, for 30 min at 37°C in the dark. Oxidized DHE intercalates within the cell's DNA and stains the nucleus a bright fluorescent red. Images were acquired using a fluorescence microscope (Olympus IX51).

4.8.11. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Procedure:

A. **RNA isolation**

i. **Homogenizing samples:** Weighed 0.2 g of cardiac tissue and added 1 ml of TRIzol reagent and homogenated using a power homogenizer.

ii. Kept the cardiac homogenate at room temperature for 5 min. and centrifuged at 12,000 × *g* for 10 min at 4°C (Note: The resulting pellet contains polysaccharides, extracellular matrix, and high molecular weight DNA, while supernatant includes the RNA).

iii. Transferred the cleared supernatant to a new autoclaved centrifuge tube.

iv. **Phase separation:** Added 0.2 ml of chloroform per 1 ml of TRIzol used for homogenization and vortexed vigorously for 15 seconds

v. Kept at room temperature for 5 min and again centrifuged at 12,000 × *g* for 15 min at 4°C (Note: The mixture separated into a lower red phenol-chloroform phase, interphase, and a colourless upper aqueous phase. RNA present only in upper aqueous phase).

vi. Transferred the upper layer by angling the tube at 45° to a new autoclaved centrifuge

vii. **RNA isolation:** Added 0.5-1.0 ml of100% isopropanol per 1 ml of TRIzol and kept at room temperature for 10 min. Centrifuged at 12,000 × *g* for 10 min at 4°C (pellet on the side and bottom of the tube).

viii. **RNA wash:** Discarded the supernatant and washed the pellet with 1 ml of 75% ethanol and vortexed briefly and centrifuged at 7,500 × *g* for 5 min at 4°C.

ix. Discarded the supernatant and collect precipitate and air dry.

x. **RNA resuspension:** Dissolved RNA pellet with the appropriate amount (20-50 µl) of DEPC-treated water (RNase-free water) by passing the solution up and down several times using a pipette.

xi. Incubated in the heat block set at 60°C for 10 min.

xii. **Determining RNA yield:** Measured absorbance at 260 nm using nanodrop instrument ($A_{260/280}$ of >1.8).

B. **Complementary DNA (cDNA) synthesis**

i. Calculated RNA volume and added 2 µl of 5* Prime Script RT master mix.

ii. Set temperatures: Stage I: 37°C for 15 min; Stage II: 80°C for 0.05 seconds; Stage III: 4°C for infinity (∞) in Thermal Cycler.

iii. Later cDNA was diluted with 150 µl of RNase-free water to increase volume.

C. **Polymerase chain reaction (PCR)**

i. Prepared 20 µl of PCR mixture according to the **Table 4.11** in PCR running plate (Note: Start the PCR at least 1.30 hr before preparation of PCR sample).

ii. Set temperatures: Stage I (initial denaturation): 95°C for 30 seconds; Stage II (proliferation): 95°C for 5 seconds; Stage III (annealing): 60°C for 30 seconds for 40 cycles in Step One Plus Real-Time PCR system.

iii. Later analysed the PCR data and was normalised to the housekeeping gene GAPDH. The sequence of primers were listed in **Table 4.12**.

Table 4.11: Preparation of PCR mixture

S.No.	Components	Volume
1	SYBR Premix Ex TaqII	10 µl
2	PCR forward primer	0.8 µl
3	PCR reverse primer	0.8 µl
4	Template	2 µl
5	dH_2O (DEPC-water)	6.4 µl
Total volume		20 µl

Table 4.12: List of primer sequence

Target gene	Primer sequence		Gene bank accession number
Nrf2	Forward	5′-GATTCGTGCACAGCAGCA-3′	XM006234397.2
	Reverse	5′-GCCAGCTGAACTCCTTAGAC-3′	
HO-1	Forward	5′-CGTGCAGAGAATTCTGAGTTC-3′	NM012580
	Reverse	5′-AGACGCTTTACGTAGTGCTG-3′	
NF-κB	Forward	5′ -GGGTCAGAGGCCAATAGAGA-3′	AF079314.1
	Reverse	5′ -CCTAGCTTTCTCTGAACTGCAAA-3′	
PGC-1α	Forward	5′ -AGGTCCCCAGGCAGTAGAT-3′	AY237127
	Reverse	5′ -CGTGCTCATTGGCTTCATA-3′	
TLR4	Forward	5′ -GTGGGTCAAGGACCAGAAAA-3′	NM019178
	Reverse	5′ -GAAACTGCCATGTCTGAGCA-3′	
NLRP3	Forward	5′ -GCTGCTCAGCTCTGACCTCT-3′	NM001191642.1
	Reverse	5′ -AGGTGAGGCTGCAGTTGTCT-3′	
ASC	Forward	5′ -TTATGGAAGAGTCTGGAGCTGTGG-3′	NM012762.2
	Reverse	5′ -AATGAGTGCTTGCCTGTGTTGG-3′	
GAPDH	Forward	5′ -TGATGACATCAAGAAGGTGGTGAAG-3′	NM017008.4
	Reverse	5′ -TCCTTGGAGGCCATGTGGGCCAT-3′	

4.8.12. Western blotting

A. **Preparation of lysate from cell culture**

i. Placed the cell culture dish on ice and aspirated the media and washed the cells with ice-cold 1×PBS.

ii. Aspirated the PBS, then added 100 µl of ice-cold lysis buffer (per well of 6 well plate or 500 µl for 10 cm diameter plate).

iii. Immediately scraped the cells off the dish using a sharp plastic cell scraper, and transferred the extract to a pre-cooled microcentrifuge tube. Kept the tubes on ice for 30 min.

iv. Centrifuged the tubes at maximum speed, i.e. 16,000 × *g* in a microcentrifuge for 15 min at 4°C.

v. Collected the supernatant and transferred to a pre-cooled centrifuge tube on ice.

B. **Preparation of lysate from tissues**

i. Weighed 50 mg of cardiac tissue, cut into small pieces and placed in a microcentrifuge tube.

ii. Added appropriate volume of ice-cold lysis buffer and homogenized with an electric homogenizer. Volumes of lysis buffer must be determined to the amount of tissue present; protein extract should not be too dilute to avoid loss of protein and large quantities of samples to be loaded onto gels (optimal concentration of protein is 1–5 mg/mL).

iii. Centrifuged the tubes at maximum speed, i.e. 16,000 × *g* in a microcentrifuge for 15 min at 4°C.

iv. Collected the supernatant and transferred to a pre-cooled centrifuge tube on ice.

C. **Denature the protein sample with loading buffer**

i. Determined the protein concentration of tissue or cell lysates samples by Coomassie Brilliant Blue staining.

ii. Next, equilibrated the sample volume according to the protein concentration. Then added 4* Loading buffer to each sample by a third of the sample volume.

iii. Heated each cell lysates at 95-100°C for 10 min and placed on ice for 5min.

iv. Stored the samples at -20°C for future use.

D. **Protein electrophoresis and Blotting**

i. Loaded 30 µg of protein into each lane of the SDS-PAGE gel, along with molecular weight marker.

ii. Run the protein on the SDS-PAGE gel with Tris-glycine-SDS running buffer (**Table 4.15**). At the beginning, run the gel at the voltage of 80V when the protein sample is running on stacking gel (upper gel; **Table 4.13; 4.14**), and then, when the protein sample run into separation gel (lower gel; **Table 4.13; 4.14**), adjusted the voltage to 110V to 130 V to speed up the running process.

iii. Transferred the protein to nitrocellulose membrane using transfer buffer (**Table 4.16**), at voltage adjusted to 110V for 2 hr or overnight.

E. **Blocking**

i. After transfer process, washed the nitrocellulose membrane with ethanol for 5 min at room temperature.

ii. Incubated the membrane in blocking buffer (5% skimmed milk in TBST or 5% BSA in TBST; **Table 4.17**) for 1 hr at room temperature.

iii. Washed three times with TBST (**Table 4.18**), each for 5 min.

F. **Primary antibody incubation**

i. Incubated membrane with primary antibody (at the appropriate dilution as recommended in the product datasheet) in 3 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.

ii. Washed three times for 10 min each with 15 ml of TBST (the washing times and duration can be adjusted according to your specific experiment).

iii. Incubated membrane with Anti-rabbit IgG, HRP-linked antibody or anti-mouse, HRP-linked antibody to detect primary antibody conjugated protein (targeted protein) in 3 ml of TBST with gentle agitation for 1 hr at room temperature.

iv. Washed three times for 10 min each with 15 ml of TBST (the washing times and duration can be adjusted according to your specific experiment).

G. **Detection of proteins**

i. Prepared ECL reagent.

ii. Incubated substrate with membrane for 1 minute, removed excess solution (membrane remains wet), wrapped in plastic and exposed to X-ray film.

Table 4.13: Preparation of 1.0 mm gel (for 2 gels)

Components	Lower Buffer pH 8.8 (Separation gel)			Upper Buffer pH 6.8 (Stacking gel)
	8%	10%	12.5%	
30% Acr/Bis	2.72 ml	3.4 ml	4 ml	0.5 ml
1.5M gel buffer	2.5 ml	2.5 ml	2.5 ml	1.0 ml (0.5 M)
H_2O	4.68 ml	4 ml	3.4 ml	2.4 ml
10% SDS	130 µl	130 µl	130 µl	50 µl
10% APS	60 µl	60 µl	60 µl	40 µl
TEMED	10 µl	10 µl	10 µl	8 µl

Table 4.14: Preparation of 1.5 mm gel (for 2 gels)

Components	Lower Buffer pH 8.8 (Separation gel)			Upper Buffer pH 6.8 (Stacking gel)
	8%	10%	12.5%	
30% Acr/Bis	4.896 ml	7.65 ml	7.2 ml	0.9 ml
1.5M gel buffer	4.5 ml	5.625 ml	4.5 ml	1.8 ml (0.5 M)
H_2O	8.424 ml	9 ml	6.12 ml	4.32 ml
10% SDS	234 µl	292.5 µl	234 µl	90 µl
10% APS	108 µl	135 µl	108 µl	54 µl
TEMED	14.4 µl	18 µl	14.4 µl	10.8 µl

Table 4.15: Preparation of SDS-PAGE Running buffer (10x) (1 L, pH 8.3)

Tris base (250 mM)	30.30 g
Glycine (1.92 M)	144.10 g
SDS (1%)	10.00 g
Distilled H_2O	1 L
Do not adjust pH with acid or base.	

Table 4.16: Preparation of Transfer buffer (10x) (1 L, pH 8.3)

Tris base (250 mM)	30.30 g
Glycine (1.92 M)	144.10 g
Distilled H_2O	1 L
Do not adjust pH with acid or base. Before use, add 20% (v/v) methanol to the working buffer. To prepare 1X buffer: 100 ml 10X transfer buffer+700 ml distilled H_2O+200 ml Methanol	

Table 4.17: Preparation of Blocking buffer (20 ml)

BSA/non-fat dry milk (5%)	1 g
TBS	20 ml

Table 4.18: Preparation of Washing buffer (1 L)

0.1% Tween	1 ml
TBS	1 L

4.9 Statistical analysis

All experimental values were represented as the mean ± standard deviation (SD) and mean ± standard error mean (SEM). Graph Pad Prism software, version 5.0 (GraphPad Software, San Diego, CA) was used for statistical analysis. One way ANOVA followed by Tukey's multiple comparison post hoc tests and Repeated measures Two-way ANOVA followed by Bonferroni post-test were employed to find the statistical relevance of the data and significance was set at $P \leq 0.05$.