4. Methods and materials:

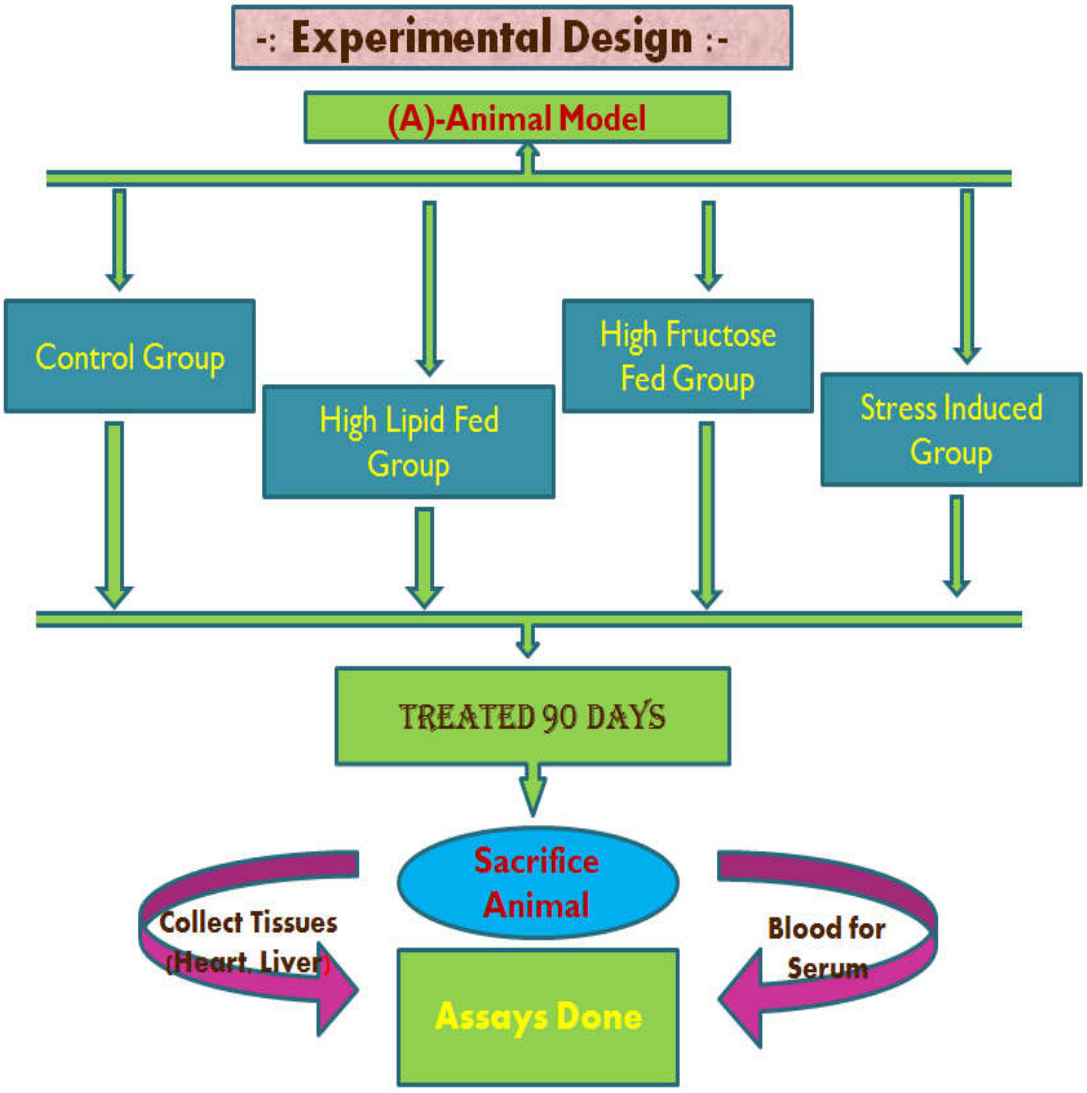
4.1. Ethical clearance: The protocol was approved by the Internal Review Board, Human and Animal Research Ethics Committee, OIST, Vidyasagar University, Midnapore on the condition that followed the approved Human Ethics Protocol strictly in accordance with 1964 Helsinki declaration and no deviation in the study was allowed without the prior written permission of the board.

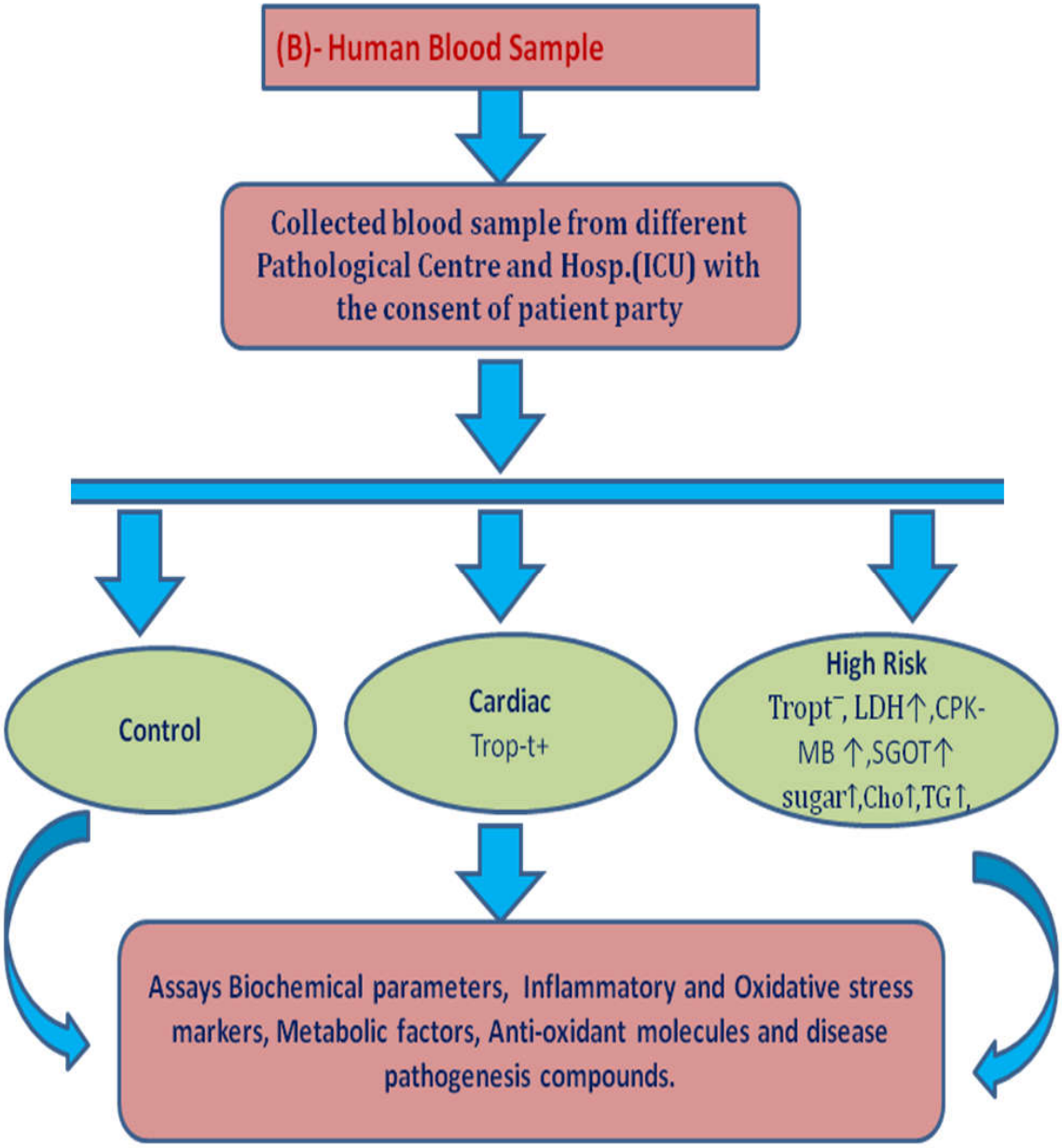
4.2. Experimental Design

On the basis of our study objectives and on the background of several literatures, we have design an experimental work plan and divide into two view point. One run on animal model another is the human blood sample.

4.2.1. Animal model: Wister male albino rats (150 ± 15g) were used in the study. Experiment starts with different iso-caloric diet like atherogenic, fructose induced with control diet and followed by esteemed experimentation.

4.2.2. On Human blood sample: For fulfill our research work we have collect the blood sample from diagnosed cardiovascular patients and whose risk factor high like diabetes, dyslipidemia, Trop-T+, LDH and CPK-MB high in blood serum from ICU patient with their family member consent and performed our esteemed biochemical, metabolic and inflammatory markers.


-: Experimental Design :-
(A)-Animal Model
Control Group
High Lipid Fed Group
High Fructose Fed Group
Stress Induced Group
TREATED 90 DAYS
Sacrifice Animal
Collect Tissues (Heart, Liver)
Blood for Serum
Assays Done


(B)- Human Blood Sample
Collected blood sample from different Pathological Centre and Hosp.(ICU) with the consent of patient party
Control
Cardiac
Trop-t+
High Risk
Tropt⁻, LDH↑,CPK-MB ↑,SGOT↑ sugar↑,Cho↑,TG↑,
Assays Biochemical parameters, Inflammatory and Oxidative stress markers, Metabolic factors, Anti-oxidant molecules and disease pathogenesis compounds.

4.3. Chemicals And Reagents:

Sodium dihydrogen phosphate (NaH2PO4), disodium hydrogen phosphate (Na2HPO4), thiobarbituric acid (TBA), reduced glutathione (GSH), 5-5′-dithiobis-2- nitro benzoic acid (DTNB), agarose (low melting point), potassium di-chromate, acetic acid, ethylene diamine tetra acetic acid (EDTA), tri-chloroacetic acid (TCA). All the biochemical kits were collected from Ranbaxy Diagnostic India Limited.

Enzymatic standard assay kit of Glucose, Triglyceride, Cholesterol, Uric Acid, CRP, SGOT, SGPT, Lactase dehydrogenase, CPK, CKP-MB from Erba, Transasia Pvt. Ltd., Beckon Dia. Pvt. Ltd., Auto-Span Pvt. Ltd. And hs-CRP, TSH, T_3, T_4 are measure by using Enzyme Linked Emmunosorbant Assay (ELISA) method by Acc-Bind, Lilak Medicine pvt. Ltd. Estriol (purity 98%), goat anti-rabbit immunoglobulin G-alkaline phosphatase and fibrinogen were obtained from Sigma-Aldrich. TNF-α, IL-6 and their primary and secondary antibody were obtained from AbCam. Polyclonal antibody against estriol was obtained from Thermo Scientific. Maxisorp plates for enzyme linked immunosorbent assay (ELISA) were obtained from Nunc, Roskilde, Denmark. analytical grade. Goat anti-rabbit HRP conjugated secondary antibody, o-phenylenediamine dihydrogen chloride (OPD), Bovine serum albumin (BSA), insulin and GLUT-4 gene specific primers and Revert Aid M-MulV reverse transcriptase (MBI Fermentas) were obtained from Sigma-Aldrich (St. Louis MO). Polyvinylidene difluoride (PVDF) membranes (Immunoblot PVDF) were purchased from

Bio-Rad (Hercules, CA). Insulin primary antibody (H-86), Glut-4 primary antibody (H-61) and TNF-α primary antibody were obtained from Santacruz Biotechnology Inc, (Santacruz, CA, USA). ELISA Maxisorb plates were from Nunc, Rosklide, Denmark. All other chemicals were of analytical grade.

4.4. Estimation of oxidative stress marker malondialdehyde (MDA)

The MDA assay was conducted following the protocol as in Buege and Aust, 1978 with a slight modification (Buege & Aust, 1978). To chelate iron and reduce its interference in the peroxidation reaction of unsaturated fatty acid, 1 mM EDTA is used in the reaction mixture. Finally, the MDA is measured and calculated using the molar extinction coefficient of MDA (1.56 x 105 cm2/ mmol) (Slater, 1984; Maiti & CA, 2000).

4.5. Estimation of antioxidant component/ precursor Non Protein Soluble Thiol (NPSH)

The NPSH in serum sample is determined by standard DTNB (5, 5'- dithiobis-2-nitrobenzoic acid) method with a little modification (Sedlak & Lindsay, 1968). In brief, the protein is precipitated by trichloroacetic acid and clear supernatant is added to 0.8M Tris-HCl (pH 9) buffer containing 20 mM EDTA and 5 mM DTNB. The contents were mixed well and absorbance read at 412 nm. The level of NPSH is determined against L-Cysteine hydrochloride standard curve.

4.6. Assay of Superoxide Dismutase (SOD) Activity by gel-zymography

A tablet of nitro blue tetrazolium (NBT) was dissolved in 30 ml water and the nondenaturing (10%) acrylamide gel (Protean-II, Bio-Rad electrophoresis system, USA) was soaked with it for 30 min with shaking. The gel was then shaken in 40 ml SOD1 (cytosolic SOD, also known as Cu-Zn SOD) solution (0.028 M tetramethylethylenediamine (TEMED), 2.8 × 10–5 M riboflavin, and 0.036 M potassium phosphate at pH 7.8) for 15 min. The soaked gel was placed on a clean acetate sheet and illuminated for 5 to 15 min. The gel became purple except at the position containing SOD1 (Steinman HM, 1978).

4.7. Experimental Animals:

Male albino rats of Wister strain weighing 150 ± 15g were used in the study. The animals were acclimatized for 1 week, in a 12:12 h light and dark cycle, in temperature and humidity controlled room. The animals were given free access to food and water. After the 1 week adaptation period, the animals were used for the study. Studies were carried out in accordance with the National Institutes of Health (NIH), USA guidelines and the Institutional Ethical concerns were maintained throughout the investigation.

4.8. High lipid and high fructose diet:

Animals were weighed, numbered and randomly segregated into three groups of six in each. All the animals were taken care of under ethical consideration and the experimental protocol was duly approved by Institutional Ethic Committee. High lipid diet or high fructose diet was supplied to two separate rat groups and the rest group was considered as the normal diet group. The specified diet was fed to the rats ad libitum along with drinking water for 90 days. . High lipid diet was prepared by mixing Indian vanaspati ghee (Rasoi; 64.13% saturated fat). Calorie value calculated from the food ingredients were as follows; carbohydrate-39.26%, protein-10.71% and fat-50.02%). Calorie value of control group and fructose feed groups were as follows; carbohydrate-69%, protein-19%, sunflower oil-12%). High fructose diet consisted of 50% fructose. All animal groups were maintained in an isocaloric (427± 6.17 Kcal/100g) dietary exposure (Tulp et al., 1979; Sheyla Leite Matos, 2005).

At the end of the experiment, all the animals were exposed to light anesthesia (by ether), and blood was collected using a disposable syringe (21-gauge needle), serum/plasma was separated. Organs required for biochemical examinations were dissected. All biological samples were preserved in -20◦C in several aliquots. Biochemical Analysis- Plasma/serum samples were utilized for the hematological and biochemical assays like total cholesterol, triglyceride, glucose and uric acids. Post-mitochondrial (10,000 X g) cytosolic fractions from liver and heart tissues were utilized for MDA and NPSH analysis. Cytosolic superoxide dismutase (Cu-Zn isoform; SOD1) activity was evaluated from both tissues by a gel zymogram.

4.9. Catalase activity assay by gel-zymography:

Catalase activity assay done by gel-zymography using 8% native gel. Chemicals used 0.003% H2O2 (30% solution vol/vol), 2% ferric chloride (wt/vol), 2% potassium ferricyanide (wt/vol). After staining, a green-blue color is developed with white band where the enzyme is present. Following separation of native protein, the catalase enzyme removes the peroxides from the area of the gel it occupies. Removal of peroxide does not allow for the potassium ferricyanide (a yellow substance) to be reduced to potassium ferrocyanide that reacts with ferric chloride to form a Prussian blue precipitate (Treadwell & WT, 1948).

4.10. Biochemical Parameter Assay:

4.10.1. Inflammatory and risk factors Estimation: Serum samples were utilized for the biochemical assays like blood sugar, total cholesterol, triglyceride, uric acid, and C reactive protein (CRP) by enzymatic methods using standard reagent kit.

4.10.2. Metabolic hormone T3, T4, TSH assay by ELISA method: Estimation of metabolic hormones and inflammatory marker like T3, T4, and TSH are assayed by ELISA (Enzyme Linked Immunosorbent Assay).

4.10.3. Cardiac marker hs-CRP determination by ELISA: The hs-CRP determination by ELISA (Enzyme Linked Immunosorbent Assay).

4.10.4. ELISA assay procedure:

Assay steps	Thyroid panel			Cardiac marker
Parameters	**T3**	**T4**	**TSH**	**Hs-CRP**
Sample dilution	-	-	-	10 µl sample in 2 ml diluents mix.
Standard/sample	50 µl	50 µl	50 µl	25 µl
Enzyme conjugate reagent	50 µl	50 µl	100 µl	100 µl
Mix	Swirl the plate for 1 minute		Swirl the plate for 20-30 seconds	
x-T3/T4 Biotin reagent	50 µl	50 µl	-	-
Mix	Swirl the plate for 1 minute		-	-
Incubation	60 min. RT (22-26°C)	60 min. RT (22-26°C)	60 min. RT (22-26°C)	15 min. RT (22-26°C)
Wash	Decant and wash 5 times with 350 µl of wash buffer			
Substrate reagent	100 µl	100 µl	100 µl	100 µl
Incubation	15 min. RT	15 min. RT	15 min. RT	15 min. RT
Stop solution	50 µl			
Readings	Mix well and read the absorbance in each well at 450 nm (using a reference wavelength of 620-630 nm to minimize well imperfections) in a microplate reader. The results should be read within 30 minutes of adding the stop solution.			

Table-1. The table descript the metabolic hormones (T3, T4 & TSH) and The cardiac marker (hs-CRP) assay done by ELISA method.. Reagent kit used Acuu-Bind ELISA microwells, Mfd: by Lilac Medicine (p) Ltd.

4.11. Anthropometric measurements:

The body mass index (BMI) of the individuals were computed using the following standard equations; BMI (kg/m2) = Weight (kg)/height (m2). Nutritional status was evaluated using internationally accepted BMI guidelines (WHO, 1995). The following cut-off points were

utilized; Grade III Thinness: BMI<16.0, Grade II Thinness: BMI=16.0–16.9, Grade I Thinness: BMI<17.0–18.4, Normal: BMI=18.5–24.9, Overweight: BMI≥25.0.

4.12. Preparation of neutrophil solution from human blood sample:

Neutrophils were isolated from the citrated blood samples using standard protocal (Bhattacharjee et al., 2012). Cell counts were determined by optical microscopy. Isolated neutrophils suspended in Hank's balanced salt solution (HBSS), pH 7.4 were incubated with different concentrations of dermcidin isoform-2 (DCN-2) for 2 h at 37°C under sterile conditions. When needed, the nucleic acids were isolated from these incubated samples for in vitro translation of TNF-α and IL-6 as described below.

4.12.1. Determination of inflammatory marker TNF-α and IL-6 in neutrophil by ELISA method:

Briefly, neutrophil was incubated with an equal volume of phosphate buffer saline (PBS) in an assay plate overnight in 4°C. Nonspecific binding was blocked by 5% bovine serum albumin in the same buffer. The samples were then washed with PBS containing Tween-20, and incubated for 2 h with diluted primary antibody in PBS (1:200) obtained from AbCam. The samples were next washed with PBS-T20 and incubated with diluted goat anti-rabbit IgG-horse radish peroxidase (1:2000) in the same buffer for 1 h. After washing they were incubated with p-nitrophenyl phosphate (1 mg/ml) in carbonate buffer (pH 9.8) containing 10 mM MgCl2. The development of color was determined at 450 nm. The amount of TNF-α and IL-6 present in the

sample was determined in an ELISA reader using respective monoclonal antibody according to the method described (Engvall & Perlmann, 1972). Level of TNF-α was checked in neutrophils from AIHD subjects as positive control reported to have high level of TNF-α. Normal neutrophil in the absence of DCN-2 was also subjected for the determination of TNF-α in control.

4.12.2. Preparation of dermcidin (DCN-2):

Dermcidin used in all experiments was prepared by repeated poly acrylamide gel-electrophoresis from the cell free plasma of acute ischemic heart disease patients in the absence of sodium dodecyl sulfate followed by the elution of protein from the gel after overnight dialysis at 4 °C (Ghosh et al., 2011).

4.12.3. Western blot of TNF-α in neutrophil solution in the presence of dermcidin:

The neutrophil solutions from different treatment groups were centrifuged and supernatant from each vial was collected separately. The supernatant was subjected for SDS-polyacralamide gel electrophoresis (SDS-PAGE). Protein bands were transferred in nitrocellulose membrane and the level of TNF-α was analysed by using goat anti-TNF-α antibody (Renart et al., 1979).

4.12.4. Identification of dermicidin by immunoblot and its quantitation by ELISA in the cell free plasma of both Type-1 Diabetes Mellitus (T1DM) and normal subjects:

The presence of dermcidin in the cell free plasma (CFP) of both normal and T1DM subjects were determined by immunoblot technique (Matsudaira P, 1987). The plasma samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (1:1) and stained with coomassie brilliant blue-30. The transfer of the separated protein bands in the plasma samples were next carried out electrophoretically to a PVDF membrane, and subjected to immunoblotting using anti-dermcidin antibody (1:500). The membrane was blocked with 5% BSA in TBS. After incubation with a HRP-linked goat anti-rabbit secondary antibody, dermcidin protein bands were visualized by using an enhanced chemiluminescence detection system (Thermo Scientific Rockford, IL). In parallel experiment, the amount of dermcidin level in both the plasma samples was quantitated by ELISA by using anti-dermcidin antibody.

4.12.5. Assay of nitric oxide in the cell free plasma (CFP) of both normal and T1DM patients:

The amount of NO in the CFP of both normal and diabetic subject was assessed by using the conversion rate of oxyhemoglobin to methemoglobin through NO using a scanning spectrophotometer (Beckman spectrophotometer). The NO content was quantitated by recording the spectral changes in the reaction mixture due to the conversion of oxyhemoglobin to methemoglobin I,e a decrease in the absorbance at 575 and 630 nm as described (Jia et al., 1996). The quantitation of NO was independently verified by chemiluminescence method (Cox & Frank, 1982).

4.11.6. Determination of plasma insulin levels in both T1DM and normal subjects by ELISA:

An enzyme-linked immunosorbant assay was performed as described (Engvall & Perlmann, 1972), to determine the plasma level of insulin in T1DM and normal subjects. Briefly, 50 µl of plasma from both the T1DM and normal subjects were incubated with equal volume of phosphate buffer saline (PBS) in the assay plate for overnight at 4 °C. The standard ELISA protocol was fallowed. The development of color was determined at 450 nm. The amount of insulin present in the sample was determined by an ELISA reader.

4.12.7. Determination of NO synthesis in dermcidin induced neutrophils:

The media solution containing neutrophils were incubated with 10 µM of 1-arginine in the presence or absence of different concentrations of DCN-2 (90 min in 37°C). The mixtures were incubated in the presence or absence of pre-incubated estriol (0.6 nM for 45 min at 37°C) with triplicate experiments for each group. To determine the NO production in the control experiment, the vehicle (HBSS buffer without neutrophils) was treated similarly under the identical conditions. Nitric oxide was assayed following the methemoglobin method, following a procedure described elsewhere, by use of a Beckman spectrophotometer (model DU6) (Karmohapatra et al,. 2007). Assay validity was confirmed by the use of an independent chemiluminescence method (Cox & Frank, 1982).