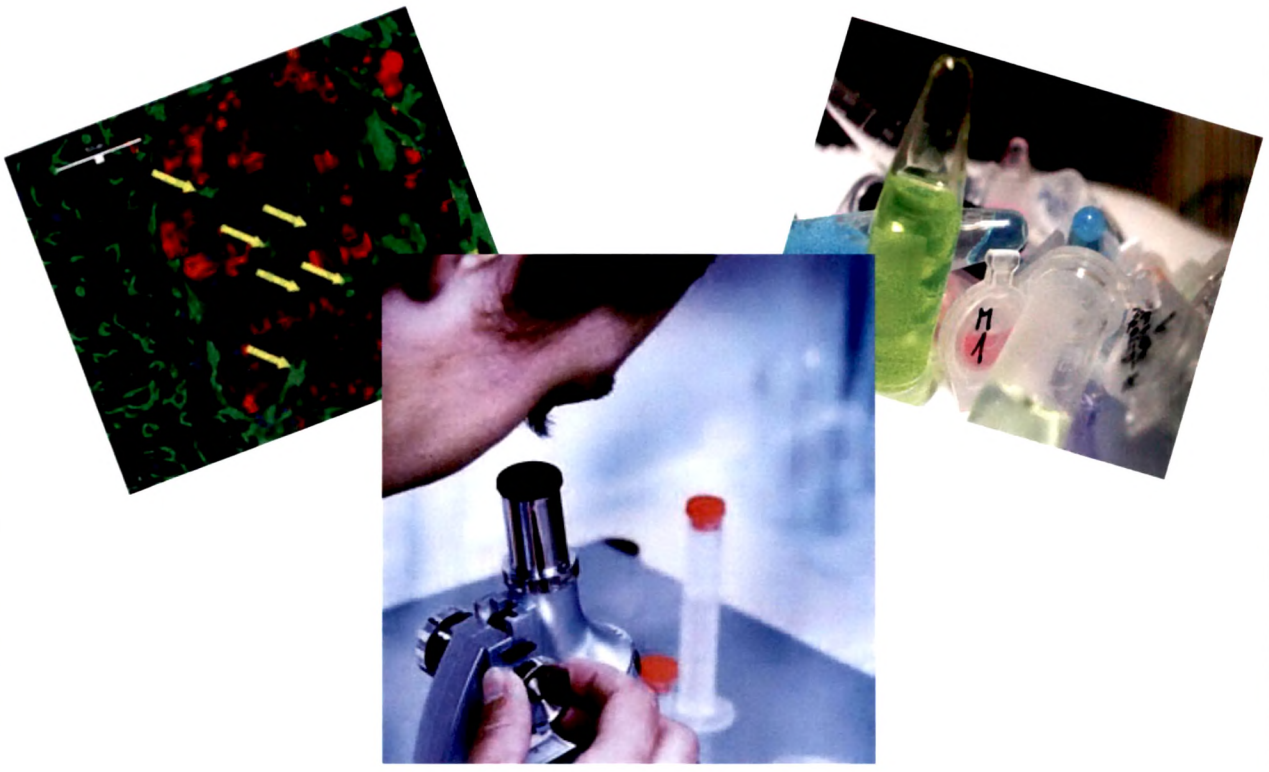
Chapter-2

Material and Methods



2.1. Chemicals

Alloxan monohydrate, Collagenase (Type XI) were procured from Sigma Chemical Co., U.S.A. and all other chemicals were of AR grade and procured from Sisco Research Laboratories Pvt. Ltd., Mumbai, India, Qualigens Fine Chemicals, Mumbai, India, Suvidhanath Laboratories, Vadodara, India and Spectrochem Pvt. Ltd., Mumbai, India. Dulbecco's Modified Eagle's Medium (DMEM), and Caspase 3 Assay Kit, Colorimetric were purchased from Sigma Co., U.S.A. Kits for serum total cholesterol, triglycerides, HDL-cholesterol and GOD-POD kits for blood glucose estimation were purchased from Monozyme India Pvt. Ltd., India and Angstrom Biotech Ltd., India. RIA kits for serum insulin estimation were procured form Board of Radio Isotope and Technology (BRIT), Mumbai, India.

2.2. Animals

Adult virgin male rats of the Charles-Foster strain weighing 250-350 g kept under controlled conditions of light (lights on from 07.00 to 20.00 h) and temperature (24 ± 2°C) and having access to food and water were used. The experimental studies were performed after the approval from the Animal Ethical Committee of Department of Biochemistry, The M.S. University of Baroda and were in accordance with the rules of ethical committee.

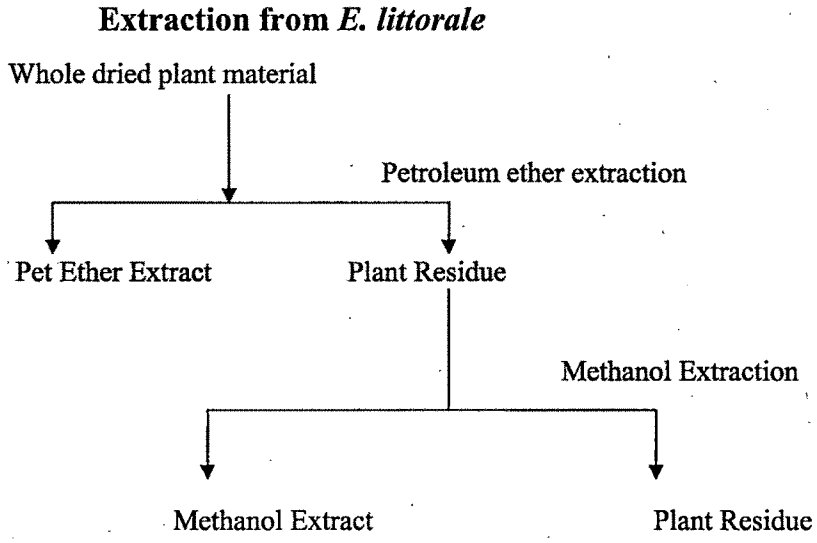
2.2.1 Induction of diabetes in animals

Male Charles foster rats (body weight 250 - 350g) were used for the study. Alloxan (120 mg/kg body weight, i.p.) was used to induce diabetes. Alloxan monohydrate, a diabetogen, specifically destroys β-cells by generating free radicals and produces diabetes like condition in the rats. After alloxan treatment animals were kept for 15 days to stabilize the diabetic condition and those animals showing blood glucose levels more than 200 mg/dl were considered as diabetic and used for the experiments.

2.3. Extract preparation of *Enicostemma littorale*

2.3.1. Methanolic extract

Authentic plant material was purchased from local market and identified at the Botany Department, M.S. University, Baroda, Voucher specimen [Oza 51,51 (a)] is present at the Herbarium of Botany Department, M.S. University, Baroda.



Whole dried plant was used for extract preparation. The plant was powdered in grinding meal. The powder was extracted with petroleum ether for 12 h in a Soxhlet apparatus. Residues were again extracted with methanol for 24 h. After the extraction, methanol was recovered by distillation and remaining traces of methanol were completely removed by keeping methanol extract at 60°C for 4 days. The yield was found to be 40% and carboxymethylcellulose solution was used as vehicle.

2.3.2 Aqueous extract:

Whole dried plant was used for extract preparation. Plant was cut into small pieces, soaked into water for 2 hours and boiled twice in water for 30 min. Residues

were removed by filtration and the combined filtrate was evaporated to obtain a desired concentration (1 g dry plant equivalent extract / ml).

2.4. Glycemic Parameters

2.4.1. Plasma glucose (Trinder et al, 1969)

Glucose is an important source of energy. Glucose concentration fluctuates only in narrow range as insulin and its counter regulatory hormones in the body maintain glucose homeostasis.

Principle: This is an enzymatic method for estimation of serum glucose levels. The aldehyde group of glucose is oxidized by the enzyme glucose oxidase (GOD) in the presence of oxygen (air) to gluconic acid with the liberation of hydrogen peroxide (H_2O_2). Peroxidase splits H_2O_2 into H_2O and active oxygen, which reacts with phenol and a chromogen 4-amino antipyrene to form a pink coloured complex, which can be estimated colorimetrically at 505 nm.

$$\text{D-Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{4 amino antipyrine} \xrightarrow{\text{POD}} \text{Red coloured quinol} + H_2O + \text{Phenol}$$

Reagents – commercially available kits contain following reagents

Enzymes (glucose oxidase - peroxidase) with chromogen and phenol

Glucose standard (100mg/dl) (Range of standard –10 –50 μg)

Procedure

Reagents	Blank	Standard	Test
Enzyme reagent	1.0 ml	1.0 ml	1.0 ml
Glucose standard	-	0.01ml	-
Plasma	-	-	0.01 ml

All the tubes were incubated for 15min at RT or for 10min at 37°C and then absorbance were read at 505 nm against blank.

Calculation - Calculation was done according to the slope calculated from the standard graph.

Units - mg/dl

2.4.2. Glycosylated Haemoglobin (Parker et al, 1981)

Protein can universally bind non-enzymatically with glucose or other sugars present in the vicinity. The degree of glycation is directly proportional to the concentration of the sugar present in the surrounding medium. RBC has longer life span (120 days) as compared to other proteins like albumin (4 days). Therefore estimation of Glycosylated Haemoglobin (Gly Hb) gives an accurate reflection of mean plasma glucose concentration over this period.

Principle: This method is specific for ketoamine-linked hexoses which form furfural when heated under strong acidic condition. This furfural reacts with 2-thiobarbituric acid (TBA) and produces a bright yellow coloured compound, which can be estimated colorimetrically at 443 nm.

Reagents

0.5 M Oxalic acid (Stable for 2 weeks at RT)

0.72g % Thiobarbituric Acid (TBA) (pH 6.0, stable for 1 weeks at RT)

Saline (0.9g % NaCl)

40 % Trichloroacetic acid (TCA)

Drabkin's reagent (pH 9.1) - 0.2g Potassium Ferricyanide [$K_3Fe(CN)_6$], 0.05g Potassium Cyanide (KCN), 0.14g Potassium dihydrogen phosphate (KH_2PO_4) in one litre of distilled water.

Hb standard solution (60 mg/dl or 65 mg/dl) was procured from market. Fructose standard 0.2 µM (Range of the standard - 0.02 - 0.12 µM)

Procedure

Haemolysate preparation -RBC sediments were washed three times with 0.9% saline. Then packed cells were lysed by adding equal amount of distilled water and ¼ part CCl_4. Mixed well and centrifuged at 3000 rpm for 10 min. Haemoglobin (Hb) was estimated with Drabkin's reagent and adjusted to 10g Hb/dl.

Reagents	Control	Test
Haemolysate	-	1ml
D/W	1ml	-
oxalic acid	0.5 ml	0.5ml
Keep in boiling waterbath for 1 hr		
chilled TCA	1ml	1ml
Centrifuge the tubes at 2000 rpm for 15min		
Supernatant	1ml	1ml
TBA	0.1ml	0.1ml
Incubated in water bath at 40°C for 30 min and read the absorbance at 443nm against the sample blank		

Calculation - Calculation was done according to the slope calculated from the standard graph.

Units - % of total Hb

2.4.3. Oral Glucose Tolerance Test (OGTT) (Al-Awadi et al, 1985)

OGTT should be administered in morning after 12 hr fasting. After that fasting blood sample was collected, and then glucose load (3 gm/Kg body wt) was given orally to rats. After glucose load, blood was collected at 30, 60 and 120 minutes. Unless the glucose concentration can be determined immediately, blood sample should be collected in containing sodium fluoride (Na F) (6mg/ml) and centrifuged to separate out the plasma.

Glucose was estimated from plasma by GOD-POD method.

2.4.4. Insulin estimation (RIA method)

Insulin is a polypeptide hormone, synthesized by pancreatic β-cells. It regulates glucose metabolism and maintain glucose homeostasis in the body.

Principle:

Radio immuno assay (RIA) is based upon the competition between unlabelled insulin in the standard or serum sample and radiolabelled (I^{125}) insulin for the limited binding sites on a specific antibody. At the end of the incubation, the antibody bound and free insulin are separated by the second antibody-polyethylene glycol (PEG) aided separation method. Insulin concentration (I^{125}) of the sample is quantitated by measuring the radioactivity (counts/minute, CPM) associated with bound fraction of sample and standards with help of gamma counter.

Reagents

Radiolabelled (I^{125}) insulin
Insulin free serum
Primary antibodies
Secondary antibodies
Polyethylene glycol (PEG)
Insulin standard
(Standard range -12.5 - 200 μU)

All these reagents are available with RIA kit in the form of dry powder. These were reconstituted with double distilled water or assay buffer as described in the kit brochure.

Procedure

Reagents	Total Counts	Nonspecific binding	Zero binding	Standard	Test
Assay buffer	-	0.4 ml	0.3ml	0.2ml	0.3ml
Insulin standard	-	-	-	0.1ml	-
Serum sample	-	-	-	-	0.1ml
Insulin free serum	-	0.1ml	0.1ml	0.1ml	-
Primary antibody	-	-	0.1ml	0.1ml	0.1ml
Mix gently and incubate all the tube at 2-4°C overnight					
(I^{125}) insulin	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
Mix gently and incubate all the tube at RT for 3hrs.					
Secondary antibody	-	0.1ml	0.1ml	0.1ml	0.1ml
PEG	1.0ml	1.0ml	1.0ml	1.0ml	1.0ml
Vortex and keep all the tubes at RT for 20 minutes then centrifuge the tubes at 1500g for 20minutes.					

Calculation

T = total counts of 100µl Insulin I^{125} only.

B0 = CPM of bound with Insulin I^{125} in absence of serum insulin or insulin standard (zero binding)

B = CPM of bound with Insulin I^{125} in presence of serum insulin or insulin standard.

% B/T and %B/B0 was calculated for insulin standards and serum samples. A logit-log graph was plotted against %B/B0 and concentration of insulin standards. Serum insulin concentration was calculated from this graph.

Unit - µU of insulin/ml of serum or ng insulin/10 islets.

2.4.5. FIRI (Fasting Insulin Resistance Index) (Harati et al., 2003)

Based on value of fasting glucose and insulin FIRI was calculated as below

$$FIRI = \frac{\text{fasting insulin} \times \text{glucose}}{2}$$

2.4.6 Aldose reductase (AR)

Aldose reductase (AR) the key enzyme of the polyol pathway, belongs to the aldo-keto reductase superfamily (Vander Jagt et al., 1990). AR has been demonstrated to play an important role not only in cataract formation in lens but also in the pathogenesis of diabetic complications such as neuropathy, nephropathy, and retinopathy. As a result of increased polyol pathway during hyperglycemia sorbitol accumulates, as it is formed more rapidly than it is converted to fructose (Brownlee, 2001). Excess intracellular sorbitol accumulation through the polyol pathway correlates with the diabetic complications. The role of polyol pathway in diabetic complications may have different mechanisms, such as; accumulation of sorbitol or fructose (Vander Jagt et al., 1990; Narayanan, 1993), myo-inositol depletion (Greene et al., 1987), or alterations in NADPH/NADP And NADH/NAD ratios (Williamson et al., 1993; Schrijvers et al., 2004). Sorbitol is also associated with myo-inositol metabolism (Greene et al., 1987).

Tissue was homogenized in 10 mM sodium phosphate buffer containing 5 mM 2-mercaptoethanol (pH 7.0) at 0°C. A cell-free extract is obtained by centrifuging the total homogenate at 17300 g for 10 min. The enzyme reaction mixture (final volume 1 mL) contains 0.4 M $(NH4)_2SO4$, 0.1 M HEPES buffer adjusted to pH 7.0 with sodium hydroxide, 10 mM DL-glyceraldehyde and 0.12 mM NADPH. A 20 µL aliquot of supernatant is added to initiate the reaction. Decrease in absorbance at 340 nm is followed spectrophotometrically. Enzymatic activity is expressed as n moles of NADPH oxidized $min^{-1}mg^{-1}$ protein.

2.5. Lipid Parameters

2.5.1. Serum Total Cholesterol & HDL cholesterol (Kit Method) (Allain et al., 1974)

Serum cholesterol level is an indicator of liver function, biliary function, intestinal absorption, propensity towards coronary artery disease & adrenal disease. Decreased HDL cholesterol levels associated with increased risk of developing coronary artery disease and other atherosclerotic complications.

Principle

$$\text{Cholesterol ester} \xrightarrow{\text{CHE}} \text{Cholesterol} + \text{Free fatty acids} + H_2O$$

$$\text{Cholesterol} \xrightarrow{\text{CHOD}} \text{Cholest} - 4 \text{ ene } 3 - \text{one} + H_2O_2 + O_2$$

$$H_2O_2 + \text{Phenol} \xrightarrow{\text{POD}} \text{Red Quinoneimine Complex} + 4 - \text{Aminoantipyrine} + H_2O$$

The colored complex is read at 505 nm against blank.

HDL Cholesterol: On addition of the precipitating reagent, which contains phosphotungstic acid, to the serum, followed by centrifugation, HDL fraction remains in the supernatant while the other lipoproteins precipitate out.

Mix and allow standing for 5 minutes at R.T. Centrifuge at 3000 rpm for 10 minutes to get a clear supernatant.

Pipette into 4 test tubes labeled Blank (B), standard (S), Total Cholesterol (TC) and HDL cholesterol (TH) as shown below:

	B	S	TC	TH
Enzyme Reagent (1)	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Cholesterol standard (3)	-	0.01 ml	-	-
Specimen	-	-	0.01 ml	-
Supernatant (from Step 1)	-	-	-	0.1 ml
D/W	0.1 ml	0.1 ml	0.1 ml	-

Mix well and incubate for 10 minutes at R.T. Read the absorbance of S, TC, TH against B at 505 nm.

Calculation

$$\text{Total cholesterol (in mg/dl)} = \frac{\text{O.D. of Test}}{\text{O.D. of Std.}} \times 200$$

$$\text{HDL Cholesterol (in mg/dl)} = \frac{\text{O.D. of Test}}{\text{O.D. of Std.}} \times 50$$

2.5.2. Serum Triglycerides (kit method) (Fossati and Prencipe, 1982)

Principle: The TG is determined after enzymatic hydrolysis with lipases. Peroxidase catalyses the conversion of hydrogen peroxide, 4-Aminoantipyrine and ESPAS to a colored quinoneimine complex measurable at 546 nm.

$$TG \xrightarrow{\text{Lipases}} \text{glycerol + fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{glycerol 3 phosphate (G 3 P)}$$

$$\text{G 3 P} + O_2 \xrightarrow{\text{GPO}} H_2O_2 + \text{dihydroxy acetone phosphate}$$

$$H_2O_2 + \text{4 amino antipyrine} + \text{ESPAS (N-ethyl N-sulfoprophyl} \xrightarrow{\text{POD}} \text{purple quinoneimine complex} + H_2O + \text{N-anisidene)} + HCl$$

Reagents

Reagent 1: Enzyme reagent (Enzyme chromogen)

Reagent 2: Triglycerides standard (200 mg/dl)

Procedure:

Enzyme		Standard	Test
Reagent 1	1.0 ml	1.0 ml	1.0 ml
Standard	--	0.01 ml	--
Sample	--	--	0.01 ml
Mix well and incubate for 10 min. at 37 °C for 15 min at R.T.			
Read at 546 nm. (Final color is brownish purple and stable for 30 min at R.T.)			

Calculation: Serum triglycerides (mg/dl) = $\frac{\text{Abs. Of Test x Conc. of Std}}{\text{Absorbance of Standard}}$

LDL cholesterol = (Total Cholesterol – HDL cholesterol – TG/5)

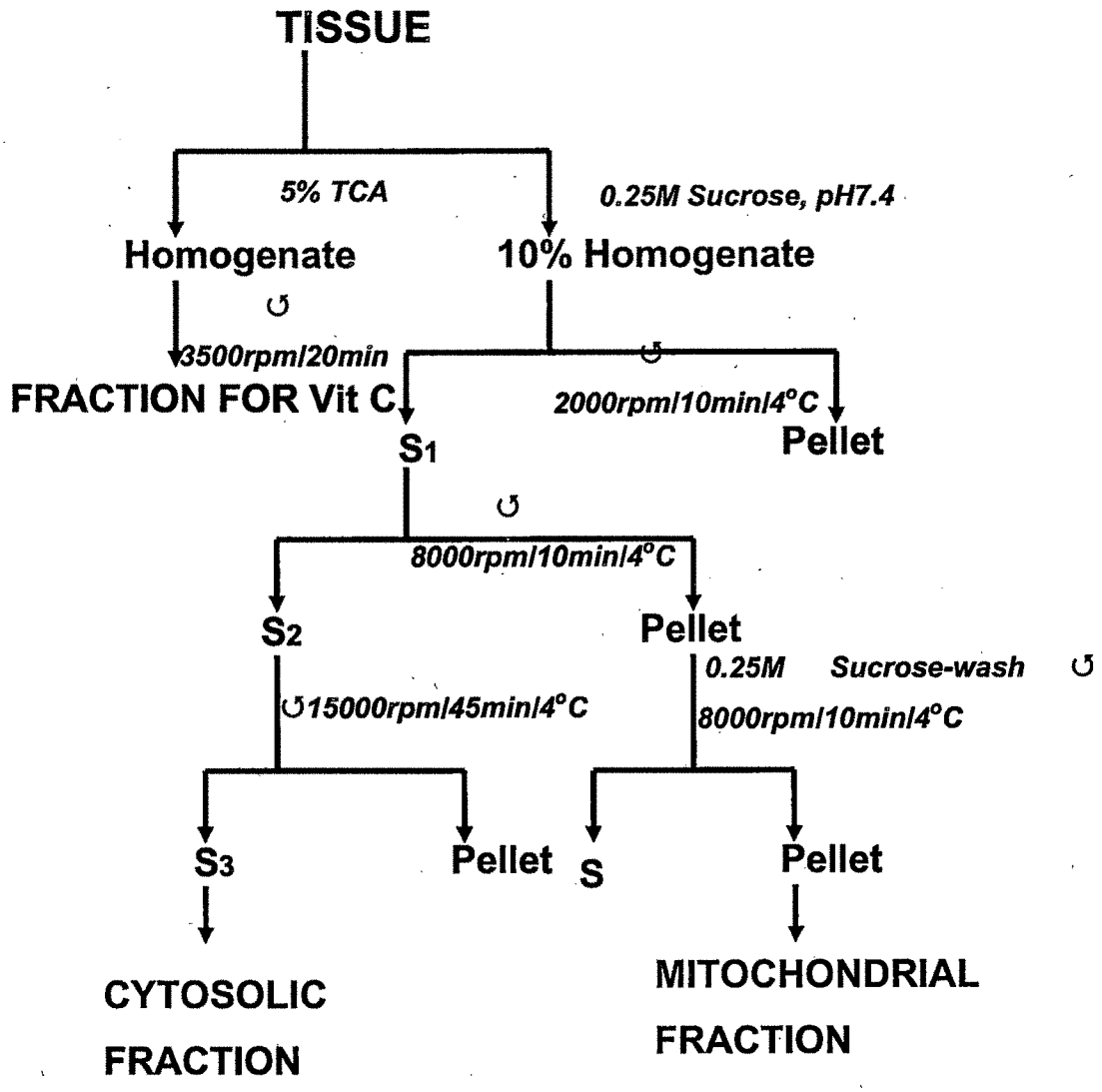
VLDL = TG/5

2.6. Preparation of mitochondrial and post-mitochondrial fraction

The tissue samples were dissected out from the animals in ice cold conditions (4°C) and brought immediately to lab for further processing according to the following protocol:

The cytosolic and mitochondrial fractions obtained were subjected to biochemical estimations/analysis of our interest.

Preparation of mitochondrial and post-mitochondrial fraction



2.7. Antioxidant parameters

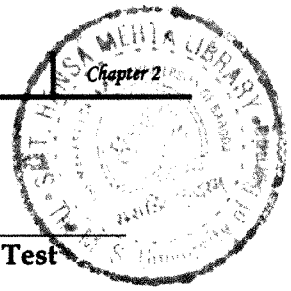
Tissues (kidney, heart, testis, and sciatic nerve) will weighed, 10% homogenized in chilled (4°C) isolation medium: 0.25 M sucrose, 10 mM Tris-HCl buffer pH 7.4, 1 mM EDTA and 250 μg BSA/ml. The isolation of mitochondria and post-mitochondrial fractions will according to the procedures describe (Swegert et al., 1999; Kaushal et al., 1999). The mitochondrial and post-mitochondrial fractions obtained will be subjected to biochemical estimations/analysis of reactive oxygen species related parameter. ROS parameters include **(a)** Reduced Glutathione **(b)** Lipid Peroxidation Levels **(c)** Superoxide Dismutase **(d)** Catalase **(e)** Glutathione Peroxidase. The activity of Acid phosphatase (ACP) has been estimated in post-mitochondrial fraction. Protein estimation will be carried out to express enzyme activity in terms of specific activity (Lowry et al., 1951).

2.7.1. Reduced Glutathione (GSH) (Beutler and Gelbart, 1985)

Principle: 5-5′ Dithiobis (2 nitrobenzoic) acid (DTNB) is a disulfide compound which is readily reduced by sulfhydryl compounds forming a highly colored yellow anion, which can be read at 412 nm.

Reagents

1. Precipitating (pipeting) reagent: 1.67g Metaphosphoric acid (MPA), 0.20g EDTA, 30g NaCl, make volume to 100ml with D/W)
2. 0.3M Na_2HPO_4.
3. DTNB: 40 mg DTNB dissolved in 100 ml 1% sodium citrate (freshly prepared).
4. PBS (0.1M, pH 7.4).
5. Standard GSH solution: 2mM GSH. (Standard range 10-100μg)



Protocol

Reagents	Reagent Blank	Substrate Blank	Enzyme Blank	Test
MPA (ml)	1.0	1.0	1.0	1.0
Aliquot (cyto/mito)	-	-	0.1	0.1
D/W (ml)	0.1	0.1	0.03	-
Keep in ice for 10', ↺3000 rpm/15', take supernatant				
Supernatant (ml)	0.4	0.4	0.4	0.4
Na_2HPO_4 (ml)	0.6	0.6	0.6	0.6
DTNB (ml)	-	0.03	-	0.03
Immediately take O.D. $_{412}$				

Unit : pmoles / mg protein

2.7.2. Lipid Peroxidation Levels (LPO) (Ohkawa and Ohishi, 1979)

Principle : Lipid peroxidation leads to the formation of an endoperoxide i.e. malondialdehyde (MDA) which reacts with thiobarbituric acid (TBA) and gives thiobarbituric reactive substance (TBARS). TBARS gives a characteristic pink color which can be measured colorimetrically at 532 nm.

Reagents

1. TBA reagent: TBA (100mg), EDTA (46mg), 20%TCA (10ml), 2.5 N HCl (5 ml) total volume was made up to 20ml with D/W (freshly prepared) .
2. 0.1 M Phosphate buffered saline (PBS, pH 7.4)
3. 10 mM Tetra methoxy propen (TMP) for standard solution. Standard rang (110 nmoles)

Procedure

Reagents	Blank	Test
Aliquot (cyto/mito) (ml)	-	0.1
D/W (ml)	1.0	0.9
TBA reagent (ml)	1.0	0.9
Keep in boiling water bath for 20', afterwards cool tubes, ↻3000 rpm/15', take supernatant & measure O.D. $_{532}$		

Unit : nmoles of MDA formed / mg protein

2.7.3. Superoxide Dismutase (SOD) (Marklund and Marklund, 1974)

Principle: The superoxide dismutase activity inhibits pyrogallol auto-oxidation under standard assay conditions, the reaction being measured at 420 nm.

Reagents

1. KPO_4 (0.2M, pH 8) (K_2HPo_4 +K_2HPO_4)
2. Pyrogallol : a pinch of pyrogallol is dissolved in 0.5N HCl, so as to achieve auto-oxidation within 0.1 to 0.120 O.D./180 sec (freshly prepared)

Protocol

Pyrogallol auto-oxidation: 0.5ml buffer + 0.450 ml D/W (autozero) + 0.050 ml pyrogallol (start)

Auto-oxidation should be between 0.1 to 0.120 O.D. /180 secs

Now, proceed as per protocol, reaction starts by addition of pyrogallol.

Reagents	Blank	Test
Aliquot (cytosol/mitochondria) (ml)	-	0.03
KPO_4 (ml)	0.5	0.5
D/W (ml)	0.45	0.42
Pyrogallol (ml)	0.05	0.05
Measure absorbance at O.D. $_{420}$		

Unit: Amount of enzyme required for 50% inhibition of pyrogallol auto-oxidation

2.7.4. Catalase (CAT) (Hugo, 1987)

Principle: Catalase is a heme containing enzyme which catalyzes dismutation of hydrogen peroxide into water and oxygen. Decomposition of hydrogen peroxide by catalase is measured spectrophotometrically at 240 nm, since hydrogen peroxide absorbs UV light maximally at this wavelength.

$$H_2O_2 \xrightarrow{CAT} H_2O + O_2$$

Reagents

1. KPO_4 (50mM, pH 7)
2. H_2O_2 (10.3mM) (freshly prepared)

Protocol

Blank : KPO_4 buffer + D/W (autozero) + H_2O_2 (start)

Test : KPO_4 buffer + D/W + Aliquot (autozero) + H_2O_2 (start)

Reagents	Blank	Test
Aliquot (cyto) (ml)	-	0.03
KPO_4 (ml)	0.5	0.5
D/W (ml)	0.45	0.42
H_2O_2 (ml)	0.05	0.05
Measure absorbance at O.D. $_{240}$		

Unit : mmoles of H_2O_2 decomposed/mg protein

2.7.5. Glutathione Peroxidase (GPx) (Hafeman et al., 1994)

Principle : Glutathione peroxidase leads to the H_2O_2 mediated oxidation of reduced glutathione, which reacts with 5-5′ Dithiobis (2-nitrobenzoic) acid (DTNB) to give yellow colour measurable at 412 nm.

Reagents

1. KPO_4 (0.4M, pH 7)
2. 30 mM Metaphosphoric acid (MPA)
3. Glutathione (2mM) (freshly prepared)
4. H_2O_2 (10mM) (freshly prepared)
5. NaN_3 (10mM) (freshly prepared)
6. Na_2HPO_4 (0.4M)
7. DTNB (40mg DTNB dissolved in 100ml 1% sodium citrate) (freshly prepared)

Protocol

Reagents	Reagent blank	Substrat blank	Enzyme blank	Test
KPO_4 (ml)	0.1	0.1	0.1	0.1
Glutathione (ml)	-	0.1	-	0.1
NaN_3 (ml)	0.1	0.1	0.1	0.1
Aliquot (cyto/mito)	-	-	0.02	0.02
D/W (ml)	0.2	0.1	0.18	0.08
H_2O_2 (ml)	0.1	0.1	0.1	0.1
Incubate for 5′ at 37°C (water bath)				
MPA (ml)	0.4	0.4	0.4	0.4
Keep in ice for 10′, ↺2000 rpm/10′, take supernatant				
Supernatant (ml)	0.6	0.6	0.6	0.6
Na_2HPO_4 (ml)	0.6	0.6	0.6	0.6
DTNB (ml)	0.03	0.03	0.03	0.03
Immediately take O.D. $_{412}$				

Calculation was done according to the slope calculated from the standard graph.

$$\text{GPx activity} = \frac{[\{\log(SB) - \log(EB)\} - \log(Test)] \times \text{Dilution}}{\text{Slope} \times \text{Aliquot volume (ml)} \times \text{Protein (mg/ml)}} \quad \text{Slope} = 0.001$$

Unit : Δlog (GSH) / min / mg protein

2.7.6 Measurement of intracellular ROS generation: DCF Fluorescence

Distribute 1.0 ml of cells into each well of 24-well culture plate. Incubate the cells for 48 h in CO_2 incubator. The cells were washed with HBSS and incubated with 100 □M carboxy-H_2DCF-DA (Molecular Probes #C-400) in the loading medium in CO_2 incubator at 37 °C for 30 min [HEPES buffered salt solution (HBSS), 100 ml : 0.6 g HEPES (25 mM), 0.7 g NaCl (120 mM), 40 mg KCl (5.4 mM), 26.5 mg $CaCl_2$ (1.8 mM), 0.21 g $NaHCO_3$ (25 mM), 0.27 g Glucose (15 mM) → adjust pH 7.4 with NaOH → filter sterilization]

[100 mM carboxy-H_2DCF-DA stock solution : 10 mg / 0.188 ml DMSO, stored at -20 °C at dark → For loading the cells with carboxy-H_2DCF-DA, stock solution is diluted with loading medium (99% basal culture media + 1% FBS) to a final concentration of 100 μM]. The cells were washed with HBSS to remove carboxy-H_2DCF-DA and replaced with 1.0 ml of HBSS containing chemical treatments The DCF fluorescence of islet was measured after 30 mins of H_2O_2 exposure, using a "Fluorescence Microscope" with excitation and emission wavelengths of 485 nm and 530 nm, respectively [F30-F0 / F60-F0 / F120-F0]

2.8. Toxicity Parameters

2.8.1. Serum glutamate pyruvate trans-aminase (SGPT) (Reitman and Frankel, 1957)

Liver tissue is rich in GPT. Normally serum GPT levels are low but in case of extensive liver damage this enzyme is liberated into the serum and hence a specific indicator of liver damage.

Principle : GPT transfers amino group from alanine to α-keto gluterate (α-KG) and convert it in to pyruvate. This pyruvate gives dark brown colour by reacting with 2,4-dinitro phenyl hydrazine (DNPH), which can be measured at 546 nm.

$$\alpha\text{-KG} + \text{Alanine} \xrightarrow{\text{SGPT}} \text{Pyruvate} + \text{Glutamate}$$

$$\text{Pyruvate} + \text{DNPH} \xrightarrow{\text{Hydrazine}} \text{Dark brown color in alkaline medium}$$

Reagents

Buffered substrate (pH 7.4) - 15g K_2HPO_4, 2.0g KH_2PO_4, 300mg α-Keto Gluterate, 17.8 g Alanine. pH was adjusted with NaOH and volume was made up to 1 liter. This is stable at 2-8°C for one month.

2,4-Dinitro-phenyl hydrazine (DNPH) - 20mg% in 1 N HCl.

0.4M NaOH

Sodium pyruvate standard - 44mg%

Sample – serum/plasma or 10% tissue homogenate (in PBS) after cell debris removal. (Standard range: 22-110 μg)

Procedure

Reagents	Control	Test
Serum/ tissue homogenate	-	0.05
Buffered substrate	0.25	0.25
Incubate for 30 min at 37C		
DNPH	0.25	0.25
Keep at RT for 20 min		
NaOH	2.5	2.5
Keep at RT for 5 min		
Serum	0.05	-
Read absorbance at 540nm		

Calculation - Calculation was done according to the slope calculated from the standard graph.

Unit - µmole of pyruvate formed/ min/L of serum or g of tissue

2.8.3. Lactate dehydrogenase assay (LDH) (King, et al., 1965)

Clinical significance

Organs rich in LDH are liver, heart and skeletal muscles. In myocardial infarction, serum LDH level begins to rise by 8-12 hours of attack, reaches to a peak within 48-72 hours and returns to normal after 1-2 weeks. Elevated serum LDH levels are also found in cirrhosis, hepatitis, hepatic metastases, hepatoma, pulmonary embolism, megaloblastic anemia, progressive muscular dystrophy and destructive renal diseases.

Principle: Lactate Dehydrogenase (LDH) catalyses the conversion of pyruvate to lactate and NADH to NAD^+. LDH activity in serum/plasma is directly proportional to the rate of decrease in the absorbance of NADH at 340nm.

$$\text{Pyruvate} + \text{NADH} \rightleftharpoons \text{Lactate} + NAD^+ + H^+$$

Sample collection, storage and stability: Serum is preferred. EDTA or heparinised plasma can also be used. Serum or plasma should be separated from the blood sample as soon as possible. Serum or plasma can be stored at 2°-8° C for one week. Do not use hemolysed or grossly contaminated samples.

Reagents

Reagent 1 (Coenzyme)	
NADH	240µmol/L
Reagent 1A (Buffer)	
Tris buffer, pH 7.20	80mmol/L
Sodium chloride	200mmol/L
Pyruvate	1.6mmol/L

Precaution, storage and stability of the reagents : When stored at 2-8 ° C and protected from light, the reagents are stable until the expiry dates stated on the labels.

Reagent reconstitution: Allow the reagents to attain the room temperature. Add 3 ml of reagent 1A into one bottle of reagent 1. Mix by gentle swirling till completely dissolved. Write the reconstitution date in the space provided on the label of bottle 1. Wait for 5 minutes before using.

Reconstituted reagent storage and stability: The reconstituted reagent is stable for 4 weeks when stored at 2-8°C.

Procedure

The samples and the reconstituted reagent should be brought to room temperature prior to use.

The following general system parameters are to be used with this kit:

General System Parameters

Reaction type	: Kinetic
Reaction Slope	: Decreasing
Wavelength	: 340nm
Flowcell Temp.	: 37 °C
Delay Time	: 60 secs
No. of readings	: 4
Interval	: 60 secs
Sample vol.	: 20 µl
Reagent vol.	: 1ml
Pathlength	: 1 cm
Factor	: 8095
Zero setting with	: Distilled Water

Set the instrument using above system parameters

Dispense into test tube	
	Test
Reconstituted Reagent	1 ml
Sample	20µl
Mix and read immediately	

Application sheets for most of the commonly used chemistry analyzers are available on request.

Note: Samples with very high LDH activity cause an excessive consumption of NADH resulting in a very low initial absorbance and /or a non-linear reaction. When this occurs, the assay should be repeated with diluted sample.

Linearity: The method is linear up to 1200 U/L. For higher values, dilute the sample suitably with 0.9% saline and repeat the assay. Apply proper dilution factor to calculate the final result.

Reference values: It is recommended that each laboratory establish its own reference values. The following values may be used as guidelines.
LDH: Serum/Plasma: 200-400 U/L (37 °C)

2.8.4. Creatine kinase (CK-MB) assays (Preus et al., 1988)

Clinical Significance: Determination of serum CK-MB activity plays a major role in the differential diagnosis of Myocardial infarction. The possibility of myocardial infarction is high when CK-MB activity ranges between 6-25% of the total CK activity. Increased CK-MB activity is also seen in cases of Myocarditis, Duchennes muscular dystrophy, polymyositis, Reye's syndrome and carbon monoxide poisoning.

Principle: The procedure involves measurement of CK activity in the presence of an antibody to CK-M monomer. This antibody completely inhibits the activity of CK-MM and half of the activity of CK-MB and Ck-BB. Then the CK method is used to quantitatively determine CK-B activity. The CK-MB activity is obtained by multiplying the CK-B activity by two.

$$\text{Creatine} + \text{ATP} \xrightarrow{\text{CK}} \text{Creatine Phosphate} + \text{ADP}$$

$$\text{ATP} + \text{D-Glucose} \xrightarrow{\text{HK}} \text{Glu-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+$$

Sample collection, storage and stability: Only serum can be used to detect the CK-MB activity. A fresh sample should be used. The enzyme activity in the serum is unstable and is rapidly lost during storage. Do not use hemolysed or grossly contaminated samples.

Reagents

Reagent 1 (Buffer/Enzymes):

Tris Buffer, pH 7.10	30mmol/L
Magnesium acetate	10mmol/L
N-Acetyle-L-cysteine	20mmol/L
ADP	2mmol/L
D-glucose	20mmol/L
Diadenosine pentaphosphate	10µmol/L
EDTA	2mmol/L
Hexokinase	≥3500 U/L
G-6-PDH	≥2000 U/L
Creatine phosphate	30mmol/L

Reagent 1A (Polyclonal Antibody): Anti-human polyclonal CK-M antibody (Goat)

Storage and stability of the reagents: When stored at 2-8° C and protected from light, the reagents are stable until the expiry dates stated on the labels.

Reagent Reconstitution: Allow the reagents to attain the room temperature. Add 3 ml of reagent 1 A to one bottle of reagent 1. Mix gently by wirling till completely dissolved. Write the reconstitution date in the space provided on the label of bottle 1. Wait for 5 minutes before using.

Reconstituted Reagent storage and stability: The reconstituted reagent is stable for 3 days when stored at 2-8 °C.

Procedure: The samples and the reconstituted reagent should be brought to room temperature prior to use.

The following general system parameters are to be used with this kit.

General System Parameters

Reaction type	: Kinetic
Reaction Slope	: Increasing
Wavelength	: 340nm
Flowcell Temp.	: 37 °C
Delay Time	: 300 secs
No. of readings	: 4
Interval	: 60 secs
Sample vol.	: 50 µl
Reagent vol.	: 1ml
Pathlength	: 1 cm
Factor	: 6752
Zero setting with	: Distilled Water

Set the instrument using above system parameters

Dispense into test tube

	Test
Reconstituted Reagent	1 ml
Sample	50µl
Mix and read immediately	

2.8.5. Creatinine (Bonsnes and Taussky, 1945)

Creatinine is the end product of creatine metabolism. It is largely formed in the muscle by irreversible and non-enzymatic removal of water from creatine phosphate. It is a waste product and excreted out from the kidney. An increased serum creatinine level is virtually a diagnostic of renal disease.

Principle – Creatinine reacts with picric acid in alkaline medium to form a reddish yellow complex. Intensity of the complex is directly proportional to the concentration of creatinine in the specimen and can be measured at 540 nm.

Reagents

Saturated Picric acid

NaOH (0.75M)

Creatinine standard solution - Stock – 100mg/dl

Working – 10mg/dl

(Standard range 10-50 µg)

Sample - 1.5 ml picric acid was added to 0.5ml serum/plasma, and tubes were centrifuged at 2000 rpm for 10min. Supernatant was taken out for creatinine estimation.

Procedure

Reagents	Control (ml)	Test (ml)
Supernatant	-	1.0
D/W	2.0	1.0
Picric acid	1.0	1.0
0.75M NaOH	1.0	1.0
Tubes kept for 20 min at RT and absorbance was recorded at 540nm		

Calculations : $\frac{\text{O.D. of test}}{\text{OD of std}} \times \text{conc. of std}$

Unit - mg/dl

2.8.7. Acid phosphatase (ACP) (Bowers & McComb, 1975)

This hydrolytic enzymes are present in high concentrations in liver, bone, placenta and intestinal epithelium. Increased ALP levels are frequently encountered as a clinical evidence of liver damage.

Principle: The enzyme reacts with p-Nitrophenyl Phosphate (PNPP) and converts it into p-Nitrophenol (PNP), which is yellow in colour. PNP in acidic medium gives bright yellow coloured phenolic ions, which can be measured at 405 nm.

Reagents

Acid buffer/substrate solution (50mM citrate buffer, pH 4.8, 5.5mM PNPP)

Tartarate (0.4M)

NaOH (0.1N)

Standard PNP solution: 1 mM stock in 0.1 N NaOH (freshly prepared)

Working standard: 0.04 mM made in 0.1 NaOH

Standard range: 0.04 - 0.16 μmols

Protocol

Reagent	Sample A	Sample B
Buffer/Substrate solution (ml)	1.0	1.0
Tartarate solution (ml)	---	0.05
Sample (ml)	0.2	0.2
Mix & incubate for exactly 30' in water bath		
Sample	2.0	2.0
Absorbance was recorded at 405 nm		

Read against blank (without tartarate) to which the homogenate is added post NaOH. The increase in extinction, ΔE over the blank is used for calculations. The activity of Prostatic ACP is obtained by the difference between the activities of Sample A and Sample B (the total ACP activity is measured in Sample A). Yellow colour stable for hours. Calculation was done according to the slope calculated from the standard graph.

Unit : IU (Enzyme required to hydrolyse 1μmole of PNP/min at pH 4.8 at 37°C).

2.8.6 Serum glutamic oxalacetic transaminase (SGOT) (Karmen et al., 1955)

Summary and explanation

AST is widely distributed with high concentrations in the heart, liver, skeletal muscle, kidney and erythrocytes. Damage or disease to any of these tissues such as myocardial infarction, viral hepatitis, liver necrosis, cirrhosis and muscular dystrophy may result in raised serum levels of AST.

Methodology:

In 1955 Karmen developed a kinetic assay procedure for AST which was based upon the use of malate dehydrogenase and NADH.

Principle

$$\text{L-Aspartate + 2-Oxoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate + L-Glutamate}$$

$$\text{Oxaloacetate + NADH} \xrightarrow{\text{MDH}} \text{L- Malate + NAD}$$

Aspartate aminotransferase (AST) catalyzes the transfer of the amino group from L-aspartate to 2-oxoglutarate to yield oxalacetate and L-glutamate. The oxaloacetate undergoes reduction with simultaneous oxidation of NADH to NAD in the malate dehydrogenase (MDH) catalyzed indicator reaction. The resulting rate of

decrease in absorbance at 340nm is directly proportional to the AST activity. Lactate dehydrogenase (LDH) is added to prevent interference from endogenous pyruvate which is normally present in serum.

Reagent composition

Active ingredients	**concentrations Concentrations**
2-Oxoglutarate	13 mM
L-Aspartate	220 mM
MDH (microbial)	>100 U/L
LDH (microbial)	>1500 U/L
NADH	>0.12 mM

pH 7.9 ± 0.1.

Reagent storage

1. Store the reagent at 2-8°C
2. The reagent is stable until the expiration date when stored at 2-8°C.

Specimen collection and handling

1. Non-hemolyzed serum is recommended. Red blood cells contain AST.
2. AST in serum is reported stable for seven days when refrigerated, one month frozen, and three days when stored at room temperature.

Assay procedure

Temperature : 37°C

Wavelength : 340 nm

Assay type : Rate/Kinetic

Direction : Decrease

Sample/Rgt ratio: 1 : 10

e.g. Sample Vol. 0.005mL (5 uL)

Reagent Vol. 0.5 mL (500 uL)

Delay/Lag time : 30 Sec

Read time : 1-3 Min

Calculation

One Unit (U/L) is defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under specified conditions.

For example:

$$\text{AST (U/L)} = \frac{\Delta\text{Abs./min} \times 1.10 \times 1000}{6.22 \times 0.10 \times 1.0} = \Delta\text{Abs/min} \times 1768$$

Where,

DAbs./min. = Average absorbance change per minute

1.10 = Total reaction volume (ml)

1000 = Conversion of U/mL to U/L

6.22 = Millimolar absorptivity of NADH

0.10 = Sample Volume (mL)

1.0 = Light path in cm

When run as recommended the assay is linear from 2 to 600 U/L.

2.9 Parameters for apoptosis

2.9.1 Estimation of Caspase activity (kit method) (Nicholson et al., 1995)

Islet cells were lysed with a lysis buffer containing 50nM Hepes, pH 7.4, 100nM NaCl, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1propane-sulphonate (CHAPS), 1nM ethylenediaminetetraacetic acid (EDTA), 10%glycerol, 10nM DL-threo-1,4-dimercapto-2,3-buanediol(DTT). Lysates were incubated with the caspase-3 substrate Ac-DEVD-pNA (N-Acetyl-Asp-Glu-Val-Asp pintroanilide; Sigma) at 37°C. After 1h, the absorbance of the lysates was measured at 405nm. Data was normalized to the absorbance to vehicle-treated cells and expressed as mean absorbance from three separate samples ± S.E.M.

Standard: pNA μmolpNA/min/ml

2.9.1. Comet assay (Gedik et al. 1992, Collins et al. 1997)

DNA single strand breaks were measured using alkaline comet assay. Glass microscope slides were frosted with 1 % normal melting point agarose (type I- A) prepared in deionised water. Islets of langerhans were resuspended in 400 µl of 0.8 % low melting point agarose (type VII A) in PBS at 37 °C and pipetted onto a frosted microscope slide precoated with 100 µl of 1 % normal melting point agarose. Slides with layers of cells in agarose were incubated at 4 °C for 10 min and then immersed in lysis solution (2.5 mol/l NaCl, 100 mmol/l Na_2EDTA, 10 mmol/l Tris, 1 % Triton, pH 10) for one hour to remove cellular membranes. After lysis, slides were placed in a horizontal electrophoresis tank containing electrophoretic solution (1 mmol/l Na_2EDTA, 300 mmol/l NaOH, pH 13) at 4 °C for 40 min (DNA unwinding). Electrophoresis was performed in the same solution at 25 V, 300 mA, 4 °C, for 30 min. The slides were washed three times for 5 min at 4 °C with neutralizing buffer (0.4 mmol/l Tris, pH 7.5) before staining with 20 µl 4´,6-diamidine-2-phenylindole dihydrochloride - DAPI (2 µg/ml).

Visual scoring: Each slide was viewed by fluorescence microscopy (Nikon) and the degree of damage was assessed visually by the appearance of formation of tail.

2.9.3 Assessment of cell death by AnnexinV-FITC/PI dual staining

To differentiate between apoptotic and necrotic cell death, dual staining with Annexin V-FITC/PI was performed using Apoptosis detection kit (Molecular Probes, USA). Phosphatidyl serine (PS) exposure on the surface of the cells is one of the characteristic features of apoptotic cells. It is also a signal for macrophages to clear such cells by phagocytosis which is important to prevent inflammation and damage to surrounding cells. PS has an affinity to bind to Annexin in a Ca^{+2} dependent manner. Annexin can bind to PS only when it is exposed outside as it cannot cross the membrane. Propidium Iodide (PI) is a DNA binding dye which can enter the cell only when membrane integrity is completely lost. Thus early apoptotic

cells will be Annexin+ and PI- while late apoptotic and necrotic cells will be Annexin+ and PI+.

~1000 islets of langerhans pelleted and washed twice with 1X PBS. *Islets of langerhans were* then suspended in binding buffer provided in the kit and incubated with Annexin V and PI for 10 minutes in dark at 22^{0}C. Fluorescence was monitored under 60X magnification using fluorescent microscope (Nikon Eclipse TE2000-S, Japan).

2.10. Parameters for thrombosis

2.10.1 Platelet aggregation. (Saldeen et al., 1999)

Rat blood was gently mixed with 3.8% sodium citrate (9:1), centrifuged at 1,200 rpm for 10 min at room temperature to obtain platelet-rich plasma (PRP) and centrifuged again at 3,000 rpm for 15 min to obtain platelet-poor plasma. Platelet count in PRP was counted and kept at about 2 to 3 10^8cells/ml. ADP (final concentration 20 mM) was used as stimulus for platelet aggregation. This concentration of ADP has been used in several experiments by others (Mehta et al., 1999; Li et al., 1999). All aggregations were conducted in a four-channel aggregometer (BIO/DATA, Horsham, Pennsylvania) in triplicate.

2.10.2 Prothrombin Time (PT) determination (kit method) (Biggs and McFarlane et al., 1962)

The arrest of bleeding depends upon primary platelet plug formed along with the formation of a stable fibrin clot. Formation of this clot involves the sequential interaction of series of plasma proteins in a highly ordered and complex manner and also the interaction of these complexes with blood platelets and materials released from the tissues. Tissue Thromboplastin, in the presence of calcium, is an activator, which initiates the extrinsic pathway of coagulation, which includes plasma coagulation factors VII, X, V, Prothrombin and fibrinogen. During oral anticoagulant

therapy most of the vitamin K dependent factors such as II, VII, IX, X, Protein C and Protein S are depressed, as also during the deficiencies of clotting factor activity which may be hereditary or acquired. Prothrombin time determination is the preferred method for presurgical screening, as a liver function test, determination of congenital deficiency of factors II, V, VII, and X and for monitoring of patients on oral anticoagulant therapy.

Reagent: UNIPLASTIN is a novel, highly sensitive, low opacity, ready to use liquid Calcified Thromboplastin Reagent, which is derived from rabbit brain.

Reagent storage and stability: Store the reagent at 2-8°C (Do not freeze). The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label. The uncontaminated reagent is stable for 1 year at 2-8°C, 1 week at 18-25°C, 2 days at 37°C.

Principle: Tissue thromboplastin in the presence of calcium activates the extrinsic pathway of human blood coagulation mechanism. When UNIPLASTIN reagent is added to normal citrated plasma, the clotting mechanism is initiated, forming a solid gel clot within a specified period of time. The time required for clot formation would be prolonged if there is acquired for congenital deficiency of factors / factor activity in the extrinsic pathway of the coagulation mechanism or reduction in the activity of Vitamin K dependent clotting factors during oral anticoagulant therapy.

Additional material required: 12×75 mm test tubes (plastic tubes are preferred), 0.1 ml and 0.2 ml pipettes, stop watch, water bath or heating block at 37°C.

Sample collection and preparation of PPP: Fasting prior blood collection provide samples with a desirable lower opacity. Withdraw blood without frothing into a eppendroff tube Transfer the blood into anticoagulated tubes. Do not delay mixing blood with anticoagulant. Avoid foam formation during mixing. Mix exactly nine

parts of freshly collected blood with one part of tri-sodium citrate (0.11 mol/l, 3.2%). Centrifuge immediately for 15 min at 1500-2000 rpm (approximately 1500 g) on a laboratory and transfer the plasma into a clean test tube. It should be ensured that the plasma is free from platelets (PPP). Plasma must be tested preferably immediately. However if the specimen are held at 22-24°C then they may be tested within 2 hours and if the specimen is held at 2-4°C then they may be tested within 3 hours.

Test procedure:

1. Bring the reagent vial to room temperature (20-30°C). Mix the content of the vial to homogenise the suspension completely.
2. Aspirate from the reagent vial enough reagent for immediate testing requirements in a thoroughly clean and dry test tube. (Plastic test tubes are preferred).
3. Prewarm the reagent and bring to 37°C before use in test procedure (5-10 min may be required depending on the reagent volume to attain 37°C before testing).
4. Recap the reagent vial and replace immediately to 2-8 °C.
5. To a 12×75 mm tube, add 0.1 ml plasma (PPP) and place the tube in a waterbath for 3-5 min at 37 °C.
6. To the tube **forcibly** add 0.2 ml of UNIPLASTIN reagent (prewarmed at 37°C for atleast 3 min) and simultaneously start a stopwatch. Shake the tube gently to mix contents.
7. Gently tilt the tube back and forth and stop the stopwatch **as soon as the first fibrin strand is visible and the gel / clot formation begins.** Record the time in 'seconds'.
8. Repeat steps 4-6 for a duplicate test on the same sample.
9. Find the average of the duplicate test values. This is the Prothrombin Time (PT).
10. **Calculations and results:** The results may be reported directly in terms of the double determination of PT of the test plasma in 'seconds'.

2.10.3 Partial thromboplastin time (APTT) (kit method) (Biggs, 1972)

Determination using Ellagic acid, as an activator: The arrest of bleeding depends upon primary platelet plug formed along with the formation of a stable fibrin clot. Formation of this clot involves the sequential interaction of a series of plasma proteins in a highly ordered and complex manner and also the interaction of these complexes with blood platelets and materials released from the tissues.

Activated Partial Thromboplastin time is prolonged by a deficiency of coagulation factors of the intrinsic pathway of the human coagulation mechanism such as factor XII, XI, IX, VIII, X, V, II and fibrinogen. Determination of APTT helps in estimating abnormality inmost of the clotting factors of the intrinsic pathway including congenital deficiency of factor VIII, IX, XI and XII and is also a sensitive procedure for generating heparin response curves for monitoring heparin therapy.

Reagent: LIQUICELIN-E is a liquid ready to use activated cephaloplastin reagent for the determination of Activated Partial Thromboplastin Time. It is a phospholipid preparation derived from rabbit brain with ellagic acid as an activator. Each batch of the reagent undergoes rigorous quality control at various stages of manufacture for its sensitivity and performance.

Reagent storage and stability: Store the reagent at 2-8 °C. Do not freeze. The shelf life of the reagent is as per the expiry date mentioned on the reagent vial label. The reagent is stable for 1year at 2-8 °C, 1 week at 18-25 °C and 2 days at 37 °C.

Principle: Cephaloplastin activates the coagulation factors of the intrinsic pathway of the coagulation mechanism in the presence of calcium ions. APTT is prolonged by a deficiency of one or more of these clotting factors of the intrinsic pathway and in the presence of coagulation inhibitors like heparin.

Sample collection and preparation: Withdraw blood without frothing into eppendrof tube. Mix exactly nine parts of freshly collected blood with one part of tri-sodium citrate (0.11mol/l, 3.2%). Centrifuge immediately for 15 minutes at 3000rpm (approximately 2000g) and transfer the plasma into a clean test tube. Plasma must be tested within three hours of blood collection. For heparin determination, platelet deficient plasma should be used, hence higher centrifugation time is required.

Additional materials required :

a. 12 X 75 mm test tubes.

b. 0.1ml, 0.2ml and 2.0ml precision pipettes.

c. stop watch.

d. water bath or heating block at 37 °C.

e. fresh normal pooled plasma.

f. $CaCl_2$ (0.025 mol/l)

Test Procedure:

1. Before use, the reagent should be mixed well by gently swirling. Do not shake.
2. Aspirate from the reagent vial enough reagent for the immediate testing requirement in a thoroughly clean and dry test tube. Bring this reagent to room temperature before prewarming at 37 °C for testing purposes.
3. Separate test tubes containing LIQUICELIN-E and TULIP calcium chloride solution should be brought at 37 °C (depending on volume, approximately 5-10 min required). Do not incubate the test plasma.
4. To a 12 X 75 mm test tube, add 0.1ml test plasma and 0.1ml LIQUICELIN-E. Shake tube briefly to mix the reagent and plasma; place tube at 37 37 °C for 3-5 mins.
5. Following incubation period add forcibly 0.1 ml of prewarmed calcium chloride into the plasma and LIQUICELIN-E mixture; simultaneously start a stop watch. Shake tube briefly to mix contents, keep it 37 °C for 20 secs.

6. Following 20 sec incubation, remove the tubes; gently tilt back and forth until a gel clot forms; stop the watch; record time.
7. Repeat step 2 to 4 for a duplicate test using the same test plasma.
8. Find average from the duplicate test values. This is the activity partial thromboplastin time (APTT of patient plasma).

Reporting Results: The result may be reported directly in terms of the double determination of the APTT of the test plasma.

2.11 Parameters for reproductive functions

2.11.1 Estimation of Vitamin C (Roe & Keuther 1943)

Principle: Ascorbic acid is oxidized by copper to form dehydro ascorbic acid and diketogulonic acid, which when treated with 2,4-Dinitrophenylhydrazone. Hydrazone in strong sulfuric acid undergoes rearrangement to form a product measurable at 520 nm .Thiourea is added to provide a mildly reducing medium, which helps to prevent the interference from non ascorbic acid chromogens.

Reagents

1) 2,4-Dinitrophenylhydrazine/thiourea/copper (DTC) solution (0.4 g thiourea, 0.05 g CuSO4.5H20, 3g Dinitrophenylhydrazine, make volume 100 ml with 9N H2SO4) (reagent stable for 1 week)
2) 65% H2SO4 (ice cold)

Protocol

Reagent blank TEST

TCA (ml) : 0.5

Aliquot (ml) : 0.5

DTC (ml) : 0.1

Mix & incubate for 3 hours at 37°C

65% H2SO4 (ice cold) 0.75

Mix well and allow to stand at room temperature for 30 min, take O.D. at 520 nm

$$\text{Vit C content} = \frac{\text{C.O.D} \times 1}{\text{Slope} \times \text{aliquot volume (ml)}} \quad \mu g \text{ Vit C/ g tissue}$$

Unit = µg Vit C / g tissue

2.11.2 17β-hydroxy steroid oxidoreductase, 3α-hydroxy steroid dehydrogenase (3α-HSD) and 3β-HSD/17β-HSD (Shivanandappa and Venkatesh, 1997)

Principle: The assay is based on the formation of NADH due to enzymatic oxidation of the steroid alcohol, which is coupled to the reduction of the tetrazolium via diaphorase present in the tissue.

Reagents

1. 50 mM Phthalate buffer (pH 3.0): 2.55 g of potassium hydrogen phthalate dissolved in a mixture of 51 ml N/10 HCl and 2.5 ml Tween 20; pH adjusted to 3.0 and the volume made upto 250 ml with distilled water.
2. 0.1 M Tris-HCl buffer (pH 7.8)
3. 0.25 M of sucrose buffer (pH 7.4)
4. 5 mM NAD
5. Color reagent: 40 mg INT, 10 mg PMS, and 0.5 ml Tween 20 were dissolved in 50 ml distilled water for the standard curve. For the enzyme assay, PMS was omitted from the reagent.
6. The substrate

17β-HSOR/HSD: 17β-estradiol was first dissolved in minimum amount of alcohol and the stock solution (1 mM) prepared in 50 ml Tris-HCl buffer.

3α-HSD/3β-HSD: DHEA was first dissolved in minimum amount of diformamide and the stock solution (1 mM) prepared in 50 ml Tris-HCl buffer.

Enzyme source: *3β-HSD /17β-HSD:* 10% testis homogenate was prepared in 0.1 M Tris- HCl buffer (pH =7.8). This was then centrifuged at 12000 g at 4° C. Supernatant was used as a source of the enzyme.

Procedure

Reagents	**Blank (ml)**	**Test (ml)**
Tris-HCl	1.0 ml	1.0 ml
NAD	0.5	0.5
Substrate	0.5	0.5
Sample	----	0.05
INT	0.250	0.250
Incubate at 37^{0}C for 1 hr		
Phthalate buffer	1.0	1.0
Centrifuged at 3000 rpm for 20 min; supernatant was read at **490 nm** against blank.		

Standard curve: 1 mM solution of NADH was prepared in distilled water. Aliquots of graded concentrations of NADH (0 to 150 nmol) were reacted with the color reagent (0.5 ml) and after color formed, 1.0 ml of phthalate buffer was added to each tube and absorbance read at 490 nm. A standard curve was prepared by plotting NADH concentration vs absorbance.

Unit: nmols of NADH formed/min/mg protein.

2.11.3 Radio Immuno Assay (RIA) of Testosterone (Tohda et al., 2001).

Principle: The procedure follows the basic principle of radioimmunoassay where there is competition between a radioactive and a non-radioactive antigen for a fixed number of antibody binding sites. The amount of I ^{125} labeled testosterone bound to

the antibody is inversely proportional to the concentration of unlabeled testosterone present. The separation of the free and bound antigen is achieved by decanting or aspirating the antibody-coated tubes.

Reagents

1. Testosterone standards (Lyophilized): Six vials, labeled A-F, containing concentrations of approximately 0, 0.1, 0.5, 2.5, 10.0 and 25.0 ng/ml testosterone in serum with sodium azide as a preservative. Vials A and B-F was reconstituted with 1.0 ml and 0.5 ml of deionized water respectively.
2. I^{125} Testosterone
3. Testosterone controls (Lyophilized)
4. Anti-Testosterone-coated tubes.

Sample- Serum.

Protocol

Reagents	Total counts	Standard	Controls	Test
Standards	---	50µl	---	---
Controls	---	---	50µl	---
Serum/tissue Sample	---	---	---	50µl
Tracer (I-125 Testosterone)	500µl	500µl	500µl	500µl
Incubate for 1 hrs at 370 temperature in a water bath				
Decant thoroughly and blot the tubes to remove any droplets adhering to the rim				
Count for 1 min in gamma counter.				

Calculation: T = Total counts of 50µl of I^{125}-testosterone. B0 = CPM of bound with I^{125}-testosterone in absence of testosterone (zero binding). B = CPM of bound with I^{125}-testos

terone in presence of testosterone standard. %B/T and % B/B0 was calculated for testosterone standards and serum/ tissue samples. A logit-log graph was plotted

against % B/B0 and concentration of testosterone standards. Serum/tissue testosterone concentrations were calculated from the graph.

Units: ng of testosterone present in ml serum or mg/g tissue.

2.11.4 Rat sperm isolation (Slott et al., 1991)

After animal sacrificed by cervical dislocation, testies were removed with adherent epididymis. Epididymis was separated from testis and were put into 2 ml pre-warmed PBS, pH 7.4. Sperm were allowed to diffuse after the epididymal tubule was pierced with a no. 11 scalpel blade and sperm was forced out of the vas deferens with fine forceps by putting pressure on the lower region of the cauda epididymis and "walking" the forceps down the vas deferens, not forcing out excess material, i.e., immature cells. The dish was shaken gently and, after 5 min of dispersion, an aliquot of sperm was used for sperm count, viability and motility.

2.11.5 Sperm Count and Viability (Eliasson R., 1977)

An aliquot of sperm was diluted 1:100 with fixative (10% formalin in PBS, pH 7.4) and counted using a haemocytometer. Sperm viability was performed by the eosin nigrosin staining. One drop of semen was mixed with two drops of 1% eosin Y. After 30 s, three drops of 10% nigrosin were added and mixed well. A smear was made by placing a drop of mixture on a clean glass slide and allowed to air dry. The prepared slide was examined using a phase contrast microscope. Pink-stained dead sperm were differentiated from unstained live sperm, and there numbers were recorded.

2.11.6 Sperm Motility (Slott et al., 1991)

Epididymal sperm motility was evaluated in the PBS, pH 7.4. A 50-µl aliquot was diluted 20 times in PBS 37°C, and transferred to a glass slide. Under a light microscope (10X magnification), a random field was chosen, and sperm classified as

motile or immotile. Sperm motility was expressed as the percentage of motile sperm per field.

2.11.7 Determination of fructose concentration from prostate and seminal plasma (Motoshima and Settlage, 1978)

The prostate and seminal vesicle was removed, weighed and stored at -20°C to determine the content of fructose. Fructose in seminal plasma reacts with resorcinol in concentrated HCl solution to form red compound under heating. 0.1 ml of fresh sample was mixed with 2.9 ml of distilled water. Then, 0.5 ml of $Ba(OH)_2$ solution (0.15 mol/L) and 0.5 ml of $ZnSO_4$ solution (0.175 mol/L) were added, mixed, and then stood for 5 min to remove seminal proteins. After centrifugation at 3000 g for 15 min, 1 ml of the supernatant was collected for determination of fructose level. The supernatant was replaced with standard fructose solution (0.28 mmol/L) to serve as standard and replaced with distilled water as blanks. Subsequently, 1 ml of resorcinol solution (8.47 mmol/L) and 3 ml of HCl (10 mol/L) were added into tubes, and maintained at 90°C for 10 min. Lastly, absorbance (A) values were read at 490 nm against blanks. Fructose concentration in seminal plasma was expressed as mmol/L: A value of test / A value of standard × 11.12.

2.12 Blood pressure measurement and vascular reactivity

2.12.1 Blood pressure measurement by tail cuff method (Gerhard Vogel, 2002)

Purpose and rational: Rats with spontaneous or experimentally induced hypertension are widely used for screening of potentially antihypertensive compounds. The indirect tail cuff method allows the determination of systolic blood pressure according to the following principle:

The cuff is quickly inflated to well above suspected systolic blood pressure; the pulse will then be obliterated. Thereafter, pressure in the cuff is slowly released and, as the pressure falls below systolic blood pressure, the pulse will reappear. The

method is analogous to sphygmomanometry in human and can be applied not only at the tail of awake rats but also in dogs and small primates. The indirect tail cuff method is widely used to evaluate the influence of antihypertensive drugs in spontaneously and experimentally hypertensive rats.

Procedure: To reduce spontaneous variations in blood pressure, animals are adjusted to the experimental cage by bringing them into the restraining cage 3–4 times before the start of the experiment for a period of 30–60 min. To measure blood pressure, a tubular inflatable cuff is placed around the base of the tail and a piezo-electric pulse detector is positioned distal to the cuff. The cuff is inflated to approximately 300 mm Hg. As the pressure in the cuff is slowly released, the systolic pressure is detected and subsequently recorded on a polygraph. Mean values in systolic blood pressure were compared with test and control groups of animals. Percent decrease in systolic blood pressure as compared to normal control under drug treatment is calculated.

2.12.2 Blood Pressure (in unconscious animals by BIOPAC)

Rats were used for measurement of blood pressure and vascular reactivity in control and experimental groups as described by Balaraman et al. (2007). After completion of treatment schedule, rats from each group were anesthetized using 1.2 g/kg (i.p.) of urethane. Tracheotomy was performed to facilitate breathing. The left common carotid artery and left femoral vein were cannulated with polyethylene tubing filled with heparinised saline (500 IU/ml) to prevent clotting. The hemodynamic parameters like systolic, diastolic and mean arterial blood pressures (SBP, DBP and MABP, respectively) were measured in the left common carotid artery using recalibrated pressure transducer SS13L and Biopac MP-30 data acquisition system (BIOPAC Systems, Inc., CA, USA). After 30 min of equilibration vascular reactivity to intravenous (i.v.) injection (via femoral vein) of adrenaline (1 μg/kg), phenylephrine (1μg/kg), isoprenaline (0.1mmol/kg), and acetylcholine (0.1 mmol/kg) were recorded. Rats received a maintenance i.v. infusion of 0.9% sodium

chloride (1 ml/h) throughout the experimental duration. All the data were analyzed using Biopac Student Lab Pro Software (Version 3.6.7).

2.13. Tests for nociceptive response

2.13.1 Tail-flick test (tail immersion test) (Gerhard Vogel, 2002)

Purpose and rational: The method has been developed to be selective for morphine-like compounds. The procedure is based on the observation that morphine-like drugs are selectively capable of prolonging the reaction time of the typical tail-withdrawal reflex in rats induced by immersing the end of the tail in warm water of 55 °C. The test is useful to differentiate central opioid like analgesics from peripheral analgesics.

Procedure: Rats are placed into individual restraining cages leaving the tail hanging out freely. The animals are allowed to adapt to the cages for 30 min before testing. The lower 5 cm portion of the tail is marked. This part of the tail is immersed in a cup of freshly filled water of exactly 55 °C. Within a few seconds the rat reacts by withdrawing the tail. The reaction time is recorded in 0.5 s units by a stopwatch. After each determination the tail is carefully dried. The cut off time of the immersion is 15 s. The withdrawal time of untreated animals is between 1 and 5.5 s. A withdrawal time of more than 6 s therefore is regarded as a positive response.

2.13.2 Formalin induced paw irritation test (Gerhard Vogel, 2002)

Purpose and rational : The formalin test in rats has been proposed as a chronic pain model which is sensitive to centrally active analgesic agents by Dubuisson and Dennis (1977).

Procedure: All groups of animals were administered 0.05 mL of 10% formalin into the dorsal portion of the front paw. Each individual rat is placed into a clear plastic cage for observation. Readings are taken at 30 and scored according to a pain

scale. Pain responses are indicated by elevation or favoring of the paw or excessive licking and biting of the paw. Analgesic response or protection is indicated if both paws are resting on the floor with no obvious favoring of the injected paw.

2.14. Sodium Potassium dependent adenosin triphosphatase (Na^+K^+ATPase) (Bonting et al., 1970)

ANSA reagent preparation (1-amino-2-naphthol-4-sulfonic acid): 15 gm of sodium metabisulphite was dissolved in 100 ml of distilled water and 20 % sodium sulphate was dissolved in 100 ml of water. 0.25 % ANSA reagent in 15 % sodium metabisulphite and 20 % sodium sulphite was taken. 250 mg of 1- amino-2-napthol, 4-sulphonic acid was dissolved in 97.5 ml of 15% sodium metabisulphite and 2.5 ml of 20% sodium sulphite was slowly added. Mix well and keep at room temp.

The incubation mixture contained 1.0 ml of tris-hydrochloride buffer (184 mM, PH 7.5), 0.2 ml of each of magnesium sulphate (50 mM), sodium chloride(650 mM),potassium chloride (50 mM), EDTA (1 mM), ATP(40 mM) and homogenate respectively. The mixture was incubated at 36°C for 15 minutes. The reaction was arrested by addition of 1.0 ml of 10 % TCA and mixed well and centrifuge.

0.5 ml of the supernatant was taken & the volume was made up to 2.5 ml with distilled water 0.5 ml ammonium molybdate and 0.25 ml of ANSA reagent was added. The colour developed in 20 min. was read using blank containing distilled water instead of sample at 620 nm. A standard graph was prepared taking different concentration of phosphorus standard (4-20 µm). The enzyme activity was expressed as µM of inorganic phosphorus liberated/mg of tissue/min.

2.15. Calcium dependent adenosine triphosphatase (Ca^{2+}ATPase)

(Hjerken et al., 1983)

The incubation mixture contained 0.1 ml of each of tris-hydrochloride buffer (125 mM, PH 7.5), calcium chloride solution (50 mM), ATP solution (10 mM) and

homogenate respectively. The mixture was incubated at 36°C for 15 minutes. The reaction was arrested by addition of 1.0 ml of 10 % TCA and mixed well and centrifuge.

0.5 ml of the supernatant was taken & the volume was made up to 2.5 ml with distilled water 0.5 ml ammonium molybdate and 0.25 ml of ANSA reagent was added. The colour developed in 20 min was read using blank containing water instead of sample at 620 nm. A standard graph was prepared taking different concentration of phosphorus standard (4-20 µm). The enzyme activity was expressed as µM of inorganic phosphorus liberated/mg of tissue/min.

2.16 Islet isolation from rat pancreas (Xia et al., 1993)

Pancreatic islets were isolated from the pancreas of adult rats by collagenase digestion. In brief the pancreas was dissected out, washed in HBSS and cut in small pieces. Before digestion all HBSS was removed and then 5 mg of collagenase (Type XI) was added with 4ml of Hank's buffer. Digestion was carried out at 37°C in shaking waterbath. Digestion was stopped after 10–15 min by adding 30 ml of ice-cold Krebs-Ringer bicarbonate (KRB) washing buffer containing 0.02 % BSA. Tubes were allowed to stand in ice for 15 min then upper layers of solution were discarded leaving 3 - 4ml of solution in the bottom. Remaining solution was washed two times with the same buffer and subject to purification.

2.16.1 Purification of islets of langerhans

- Quickly decant the supernatant and centrifuge at 453g. After the spin, before decanting the supernatant, remove the foam layer on the top of the media with a standard Pasteur pipet. Then, quickly decant the supernatant and remove the last drop of media from the tube with the Pasteur pipet.
- Add 4 mL of 25% Ficoll to each tube using a disposable pipet, and vortex the tube at approximately three-quarters speed. Using the Pasteur pipet, gently remove any mucin from the mixture. Mucin is the byproduct of the collagenase digestion,

which appears as a gelatinous body that should be removed from the tissue mixture. To remove it, gently swirl a Pasteur pipet in the mixture. The mucin will adhere to the pipet and can be discarded.

- Note that mucin will not always be present in each digestion and can vary from tube to tube.
- Once the mucin is removed, prepare a Ficoll step gradient by slowly layering 2 mL 23% Ficoll, 2 mL of the 20.5% Ficoll, and 2 mL of 11% Ficoll to each tube. Spin the tubes at 800*g* for 12 min at room temperature with no brake.
- Once the spin has completely stopped, return the tubes to the hood. Using the Pasteur pipet, remove islets from the 11–20.5% interface and place into one to two sterile 15-mL-thick-walled glass conical tubes containing 2 mL HBSS. Repeat this procedure for the 20.5–23% interface and place islets into one or two separate conical tubes. Following transfer of material at each interface, fill each conical tubes with HBSS to a final volume of approx 12 mL.
- Resuspend the pellet by pipetting up and down with a Pasteur pipet until the Ficoll is completely mixed with the HBSS. Centrifuge the tubes at 805*g* for 20–30 s and stop with the brake. Decant the supernatant and repeat this procedure two additional times.
- Add 6 mL RPMI to the pellet and resuspend the islets using a Pasteur pipet. Spin the tubes for 5 s (including acceleration time) and immediately stop the spin. Decant the supernatant of each tube into a separate Petri dish and save. Repeat this washing step two more times, decreasing the centrifugation time by 1 s for each wash.
- Once the washes are complete, add 4 mL RPMI media to each tube, and using the pipet, transfer the remaining pellet into a separate Petri dish.
- Using a flame-pulled Pasteur pipette and dissecting microscope, remove all of the duct and acinar tissue that remain in each dish. This can be accomplished by either selectively moving the islets to new, clean Petri dishes or swirling the plate and sucking off the acinar and ducts and discarding them into a waste container. Replace RPMI as needed during the cleaning process. The preparation should be

free of as much extraneous tissues as possible to ensure optimum islet culture conditions.

- Purified Islets were cultured in RPMI 1640 media (Gibco, Grand Island, NY, USA) supplemented with 10% FCS (Trace Biosciences PTY Ltd., New South Wales, Australia) and antibiotics (penicillin 200 U/mL and streptomycin 0.2 mg/mL) in culture grade Nunclon flasks at 37 °C, 5% CO_2 for 48 hours.
- Divide the total pooled islets (1000 islets/rat) into four fresh 15-mm Petri dish.

2.16.2. Islet cell dispersion

Islets were then disrupted into single cells by mild enzymatic trypsin digestion, applying 0.15% and mechanical forces by simultaneous pipetting through a Pasteur pipette for approximately 5 minute (Joseph et al., 2004). Islet cell viability was determined by Trypan Blue exclusion tests. The single cells were resuspended in RPMI-1640 medium.

2.17. Histology

Kidney was removed and fixed in Bouins fixative. Histological examination was carried out by standard histological techniques. Sections of 5μm thickness were cut and stained with hematoxylin: eosin. Histological observations were made under the light microscope.

2.18. Statistical analysis

Statistical analyses of data was done by Student's test, one-way analysis of variance and all groups were compared by means of Bonferroni, with significance set at $p<0.05$. All values represent the mean ± S.E.M. Data were analyzed using PRISM Graph Pad software (version 3.03).

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