6. METHODOLOGY

a. Objective 1: To determine the antioxidant potential of naringenin using *in vitro* antioxidant assay methods.

In order to determine whether naringenin could inhibit reactive oxygen and nitrogen species production, the levels of hydroxyl (*•OH*), superoxide (*O_2*-•), Hydrogen peroxide (*H_2O_2*) and nitrosyl radicals (NO) were assayed according to the protocols described by Kunchandy and Rao (1990) (258), Sanchez-Moreno et al. (1999) (259), Long (1999) (260), and Garratt (1964) (261), respectively. In addition, the ability of naringenin to scavenge DPPH radicals was assessed according to Burits and Bucar (2000) (262). Furthermore, lipid peroxidation activity of naringenin was carried out by the method described by Ohkawa et al. (1979) (263).

**(I) Hydroxyl radical scavenging assay**

The hydroxyl scavenging capacity of naringenin was quantified by Fenton reaction as described by Kunchandy and Rao (1990) (258). The reaction mixture containing 2.8mM of 2-deoxy-2-ribose in phosphate buffer (pH 7.4), 100µM FeCl_3, 100µM EDTA, 1mM H_2O_2, 100µM ascorbic acid and naringenin (0-1836.5µM) was incubated for 1h at 37°C. Then 0.5ml of the reaction mixture was first added to 1ml 2.5% TCA. Next, 1ml 1% TBA was added and the mixture further incubated at 90°C for 15min and the absorbance measured at 532nm (258). Percentage inhibition was calculated by comparing the test and blank solutions. The effect of hydroxyl scavenging was compared with tocopherol (0-1160.9µM).

**(II) Hydrogen peroxide scavenging assay**

The H_2O_2 scavenging activity was measured using FOX reagent (260). Briefly, an aliquot of 50mM H_2O_2 was incubated with increasing concentrations (0-1836.5µM) of the naringenin in 1:1 (volume/volume) for 30min at room temperature. After incubation, 90µL of this mixture is mixed with 10µl methanol and 0.9ml of FOX reagent (prepared by mixing 9 volumes of 4.4mM BHT with 1 volume of 1mM xylene orange and 2.56mM ammonium ferrous sulfate in 0.25M sulfuric acid). The reaction mixture was vortexed and further incubated for 30min at room temperature. The absorbance of the developed ferric-xylene orange complex was measured at 560nm (260). The hydrogen peroxide destroying the ability of naringenin was compared with the standard antioxidant vitamin-C (0-1135.6µM). The scavenging activity was calculated based on the formula: % Hydrogen peroxide scavenging = (A_{control} - A_{sample})/A_{control} X100.

**(III) Scavenging of superoxide radical**

The superoxide anion scavenging ability of naringenin was measured according to the method Sanchez-Moreno et al. (1999) (259), by estimating the inhibition of generation of O_2^- . Ten millimolar potassium superoxide (5.0µL) and dry DMSO (500µL) were allowed to stand in contact for 24h and the solution filtered immediately before use. The filtrate (200µl) was added to 2.8ml of an aqueous solution containing 500µM nitroblue tetrazolium (NBT), 10µM EDTA and 10mM potassium phosphate buffer (pH-7.4). Naringenin (0-1836.5µM) was added and the absorbance recorded at 560nm against a control in which pure DMSO was added. The results were compared with the reference antioxidant quercetin (0-1654.3µM).
(IV) Nitric oxide radical scavenging assay

The nitric oxide generation capacity was quantified by the Griess Ilosvays reaction using sodium nitroprusside (SNP) (261). The reaction mixture containing 10mM SNP in phosphate buffer (pH 7.4 100mM) and increasing concentration of the naringenin (0-1836.5μM) were incubated for 2h at room temperature. Then 1ml of 0.33% sulfanilamide in 20% glacial acetic acid were added and allowed to stand at room temperature. After 5min, 1ml of 0.1% naphyl ethylenediamine dihydrochloride (NED) was added and the mixture incubated for additional 30min. The color developed was measured spectrophotometrically at 540nm. The results were compared with the reference antioxidant vitamin-C (0-2839μM).

(V) Determination of DPPH radical scavenging activity

The ability of naringenin to scavenge DPPH radicals was assessed according to Burits and Bucar (2000) (262). Three milliliters of 60µM DPPH in ethanol was added to increasing concentration of naringenin (0-367.3mM) and then incubated at room temperature in dark condition for 15min. The decrease in the absorbance was read at 517nm using spectrophotometer (UV-1800 UV-VIS spectrophotometer, Shimadzu, Japan). Vitamin C (0-567.79mM) was used as a standard. Comparing the absorbance values of control containing only solvent and DPPH, with the tests having increasing concentrations of naringenin and DPPH, the percentage radical scavenging activity was calculated by using the formula: 

\[ \text{Inhibition} \% = \left( \frac{A_{Control} - A_{Sample}}{A_{Control}} \right) \times 100 \]

All determinations were performed in triplicates.

(VI) Inhibition of lipid peroxidation by thiobarbituric acid (TBA) assay

i. Preparation of rat brain homogenate

Adult male Wistar rats weighing about 200-250g were used for this study after obtaining clearance from IAEC (JSSMC/IAEC/18/5675/DEC2013). The animals were fed ad libitum with normal research laboratory pellet diet (Hindustan Lever Ltd., India) and water; and maintained in the controlled environment with a standard temperature of (25±2ºC) and humidity with an alternating 12h light/12h dark cycle. The animals used in the present study were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India. The rats were deprived of food 12h prior to sacrifice. The animals were euthanized, and the whole brain (except cerebellum) removed quickly. Next, the whole brain was processed to get 10% homogenate in 0.15M KCl using Teflon homogenizer. The homogenate was filtered to get the clear solution and used as a source of polyunsaturated fatty acids for determining the extent of lipid peroxidation (263).

ii. Lipid peroxidation determination

Lipid peroxidation activity of naringenin was carried out by modified method of Ohkawa et al. (1979) (263). TBA reacts with malondialdehyde (MDA) to form a pink colored chromogen, which is detected spectrophotometrically at 532nm. Naringenin (0-1836.5μM) was added to the brain homogenate (0.5ml), and the mixture incubated with 0.15M KCl for 30minutes. Lipid peroxidation was initiated by adding 100μl of the 15mM FeSO₄ solution. The reaction mixture was further incubated at 37ºC for 30min. An equal volume of 1:1 TBA: TCA (1ml) was added to the above solution followed by the addition of 1ml BHT. This final mixture was heated on a water bath for 20min at 80ºC and cooled, centrifuged and absorbance read at 523nm using a spectrophotometer. The percentage inhibition of lipid peroxidation was calculated by comparing the absorbance of the test with those of controls as per the formula: Inhibition (%) = \left( \frac{A_{Control} - A_{Sample}}{A_{Control}} \right) \times 100.
Objective 2: To study the effect of naringenin on Nrf2 activation in MIN6 pancreatic β-cell line.

(I) Culturing of MIN6 cells

MIN6 is a mouse insulinoma cell line procured from National Centre for Cell Science (NCCS), Pune, India. MIN6, display many important characteristics that are similar to pancreatic islets (264). For example, MIN6 cells are known to exhibit glucose-stimulated insulin secretion (GSIS) (265).

MIN6 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100U/mL penicillin, 100µg/mL streptomycin, and 2.0mM glutamine (Purchased from GE Healthcare, Little Chalfont, United Kingdom) in a humidified carbon dioxide incubator maintained at 37°C with 5% CO₂. MIN6 cells with passage number between 5 to 20 were used for all the experiments (266).

(II) Cell viability assay

The effect of naringenin on cell viability was measured using an MTT assay (267). Experimentally first, MIN6 cells (2 X 10⁴ cells/well in 100µL) plated in 96-well plates, were allowed to grow for 24h in a CO₂ incubator. Next, the growing cells were exposed to increasing concentrations (0-200µM) of naringenin (Sigma Chemical Company, Missouri, USA) for 24h at 37°C. After treatment, cells were replenished with 90µL phenol-red free media containing 10µL MTT (5mM in DMEM) and incubated for additional 3h in the CO₂ incubator. The media was aspirated carefully without disturbing the precipitate. The precipitate was dissolved in 50µL DMSO by keeping at 37°C for 20min. The absorbance of the solution was measured at 540nm using a plate reader (Infinite 1000, Tecan, Mannedorf, Switzerland). The experiments were performed in triplicate. The relative cell viability (%) compared to control cells treated with DMSO was calculated using: Cell viability (%) = \( \frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{control}} - A_{\text{blank}})} \times 100 \). 35% of cell death was found at 200µM, hence further studies we restricted the concentration up to 100µM.

To elucidate the protective role of naringenin on STZ-induced cytotoxicity, first, the cells were pretreated with increasing concentration of naringenin (0-100µM) for 24h. Next, the naringenin-treated cells were exposed to 10mM STZ for 1h and the number of viable cells estimated using MTT assay. All experiments were performed in triplicates.

(III) Luminometric evaluation of Nrf2-Keap1 complementation system with naringenin.

2X10⁴ MIN6 cells/ml were transiently transfected with Nrf2–Keap1 reporter complementation system in a 12-well plate using Lipofectamine 2000 as suggested by the manufactures protocol (Invitrogen, Carlsbad, California). Six hours after transfection, the media was replaced with a fresh batch of medium, and cells treated with naringenin (25, 50, 100µM) for 24 hours. Control and treated cells were lysed in 1X lysis buffer (pH 7.8; Promega, Madison, WI) containing 2mM DTT, 2mM 1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid, 10% glycerol and 1% Triton X-100 by shaking for 10 min. Protein lysates were collected, and the debris separated by centrifuging the lysates for 5min at 10,000g at 4°C. Total protein content in the supernatant was estimated using Bradford reagent (Bio-Rad Laboratories Inc, Hercules, CA). Next, 100µL luciferase substrate reagent, prepared by mixing 10ml of luciferase assay buffer with the lyophilized Luciferin (Promega, Madison, WI), was added to the 20µL of supernatant containing 172.38µg of protein and the luciferase activity measured using a luminometer (Promega, Madison, WI). The sensor system identifies the potential of naringenin to promote the dissociation of the Nrf2–Keap1 complex. A drop in luciferase signal is inversely proportional to Nrf2 activation. The results were presented as fold change of three independent experiments.
(IV) Nuclear and cytosolic fractionation

To study the effect of naringenin on Nrf2 translocation, nuclear and cytoplasmic extracts were prepared using a commercially available nuclear extraction kit (Pierce NE-PER®) as per the manufacturer's instructions (Pierce, Rockford, IL, USA). In brief, cells were homogenized in CER-I buffer using a homogenizer in the pre-extraction buffer and incubated on ice for 15min. The cellular homogenate was centrifuged at 10,000Xg for 10min at 4°C and the supernatant containing cytoplasmic fraction separated. Next, the pellet containing nuclei was suspended in NER buffer (supplemented with protease inhibitors) and the nuclear fraction generated by thorough vortex mixing for 1min with the break for every 10min for 40min. The vortexed samples were centrifuged at 16000Xg for 15min at 4°C, and the supernatant solution was collected as a nuclear fraction and used for western blotting.

(V) Western blotting

The total protein content in the cytosolic and nuclear fractions was estimated using Bradford method (268). BSA was used as a standard for preparing a concentration curve. Principally, The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye shifts from 465nm to 595nm when binding to proteins, mainly to basic and aromatic amino acid residues, especially arginine. The formation of blue protein-dye was detected at 595nm using bio-rad smartspec plus spectrophotometer.

Reagents

1x Dye Reagent-1L of dye solution containing methanol and phosphoric acid; BSA Standard-2 mg/ml.

Procedure

Pipet standard and protein sample (20μl) solution into the 1.0ml disposable cuvettes. Add 1.0ml of 1x dye reagent to each cuvette and vortex. Incubate the samples for 5min at room temperature. Measure the absorbance of the standard (BSA), blank, and samples using spectrophotometer at 595nm. A standard curve was prepared by plotting the 595nm values (y-axis) versus their concentration in μg/ml on the x-axis.

About 100μg protein was denatured using sample buffer consisting of 31.5mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS and 0.005% bromophenol blue at 95°C for 5min, and separated on an SDS-PAGE gradient gel (Biorad, PA, USA). The separated proteins were electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) and probed with primary and secondary antibodies against Nrf2, caspase-3, β-actin and lamin-B (Santa Cruz Biotechnology, CA, USA).

The blots were developed using chemiluminescence (ECL) kit (Biorad, PA, USA) and the signals were captured using gel documentation system (GBOX, Syngene, UK).

(VI) ARE-Luciferase reporter gene assay

NQO1-ARE-Luc and GST-ARE-Luc reporter constructs, provided by Donna D. Zhang (College of Pharmacy, University of Arizona, Tucson, AZ) to Dr. Ramkumar, were used for cell-based reporter gene assay. For more details about the construction of these reporter plasmids refer Ramkumar et al. (2013) (257). ARE-Luc constructs (500ng/well) was transiently transfected into MIN6 cells in 12-well plates using Lipofectamine-2000 as described previously (257). After six hours of transfection, the media was changed and the cells were exposed to 25, 50, and 100μM naringenin for 24h. The cell lysates were collected and the luminometric assay was carried out according to Ramkumar et al. (2013) (257). The increase in the luciferase activity compared to control DMSO-treated cells was represented (257). The results are the averages of at least three independent experiments.
(VII) Statistical analysis

All data were expressed as mean ± SEM of three separate experiments (n = 3). The statistical significance was evaluated by One-way analysis of variance (ANOVA) using SPSS version 20 (SPSS, Cary, NC, USA) followed by Tukey's post hoc test. P<0.05 was considered significant.
c. Objective 3: To evaluate the ability of naringenin-induced Nrf2 to mitigate STZ-triggered pancreatic β-cell apoptosis in MIN6 cells.

(I) Measurement of intracellular reactive oxygen species (ROS)

Levels of intracellular ROS were determined by flow cytometry using an oxidation-sensitive fluorescent dye, 2,7-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) (269). Experimentally, first, MIN6 cells (2 X 10$^4$ cells/well in 100µL) were treated with 50µM and 100µM naringenin in complete medium for 24h. Next, naringenin-treated cells were exposed to 10mM STZ for 1h. Then the cells were incubated with 20µM H$_2$DCFDA (10µL) for 30min at 37°C. The reaction was stopped by washing with phosphate-buffered saline (PBS) containing 10% fetal calf serum (FCS). Cells were pelleted by centrifugation at 800Xg for 10min, washed, and resuspended in 1ml PBS. The resultant fluorescence intensity was measured by FACS analysis (BD Biosciences, CA, USA). Data was analyzed using BD Cell Quest™ Pro Analysis software and the shift in fluorescence intensity caused by DCF production, which is an indicator of ROS generation, represented as a histogram.

(II) Annexin-V-FITC/PI double staining and analysis by flow cytometry

To evaluate the effects of naringenin for protecting MIN6 cells from streptozotocin-induced cell death, the FITC annexin V/Dead Cell Apoptosis Kit with FITC annexin V and PI for Flow Cytometry (Invitrogen), was used (270). Experimentally, MIN6 cells were grown in 6-well plates and treated with naringenin (50 & 100µM) for 24h. Next, naringenin-treated cells were exposed to 10mM STZ. After 1h, the cells were trypsinized, collected and washed with cold PBS. The supernatant was discarded and the cells resuspended in 200µL of the 1X binding buffer, containing 50mM HEPES, 700mM NaCl, 12.5mM CaCl$_2$, pH 7.4. To 100µL cell suspension, 5µL annexin-V-FITC and 1µL of PI solution (100µg/mL) were added. Then, the cells were incubated for 15min at room temperature in dark, and 400µL of 1X annexin-binding buffer added. The stained cells were analyzed by flow cytometry using Cell Quest™ Pro Analysis software.

(III) Statistical analysis

All data were expressed as mean ± SEM of three separate experiments (n = 3). The statistical significance was evaluated by One-way analysis of variance (ANOVA) using SPSS version 20 (SPSS, Cary, NC, USA) followed by Tukey’s post hoc test. P<0.05 was considered significant.
d. Objective 4: To assess the efficacy of Nrf2-upregulating naringenin for protecting mice from STZ-induced diabetes.

(I) Animals and diet

Male *Swiss albino* mice (25-30g b.w) were procured from the central animal facility, JSS Medical College, Mysuru, India. The Institutional Animal Ethics Committee (JSSMC/IAEC/18/5675/DEC2013) approved the study protocol and the animals were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India. The animals were housed in the controlled environment with standard temperature (25±2ºC) and humidity with an alternating 12h light/12h dark cycle. The animals were fed with a balanced commercial diet and water *ad libitum*.

(II) Experimental induction of diabetes

Experimental diabetes was induced by multiple low-dose streptozotocin (MLD-STZ) (266). Briefly, STZ was dissolved in freshly prepared 0.1M citrate buffer (pH 4.5) and injected intra peritoneally (i.p.), at a dose of 50mg/kg/day for 5 consecutive days (266). Blood (50µL) was collected from the tail vein of mice and used to estimate glucose using a glucometer (Accu-Check Active). After two weeks, mice with moderate diabetes, as evidenced by hyperglycemia (i.e, blood glucose concentration range of 250-300mg/dL) were selected for conducting the anti-diabetic effects of naringenin.

(III) Experimental procedure

A total of 36 mice (24 diabetic mice, 12 normal mice) were used for this study. The mice were divided into 6 groups of 6 mice each.

- **Group 1:** Normal control mice received vehicle (0.5% carboxy methyl cellulose) orally.
- **Group 2:** Normal mice received oral administration of naringenin (100mg/kg b.w) suspended in 0.5% carboxy methyl cellulose (CMC) for 45 days
- **Group 3:** STZ control mice received STZ (dissolved in 0.1M citrate buffer, pH 4.5) intraperitoneally (i.p.), at a dose of 50 mg/kg/day for 5 consecutive days
- **Group 4:** STZ mice received oral administration of naringenin (50mg/kg b.w) suspended in 0.5% carboxy methyl cellulose (CMC) for 45 days
- **Group 5:** STZ mice received oral administration of naringenin (100mg/kg b.w) suspended in 0.5% carboxy methyl cellulose (CMC) for 45 days
- **Group 6:** STZ mice received oral administration of glibenclamide (600µg/kg b.w) suspended in 0.5% carboxy methyl cellulose (CMC) for 45 days
At the end of the experiment (after 24h of last naringenin administration), the animals were deprived of food overnight and euthanized using chloroform and sacrificed by decapitation (271). Blood was collected by cardiac puncture in all experimental animals and allowed for clotting. Serum was separated by centrifuging at 3000rpm for 15 min at room temperature and stored at −20°C until analysis. Pancreas, liver, and kidney were quickly excised from the experimental animals and washed in ice-cold saline, patted dry and weighed. In addition, tissues were collected in 10% formalin solution and immediately processed for histological study by the paraffin technique (272). A 10% tissue homogenate of all tissues was prepared in 0.1M sodium phosphate buffer (pH 7.4) and centrifuged at 3000Xg for 10min. The resultant supernatant was used as an enzyme source for the estimation of various parameters.

(IV) Measurement of body weight, food, and water intake

Body weight (in grams) of mice from all experimental groups was monitored every week for 45 days at a fixed time. Food and water intake of mice was also monitored on a daily basis for 45 days. Fixed amount of mice chow and water were given to each mouse and replenished the next day. The dosage was adjusted every week, according to the changes in the body weight to maintain similar dose/kg b.w of mice in each group over the entire period of study.

(V) Measurement of blood glucose and serum Insulin levels

Blood samples from the tail vein of each mice were collected and analyzed for glucose (mg/dL) by Accu-Check active Glucometer. Even though blood glucose levels were measured for every 3 days till the end of the experimental period for 45 days, the results were shown for every 6 days. Serum insulin level was determined using a commercially available ELISA kit from Thermo Scientific, USA.

(VI) Intraperitoneal glucose tolerance test (IPGTT)

IPGTT was carried out at the end of the experimental period with minor modifications to the method described by Goren et al. (2004) (273). Briefly, after an adequate fasting period
(12h), the mice were injected intraperitoneally with glucose at 1g/kg body weight and blood (collected from a tail vein) glucose levels measured at 30, 60, 90 and 120min.

(VII) Estimation of liver function tests

The activities of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT) in serum were measured using commercially available kits (Coral Clinical System, Goa, India).

i. Estimation of serum glutamate oxaloacetate transaminase (SGOT) activity

The SGOT level was determined by a modified IFCC method with SGOT kit, from Coral Clinical Systems, Goa, India. Principally, SGOT catalyzes the transfer of amino group between L-aspartate and α-ketoglutarate to form oxaloacetate and glutamate. The oxaloacetate formed reacts with NADH in the existence of malate dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance at 340nm, which is proportional to the SGOT activity.

\[
\text{SGOT: } \text{L-Aspartate} + \alpha - \text{Ketoglutarate} \rightarrow \text{Oxaloacetate} + \text{L-Glutamate}
\]

\[
\text{MDH: } \text{Oxaloacetate} + \text{NADH} + \text{H}^+ \rightarrow \text{Malate} + \text{NAD}^+
\]

Reagents
L1: Enzyme Reagent
L2: Starter Reagent

Procedure: 1000μL working reagent was added to the 100μL sample in a test tube and mixed well. The reaction mixture was incubated for 1 minute at 37°C and the initial absorbance read at 340nm. After 1 minute the absorbance was recorded at 1st, 2nd and 3rd minute, then mean absorbance change per minute (ΔA/minute) calculated.

Calculation: SGOT activity in U/L at 37°C = \(\Delta A \div \text{min} \times 1746\). Where 1746 is the factor of system parameters.

ii. Estimation of serum glutamate pyruvate transaminase (SGPT) activity

The SGPT level was determined by a modified IFCC method with SGPT kit, from Coral Clinical Systems, Goa, India. Principally, SGPT catalyzes the transfer of amino group between L-alanine and α-ketoglutarate to form pyruvate and L-glutamate. The pyruvate formed reacts with NADH in the presence of lactate dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance at 340nm, which is proportional to the SGPT activity.

\[
\text{SGPT: } \text{L-Alanine} + \alpha - \text{Ketoglutarate} \rightarrow \text{Pyruvate} + \text{L-Glutamate}
\]

\[
\text{LDH: } \text{Pyruvate} + \text{NADH} + \text{H}^+ \rightarrow \text{Lactate} + \text{NAD}^+
\]

Reagents
L1: Enzyme Reagent
L2: Starter Reagent

Procedure: 1000μL of working reagent was incubated with 100μL of the sample in a test tube for 1 minute at 37°C. The initial absorbance is read at 340nm, after 1 minute and the reading repeated at 1st, 2nd and 3rd minutes. The mean absorbance change per minute (Δ A/minute) calculated.

Calculation: SGPT activity in U/L at 37°C = Δ A / min X 1746. Where 1746 is the factor of system parameters.

iii. Estimation of alkaline phosphatase (ALP) levels

Serum ALP level was determined by modified Kind & King's method with ALP kit, from Coral Clinical Systems, Goa, India. Principally, ALP is an enzyme that hydrolyses di sodium phenyl phosphate to form phenol at an alkaline pH 10.0. The phenol reacts with 4- aminoantipyrine in the presence of potassium ferricyanide, as an oxidizing agent, to form a red coloured complex. The intensity of the colour formed is directly proportional to the activity of ALP present in the sample.

\[
\text{Di sodium phenylphosphate} + \text{H}_2\text{O} \xrightarrow{\text{ALP}} \text{Phenol} + \text{di sodium hydrogen hosphate} \\
\text{pH 10.0}
\]

\[
\text{Phenol} + 4\text{-Aminoantipyrine} \xrightarrow{\text{K}_3\text{Fe(CN)}_6} \text{Red coloured complex}
\]

Reagents

L1: Buffer reagent
L2: Substrate reagent
L3: Colour reagent
S: Phenol Standard (10mg/dL)

Procedure: Pipetted 1.0ml distilled water into four clean dry test tubes labeled as Blank (B), Standard (S), Control (C) and Test (T). 0.05ml of distilled water was added to blank (B). Then 1.0ml buffer reagent (L1) and 0.10ml substrate reagent (L2) were added to each test tube, mixed well and allowed to stand for 3min at 37°C. Next, 0.05ml of phenol standard was pipetted into S test tube and 0.05ml of sample into T test tubes. Mixed well and allowed to stand at 37°C for 15min. Then 1.0ml of colour reagent (L3) added to each test tube. Sample control was prepared by the addition of 0.05ml of the sample to control (C) test tube. Contents of each tube were mixed well and absorbance read at 510nm.

Calculation

The activity of ALP (in U/L) was calculated by using formula, \([\text{Abs (T)} - \text{Abs (C)} / \text{Abs (S)} - \text{Abs (B)}] \times 10.\)
iv. Estimation of γ-glutamyl transferase (GGT)

Serum γ-glutamyl transferase level was determined by carboxy substrate method with γ-glutamyl transferase kit, from Coral Clinical Systems, Goa, India. Principally, GGT catalyzes the transfer of amino group between L-γ-glutamyl-3-carboxy-4-nitroanilide and glycylglycine to form L-γ-glutamyl glycylglycine and 5-amino-2-nitrobenzoate. The amount of formation of 5-amino-2-nitrobenzoate was measured as an increase in absorbance at 405nm, which is proportional to the GGT activity in the sample.

\[
\text{L-g-Glutamyl-3-carboxy-4-nitroanilide} \xrightarrow{\text{GGT}} \text{L-γ Glutamylglycylglycine} + \text{Glycylglycine} + \text{5-amino-2-nitrobenzoate}
\]

Reagents

L1: Buffer Reagent
L2: Substrate Reagent

Procedure: 1000μL of working reagent was added to 100μL of the sample in a test tube, mixed well and incubated for 1 min at 37°C. The initial absorbance was read at 405nm, and after 1 minute the readings were measured at 1st, 2nd and 3rd minutes, then mean absorbance change per minute (Δ A/minute) calculated.

Calculation: GGT activity in U/L at 37°C= Δ A/ min × 1158. Where 1158 is the factor of system parameters.

(VIII) Estimation of serum lipid profile

The collected blood was used for the estimation of serum lipid profile. Cholesterol (TC), triglycerides (TG) and high-density lipoprotein (HDL)-cholesterol were determined in the serum samples using commercially available kits (Coral Clinical System, Goa, India). Phospholipids and free fatty acids were determined according to the protocols described by Stewart et al. (1980) (275) and Falholt et al. 1973 (276), respectively. Very low-density lipoprotein (VLDL)-cholesterol and low-density lipoprotein (LDL)-cholesterol were calculated by Friedwald formula. VLDL= TG/5; LDL= TC-(HDL+VLDL).

i. Estimation of serum cholesterol

Serum cholesterol levels were determined using CHOD / PAP method with cholesterol kit, from Coral Clinical Systems, Goa, India. Principally, cholesterol esterase hydrolyses the cholesterol esters into free cholesterol. The free cholesterol is oxidized by cholesterol oxidase to form hydrogen peroxide, which further reacts with 4-aminoantipyrine and phenol and by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. The absorbance of quinoneimine was measured spectrometrically at 505nm and was proportional to cholesterol concentration in the sample.

\[
\text{Cholesterol esters} + \text{H}_2\text{O} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty Acids} \\
\text{Cholesterol} + \text{O}_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestenone} + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + 4 \text{ Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Red quinoneimine dye} + \text{H}_2\text{O}
\]

Reagents
L1: Enzyme Reagent 1  
L2: Enzyme Reagent 2  
S: Cholesterol Standard (200mg/dl)

Procedure: Pipetted 1.0ml working reagent into three clean dry test tubes labeled as Blank (B), Standard (S) and Test (T). 0.01ml of distilled water was added into the blank (B). Then 0.01ml of cholesterol standard into a standard test tube and 0.01ml of sample into T test tubes were added. Mixed well and allowed to stand at 37ºC for 15min. Measured the absorbance of standard and tests against blank at 505nm.

Calculation

\[ \text{Cholesterol in mg/dl} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 200 \]

ii. Estimation of serum triglycerides

Serum triglycerides level was determined by GPO / PAP method with triglycerides kit, from Coral Clinical Systems, Goa, India. Principally, lipoprotein lipase hydrolyses serum triglycerides to glycerol and free fatty acids. In the presence of ATP and glycerol kinase (GK), the glycerol is converted to glycerol-3-phosphate, which is oxidized by the enzyme glycerol phosphate oxidase to yield hydrogen peroxide. Further, the hydrogen peroxide reacts with phenolic compound and 4-aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye which is measured at 505nm. The intensity of the colour formed is directly proportional to a number of triglycerides present in the sample.

\[ \text{Triglycerides} \xrightarrow{\text{Lipoprotein lipase}} \text{Glycerol + Free fatty acids} \]
\[ \text{Glycerol + ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol 3-phosphate + ADP} \]
\[ \text{Glycerol 3-phosphate + O}_2 \xrightarrow{\text{Peroxidase}} \text{Dihydroxyacetone phosphate + H}_2\text{O}_2 \]
\[ \text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine + Phenol} \xrightarrow{\text{Peroxidase}} \text{Red quinoneimine dye + H}_2\text{O} \]

Reagents

L1: Enzyme Reagent 1  
L2: Enzyme Reagent 2  
S: Triglycerides Standard (200mg/dl)

Procedure: Pipetted 1.0ml working reagent into three clean dry test tubes labeled as Blank (B), Standard (S) and Test (T). First, 0.01ml of distilled water was added into the blank (B), then 0.01ml of triglyceride standard was added into test tube labeled “Standard”. Similarly, 0.01ml sample was added into T test tubes added. Mixed well and allowed to stand at 37ºC for 5min. Measured the absorbance of standard and tests against blank at 505nm.

Calculation

\[ \text{Triglycerides in mg/dl} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 200 \]

Where 200 is the standard concentration
### iii. Estimation of serum high-density lipoprotein (HDL)-cholesterol

Serum high-density lipoprotein (HDL)-cholesterol levels determined by PEG / CHOD-PAP method with HDL cholesterol kit, from Coral Clinical Systems, Goa, India. Principally, when the serum reacts with polyethylene glycol (contained in the precipitating reagent), all the VLDL and LDL are precipitated. The HDL remains in the supernatant was assayed as a sample for cholesterol using the cholesterol (CHOD/PAP) reagent.

**Reagents**

L1: Enzyme Reagent 1  
L2: Enzyme Reagent 2  
L3: Precipitating Reagent  
S: HDL Standard (25mg/dl)

**Procedure:** Pipetted 1.0ml working reagent into three clean dry test tubes labeled as Blank (B), Standard (S) and Test (T). 0.05ml of distilled water was added into blank (B). Then 0.05ml of HDL standard was into a standard test tube and 0.05ml of sample into T test tubes added. Mixed well and allowed to stand at 37°C for 5min. Measured the absorbance of standard and tests against blank at 505nm.

**Calculations**

\[
\text{HDL cholesterol in mg/dl} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 25 \times 2
\]

Where 25 is the standard concentration; 2 is the dilution factor

### iv. Low-density lipoprotein cholesterol (LDL - C)

Low-density lipoprotein cholesterol (LDL - C) was estimated by Friedwald et al. (1972) (274) formula which states: LDL cholesterol = TC – (VLDL + HDL cholesterol).

### v. Very low-density lipoprotein cholesterol (VLDL - C)

Very low-density lipoprotein (VLDL) estimated by Friedwald et al. (1972) (274) formula which states: VLDL cholesterol = TG/5.

### vi. Estimation of Phospholipids

Serum phospholipids were estimated colorimetrically by the method of Stewart (1980) (275). Principally, the red inorganic compound ammonium ferrothiocyanate is insoluble in chloroform, but it forms a complex with phospholipids which are freely soluble in chloroform. When the chloroform solution containing phospholipid is mixed intimately with ammonium ferrothiocyanate at room temperature, a coloured complex (Amax=472nm) was formed which partitions in the chloroform phase.

**Reagents**

1. Preparation of ammonium ferrothiocyanate solution (0.1M) - 27.03g of ferric chloride hexahydrate and 30.4g of ammonium thiocyanate were dissolved in double distilled water, and the volume was made up to 1L.  
2. Anhydrous sodium sulfate.
3. Standard makeup: 10ml of a solution of phospholipid in chloroform at a concentration of 0.1mg/ml.

Procedure: To the 500μl of the sample, 2ml chloroform, and 2ml 0.1M ammonium ferrothiocyanate reagent were added, gently mixed, allowed to stand for 5min and centrifuged at 3000 rpm. The absorbance of the lower chloroform layer was read at 472nm. Assay with standard dipalmitoyl phosphatidylcholine (DPPC) was run simultaneously for plotting the standard graph and calculations of phospholipid content.

vii. Estimation of free fatty acids

Serum free fatty acids were estimated by the method of Falholt et al. (1973) (276). Principally, free fatty acids were extracted with the chloroform-heptane-methanol mixture to eliminate interference from phospholipids and the extract was shaken vigorously with a high-density copper reagent. The copper soaps remained in the upper organic layer, from which an aliquot was taken and copper content was determined colorimetrically by treating with diphenylcarbazide.

Reagents

2. Stock copper solution - 500mM: 12.07g copper nitrate was dissolved in 100mL of distilled water.
3. Triethanolamine solution: 1M: 1mL of triethanolamine was made up to 100mL with distilled water.
4. Sodium hydroxide - 1M: Take 40g NaOH, and dissolve in 1.0L water.
5. Copper reagent: 10mL of the copper solution was mixed with 10mL of 1M triethanolamine and 6.0mL of 1M sodium hydroxide and made up to 100mL. To this, 33g of sodium chloride was added and the pH was adjusted to 8.1 before use.
6. Diphenyl carbazide solution: This solution was prepared immediately before use by adding 40mg of diphenyl carbazide and 10mL of ethanol followed by 0.1mL of triethanolamine solution.
7. Standard palmitic acid: A solution containing 2mg/mL of palmitic acid in chloroform was used as stock. Working standard containing 200μg/mL was prepared by diluting 1.0mL of stock to 10mL with chloroform.

Procedure: An aliquot of 0.5mL of lipid extract was evaporated to dryness and dissolved in a 6.0ml chloroform-heptane-methanol solvent. Then 2.5ml of copper reagent was added. All the tubes were shaken vigorously for 90sec and were kept aside for 15min. Then, the tubes were centrifuged and 3.0mL of the upper copper layer was transferred to another tube containing 0.5mL of diphenyl carbazide and mixed carefully. The color developed was read at 540nm against a reagent blank containing 3.0mL solvent and 0.5mL diphenyl carbazide. The free fatty acid content was expressed as mg/dl of serum.

(IX) Estimation of lipid peroxidation in pancreas, liver, and kidney

The pancreas, liver and kidney homogenates were assayed for thiobarbituric acid-reactive substance (TBARS) and lipid hydroperoxides (LPO) by the methods of Niehaus and Samuelson (1968) (277) and Jiang et al. (1992) (278).

i. Estimation of thiobarbituric acid reactive substances (TBARS)
Free radical attack on polyunsaturated fatty acids produces the sequence of reactions resulting in the formation of both conjugated dienes and lipid peroxides (279). Therefore, the extent of lipid peroxidation was assessed by measuring the amount of MDA formed (279). MDA reacts with thiobarbituric acid to form a chromogenic adduct with two molecules of TBA, which was measured at 535nm (280). Hence, the TBARS assay is conducted to assess the levels of lipid peroxidation by the method of Niehaus and Samuelson (1968) (277).

**Reagents**

1. Tris - HCl buffer - 0.025M, pH 7.5
2. 15% w/v trichloro acetic acid (TCA)
3. 0.375% w/v thiobarbituric acid (TBA)
4. 0.25N hydrochloric acid (HCl)
5. TCA-TBA-HCl - reagent (1:1:1)
6. Stock standard: 4.8nmoles of 1,1’,3,3’-tetramethoxy propane was prepared for the preparation of the stock solution.
7. Working standard: the Stock solution was diluted to get a concentration of 48 nmol/ml.

**Procedure:** To 1.0ml of tissue homogenate in potassium chloride buffer, 2ml of TBA:TCA:HCl reagent (TBA 0.375%, 15% trichloroacetic acid, and 0.25N hydrochloric acid in ratio 1:1:1 v/v) was added and boiled for 15min and cooled. The mixture was then centrifuged at 10,000rpm for 5min, the absorbance of the supernatant solution measured at 535nm against a reagent blank and expressed as mM per 100g tissue.

**ii. Estimation of lipid hydroperoxides (LPH)**

Lipid hydroperoxide level in the tissue was estimated by the method developed by Jiang et al. (1992) (278). The oxidation of ferrous ion (Fe²⁺) under acidic conditions in the presence of xylenol orange leads to the formation of a chromophore, which was read at 560nm.

**Reagents**

Fox reagent: 88mg butylated hydroxytoluene (BHT), 7.6mg xylenol orange and 9.8mg ammonium iron (II) sulfate were added to 90ml methanol and 10ml H₂SO₄ (250mM) mixture.

**Procedure:** Briefly, 0.2ml of tissue homogenate was mixed with 1.8ml of Fox reagent and incubated for 30min at room temperature and the absorbance read at 560nm. Lipid hydroperoxide was expressed as mM per 100g tissue.

**(X) Determination of antioxidant status in pancreas, liver, and kidney**

The activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione-s-transferase (GST) and the concentration of reduced glutathione (GSH) in the pancreatic, liver and kidney tissues of the mice were determined as described below.

**i. Superoxide dismutase (SOD)**

The SOD activity in the tissues was assayed according to the method of Kakkar et al. (1984) (281). The assay was based on the inhibition of formation of NADH phenazine methosulphate-nitroblue tetrazolium formazan (281). The reaction was initiated by the addition of NADH. After incubation for 90sec, the reaction was stopped by the addition of glacial acetic acid. The colour formed at the end of the reaction was extracted into butanol.
layer and measured at 560nm.

Reagents

1. Sodium pyrophosphate buffer - 0.02M (pH 8.3)
2. Phenazine methosulphate (PMS) - 186µM
3. Nitroblue tetrazolium (NBT) - 300µM
4. NADH - 780µM
5. Glacial acetic acid
6. Chloroform
8. Ethanol
9. n - butanol

Procedure: First, the assay mixture, containing 0.5ml of tissue homogenate, 1ml of water, 2.5ml of ethanol and 1.5ml of chloroform, was centrifuged at 2000rpm for 5min at room temperature. Next, 0.5ml of the supernatant was mixed with 1.2ml of sodium pyrophosphate buffer (0.025M, pH 8.3), 0.1ml of phenazine methosulphate (PMS) and 0.2ml of NBT (300µM). The reaction was initiated by the addition of 0.2ml of NADH (780µM) diluted to 3.0ml with water. The mixture was incubated at 30°C for 90sec and arrested by adding 1.0ml of glacial acetic acid. The reaction mixture was then combined with 4.0ml of n-butanol, allowed to stand for 10min and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560nm. One unit of enzyme activity is defined as the enzyme, which gave 50% inhibition of NBT reduction in one minute under the assay condition and expressed as specific activity in units/mg protein.

ii. Catalase (CAT)

The catalase activity was assayed by the method of Sinha et al. (1972) (282). Dichromate in acetic acid was reduced to chromic acetate when heated in the presence of hydrogen peroxide with the formation of perchromic acid as an unstable intermediate. Resulted chromic acetate was measured at 620nm. Catalase was allowed to split H₂O₂ for different periods of time. The reaction was stopped by the addition of dichromate-acetic acid mixture and the remaining H₂O₂ was determined by measuring chromic acetate colorimetrically.

Reagents

1. Phosphate buffer: 0.01M, pH 7.0
2. Hydrogen peroxide: 0.2M
3. Potassium dichromate: 5%
4. Dichromate-acetic acid reagent: 1:3 ratio of potassium dichromate was mixed with glacial acetic acid. From this 1ml was diluted again with 4ml of acetic acid.
5. Standard hydrogen peroxide: 0.2mM

Procedure: Briefly, 0.1ml of tissue homogenate was added to the reaction mixture containing 0.9ml of phosphate buffer (0.01M, pH 7.0), 0.4ml of H₂O₂ (0.2M), and 0.4ml H₂O, incubated for the different time period 15, 30, 45 and 60 seconds. The reaction was stopped by the addition of 2ml of dichromate/acetic acid mixture, which was prepared by mixing 5% potassium dichromate with glacial acetic acid (1:3 by volume). All the tubes were heated in boiling water both for 10min and the absorbance was read at 620nm. Catalase activity was expressed in terms of µmoles of H₂O₂ consumed/min/mg protein.

iii. Glutathione peroxidase (GPX)

The glutathione peroxidase activity was determined by the method of Rotruck et al. (1973) (283). GPX catalyzes the decomposition of H₂O₂ or other peroxides (-OOH) with the simultaneous oxidation of glutathione into GSSG. A known amount of enzyme preparation
was allowed to react with H$_2$O$_2$ in the presence of GSH for a specified time period (for 10 min at 37°C). Then the remaining GSH content was measured by the method of Ellman (1959) (284).

Reagents

1. Tris buffer: 0.4M, pH 7.0
2. Sodium azide solution: 10mM
3. TCA: 10%
4. EDTA: 0.4mM
5. H$_2$O$_2$ solution: 0.2mM
6. Glutathione solution: 2mM

Procedure: Briefly, the reaction mixture consisting of 0.2ml of EDTA (0.8mM), 0.1ml of sodium azide (10mM), 0.2mL of tris-buffer and 0.5ml of tissue homogenate was incubated at 37°C for 10 min, along with a control containing all reagents except homogenate. The reaction was terminated by the addition of 0.5ml of 10% TCA and the tubes were centrifuged at 2000rpm. The 1ml supernatant was assayed for GSH by the method of Ellman (1959) (284). The activity of GPX was expressed as µmoles of glutathione oxidised/minute/mg of protein.

iv. Glutathione-s-transferase (GST)

The GST activity was determined by the method of Habig et al. (1974) (285). GST catalyzes the transfer of GSH moieties to acceptor molecules, thereby make the conjugated product more water soluble. The conjugate formed between glutathione and 1-chloro-2,4-dinitrobenzene was read at 340nm.

\[ 2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O} \]
Reagents

1. Phosphate buffer - 0.3M (pH 6.5)
2. Reduced glutathione - 30mM
3. 1-chloro-2,4-dinitrobenzene (CDNB) - 30mM was prepared in ethanol

Procedure: Experimentally the reaction mixture containing 1.0ml of 0.3M phosphate buffer (pH 6.5), 0.1ml of 30mM CDNB and 0.1ml of tissue homogenate was made up to 2.5ml with water. The reaction mixture was pre-incubated at 37°C for 5min and 0.1ml of 30mM GSH was added. Change in absorbance at 340nm was observed for 3min at 30-second interval. The activity of glutathione S-transferase was expressed as μmoles of CDNB conjugate formed/minute/mg of protein.

v. Reduced glutathione (GSH)

The concentration of total reduced glutathione content was assayed by the method of Ellman's (1959) (284). The 5, 5'-dithio-2-nitro benzoic acid (DTNB) reacts with the compounds containing sulphydryl group's leads to the development of a yellow colour and the absorption maxima are measured at 412nm.

Reagents

1. Phosphate buffer: 0.1M, pH 8.0
2. TCA: 5%
3. Ellman's reagent: 34mg of DTNB in 10mL of 0.1% sodium citrate.
4. Disodium hydrogen phosphate: 0.3M
5. Standard glutathione solution: 100mg GSH in 100mL H20.
6. Working standard: Stock was diluted to get a concentration of 100μg/mL.

Procedure: Briefly, 0.5ml of tissue homogenate was de-proteinized with 2ml of 5%TCA and centrifuged at 2000rpm for 5min at room temperature. 1ml of the resulted supernatant was mixed with 3.0ml phosphate Buffer (0.1M, pH 8), 0.5ml of Ellman's reagent and 4.0ml of 0.3M disodium hydrogen phosphate. The developed yellow colour was read at 412nm using spectrophotometer. The amount of glutathione was expressed as mg/100g tissue.

(XI) Estimation of carbohydrate metabolic enzymes and glycogen levels in liver

i. Assay for hexokinase

Hepatic hexokinase activity was determined by the method of Brandstrup et al. (1957) (286). Principally, hexokinase converts D-glucose and ATP to glucose 6-phosphate and ADP. The residual glucose was reacted with an O-toluidine reagent to form green colour, which was measured at 640nm.

Reagents

1. Glucose solution: 0.005M
2. ATP solution: 0.72M
3. Magnesium chloride solution: 0.05M
4. Dipotassium hydrogen phosphate solution: 0.0125M
5. Potassium chloride solution: 0.1M
6. Sodium fluoride solution: 0.5M
7. Tris-HCl buffer: 0.01M, pH 8.0

Procedure: The reaction mixture in a total volume of 5.0mL containing 1.0mL of glucose solution, 0.5mL of ATP, 0.5mL of magnesium chloride, 0.5mL of dipotassium hydrogen phosphate solution, 0.4mL of potassium chloride, 0.1mL of sodium fluoride solution and 2.5mL of Tris-HCl buffer (pH 8.0) was preincubated at 37°C for 5min. The reaction was initiated by the addition of 1.0mL of liver tissue homogenate. A 1.0ml aliquot of the reaction mixture was immediately transferred to the tube containing 1.0mL of 10% TCA which was considered as zero time. A second aliquot was removed after 30min incubation at 37°C and added to tubes containing 1.0mL of 10% TCA. All the tubes were centrifuged to remove the precipitated protein and the remaining glucose in the supernatant was estimated by the O-toluidine method of Sasaki et al. (1972) (287). The supernatant was mixed with 4ml of O-toluidine reagent and was kept in boiling water bath for 15min. The green colour developed was read colorimetrically at 640nm. A reagent blank was run with each test and the difference between the two values gave the amount of glucose phosphorylated. The activity of hexokinase was expressed as μmoles of glucose phosphorylated per min/mg protein.

ii. Assay for glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase was assayed in the liver homogenate as per the method of Ells and Kirkman (1961) (288). Principally, the enzyme assay involves measurement of the increase in absorbance when NADP reduces to NADPH. This reaction takes place when electrons are transferred from glucose-6-phosphate to NADP in the reactions catalyzed by glucose-6-phosphate dehydrogenase.

Reagents

1. Tris-HCl buffer: 0.05M, pH 7.5
2. Magnesium chloride: 0.1M
3. NADP+: 0.1M
4. 2, 6-Dichlorophenol indophenol: 0.01% in distilled water (freshly prepared)
5. Phenazine methosulphate: 0.005% in distilled water (freshly prepared)
6. Substrate: 0.02M glucose 6-phosphate solution

Procedure: The reaction mixture in a total volume of 5.0mL containing 1.0mL of tris buffer, 0.1mL of magnesium chloride, 0.1mL of NADP+, 0.5mL of phenazine methosulphate, 0.4mL of the dye solution and the requisite amount of the enzyme extract was allowed to stand for 10min at room temperature to permit the oxidation of endogenous materials. The reaction was initiated by the adding of 0.5mL of glucose 6-phosphate. The absorbance was read at 640nm against water blank at one min intervals for 3-5min in a UV spectrophotometer. The enzyme activity was calculated in units by multiplying the change in OD/min by the factor 6/17.6, which is the molar extinction co-efficient of the reduced enzyme activity.

Change in OD/min x molar extinction co-efficient x temperature correction factor (Tf).

Temperature correction factor at 37°C is 0.76 and molar extinction co-efficient of NADPH is 6/17.6. The activity of glucose-6-phosphate dehydrogenase was expressed as 10^-4 mlU/mg of protein.
iii. Assay for glucose-6-phosphatase

Glucose-6-phosphatase was assayed in the liver homogenate by the method of Hikaru and Toshitsugu (1959) (289). Principally, it is based on the conversion of glucose 6-phosphate to glucose with the liberation of inorganic phosphorus. The inorganic phosphorus was estimated using Fiske and Subbarow method using ammonium molybdate and amino naphthol sulphonic acid (290). The blue phosphomolybdus complex formed was measured at 680nm.

Reagents

1. Maleic acid buffer: 0.1M, pH 6.5
2. Glucose 6-phosphate: 0.01M in distilled water.
3. Ammonium molybdate solution: 2.5g ammonium molybdate dissolved in 100mL of 3N sulphuric acid.
4. Aminonaphthol sulphonic acid (ANSA) reagent: 500g of ANSA was dissolved in 195mL of 15% sodium bisulphite and 5mL of 20% sodium sulphite was added to it. The solution was filtered and stored in a brown bottle.
5. TCA: 10%

Procedure: The reaction mixture containing 0.3mL of citrate buffer (0.1M, pH 6.5), 0.5 mL of glucose-6-phosphate and 0.2mL of liver tissue homogenate was incubated at 37°C for 1h. Next, 1.0mL of 10% TCA was added to terminate the enzyme activity. The suspension was centrifuged and the phosphorus content of the supernatant was estimated by the method of Fiske and Subbarow (290). To 1.0ml of the supernatant, 1ml of ammonium molybdate and 0.4ml of ANSA were added and incubated for 20min. The blue colure developed was read at 680nm. A tube devoid of the enzyme served as control. A sequence of standards containing 8-40μg of phosphorus was treated similarly along with a blank containing only the reagent. The activity of glucose-6-phosphatase was expressed as μmoles of Pi liberated/min/mg protein.

iv. Assay for fructose-1,6-bisphosphatase

Fructose-1,6-bisphosphatase in the liver homogenate was assayed by the method of Gancedo and Gancedo (1971) (291). Principally, fructose-1,6-bisphosphatase catalyzes the dephosphorylation of fructose-1,6- bisphosphate to fructose-6-phosphate in presence of Mg²⁺. The liberated phosphorus gives a blue colour with ammonium molybdate and ANSA, which was measured at 680nm.

Reagents

1. Tris-HCl buffer: 0.1M, pH 7.0
2. Substrate: Fructose 1,6- bisphosphate, 0.05M
3. Magnesium chloride: 0.1M
4. Potassium chloride: 0.1M
5. EDTA solution: 0.001M
6. TCA: 10%
7. Molybdic acid: 2.5% ammonium molybdate in 3N sulphuric acid.
8. ANSA reagent: As above.
9. Phosphorus stock: 35.1mg of potassium dihydrogen phosphate was dissolved in 100mL of distilled water (80μg/mL).
Procedure: The assay mixture in a final volume of 2.0ml containing 1.0ml tris buffer, 0.4ml of the substrate, 0.1mL each of magnesium chloride, 0.2mL potassium chloride, 0.1mL of EDTA and 0.2mL of enzyme homogenate was incubated at 37°C for 5min. The reaction was terminated by adding 10% TCA. The suspension was centrifuged and the supernatant was used for phosphorus determination by the method of Fiske and Subbarow (290). To 1.0ml of the supernatant, 1.0ml of ammonium molybdate and 0.4ml of ANSA were added and incubated for 20min. The blue colour developed was read at 680nm. The activities fructose-1,6-bisphosphatase was expressed as μmoles of Pi liberated/h/mg protein.

v. Glycogen

Liver glycogen was extracted and estimated by the method of Morales et al. (1975) (292). Principally, glucose is dehydrated by sulphuric acid to furfural derivative which then complexes with anthrone to give a green coloured complex, which is read at 620nm (292).

Reagents

1. Potassium hydroxide: 30%
2. Anthrone reagent: 0.2% of anthrone in concentrated sulphuric acid was prepared just before use.
4. Ammonium acetate: 1M.
5. Stock standard: 1mg/ml -100mg of anhydrous D-glucose was dissolved in 100mL of distilled water containing 0.01% benzoic acid.
6. Working standard: 100μg/ml -10ml of the stock standard was diluted to 100ml with distilled water.
7. Deproteinizing agent: 5mg of TCA (Tri Chloro Acetic Acid) was dissolved in 100ml of distilled water.

Procedure: The alkali extract of the tissue was prepared by digesting 50mg of fresh tissue with 3.0mL of potassium hydroxide (30%) solution in boiling water bath for 15min. The tubes were cooled and a drop of 1.0M ammonium acetate was added to precipitate glycogen and left it in a freezer overnight for complete precipitation. The precipitated glycogen was collected by centrifuging at 3000rpm for 20min. Then, the precipitate was dissolved with the aid of heating and again the glycogen was reprecipitated with alcohol and 1.0M ammonium acetate was added and centrifuged. The absolute precipitate was dissolved in saturated ammonium chloride solution by heating in a boiling water bath for 5min and 4.0mL of anthrone reagent was added and allowed to cool. The tubes were shaken well, covered with marble caps and heated in a boiling water bath for 20min and cooled. The absorbance was read at 620nm against reagent blank. The glycogen content was calculated from the amount of glucose present in the sample and expressed as mg/100g of tissue.

vi. Estimation of protein

The total protein content in the tissue homogenate was measured by the method of Lowry et al. (1951) (293) with BSA as standard. Principally, the CONH groups in the protein react with copper sulphate in an alkaline medium to give a blue coloured complex. The tyrosine and tryptophan residues of protein cause reduction of the phosphomolybdate and phosphotungstate components of Folin Ciocalteau reagent to give bluish products. The absorbance of which is measured at 660nm.

Reagents

1. Alkaline copper reagent
- Solution A: 2% sodium carbonate in 0.1N NaOH
- Solution B: 0.5% copper sulphate
- Solution C: 1% sodium potassium tartarate

50mL solution A was mixed with 0.5mL solution B and 1mL solution C just before use.

2. Folin's phenol reagent: 100g sodium tungstate, 25g sodium molybdate, 700mL water, 50mL 85% orthophosphoric acid and 100mL concentrated HCl were taken in a 1500mL round bottomed flask. The mixture was refluxed gently for 10h. To this 150g lithium sulphate, 50mL water and a few drops of bromine were added and the mixture was boiled for 15min to remove excess bromine. This was diluted 1:2 with double distilled water just before use.

3. Standard bovine serum albumin (BSA): A stock solution was prepared by dissolving 100mg of BSA in 100mL water in a standard flask (293). Small quantities of NaOH were added to complete the dissolution of BSA. 10mL of the stock was diluted to 100mL, to obtain a working standard concentration of 100μg/mL.

Procedure: 0.1mL of tissue homogenate was made up to 1mL with saline, then 1mL 10% TCA was added. The mixture was centrifuged at 160×g for 10min, the supernatant was discarded and the precipitate was dissolved in 1mL of 0.1N NaOH. From this, aliquots were taken for the estimation. 4.5mL of alkaline copper reagent was added to all the tubes and the contents were allowed to stand at 37°C for 10min. Then 0.5mL dilute folin's phenol reagent was added and mixed. The colour intensity was measured in a spectrophotometer at 660nm. A series of standards with a concentration range of 20-100μg and a blank were processed in a similar manner. Values are expressed as mg/gm tissue.

(XII) Histological examination of pancreas, liver, and kidney collected from control and STZ-treated animals

i. Preparation of the paraffinized tissues

Pancreas, liver, and kidney were collected from control and experimental mice and fixed in 10% buffered formalin for 24h (272). The tissues were then dehydrated with alcohol (50% to 100%), xylene and liquid paraffin at 58°C before being embedded in paraffin. Finally, the tissues were embedded in an embedding ring (272). Histopathological examination of pancreas and liver were performed on paraffin-embedded pancreatic and hepatic sections following hematoxylin and eosin (H&E) staining technique.

ii. Immunohistochemical staining for insulin

Paraffin sections were serially cut at the 5µm thickness and placed on microscope slides coated with poly-L-lysine (Sigma, USA). The tissue was fixed on the microscope slide in a 37°C oven for 1h. Then, the tissues were deparaffinized by rinsing in xylene three times and dehydrated with rinsing in absolute alcohol for two times and rinsing two times in 95% ethanol for 3min each before being washed with phosphate buffer saline (PBS) and distilled water for 5min each. The tissue sections were then incubated for 15min in 3% H₂O₂ in methanol to quench the endogenous peroxides. The sections were then washed with PBS for 5min and excess PBS was wiped from surrounding regions. The sections were blocked by incubating with diluted normal serum for 20min and excess serum was blotted from the sections. For insulin detection, sections were incubated with primary antibody (Rabbit polyclonal anti-insulin antibody from Cell Signaling, MA, USA) which is diluted to 1:100 in PBS for 60min and sections washed with PBS for 5min. Excess PBS was wiped from the slides. Then, the sections were treated by an indirect immunoperoxidase technique using a One-Step Polymer-HRP Detection kit (Biogenex, Netherlands) with secondary antibodies using hematoxylin as the counter stain. Evaluation of immunohistochemical staining was
made by examination of 10 islets for each group of mice using a Confocal Scanning Microscopes (Carl Zeiss, Germany).

(XIII) Statistical analysis

All data were expressed as mean ± SEM of six separate experiments (n = 6). The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 20 (SPSS, Cary, NC, USA) followed by Tukey’s post hoc test. P<0.05 was considered significant.