Chapter-3

Materials & Methods

1. Chemicals and Reagents

Streptozotocin (STZ), coomassie blue G250, reduced glutathione (GSH), oxidized glutathione (GSSG) and pyrogallol were purchased from Sigma–Aldrich Chemicals Pvt. Ltd. (New Delhi, India). Methanol (AR grade), ethanol, bovine serum albumin, hydrogen peroxide, ethylene diamine tetra acetic acid, tris-buffer, thiobarbituric acid and tricarboxylic acid were purchased from E Merck Ltd. (Mumbai, India). Sulphuric acid, phosphoric acid, potassium hydroxide, sodium phosphate monobasic, sodium phosphate dibasic, reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxidized nicotinamide adenine dinucleotide phosphate (NADP), were obtained from SRL India. Primary antibodies used were (1) mouse monoclonal antibody to human cellular fibronectin which cross reacts with human, mouse, rat, dog, and monkey (Abcam, Cambridge, MA, USA), (2) Rabbit polyclonal antibody to collagen type IV which reacts with human, mouse, rat, cow, and pig (Abcam, Cambridge, MA, USA), (3) rabbit polyclonal antibody to TGF-β which detects TGF- β1, TGF- β2, and TGF- β3 of mouse, rat, and human origin (Santacruz Biotechnology, Santacruz, CA, USA).TRF from palm oil was purified in our lab by standardized method mentioned in section 4, protected from light, and sealed under a nitrogen atmosphere. Tocopherol and tocotrienol standards were obtained as isomer kits from Merck (Darmstadt, Germany). HPLC grade solvents (n-hexane and 1, 4 dioxane) were also purchased from Merck. Other chemicals used in the study were of analytical grade of highest purity, and procured from standard commercial sources.

2. Instruments

Following equipments were used in the study: Waters Alliance HPLC system (Waters, Milford, MA, USA), Spectrophotometer (Perkin Elmer Bio-Lambda 20 and Optizen 3220 UV-Bio), Cooling centrifuge (Sorvall, RT- 6000B and REMI 30), Boiling water bath (Varco), Deep freezer (-80^{0}C and -20^{0}C), Electronic balance (Sartorius and Precisa), Water bath shaker (NSW-133), Homogenizer (Heidolph Diax 900), pH meter (Merck), Oven (IFB), Refrigerator (Godrej), Incubator (Scientific Systems).

3. Collection and storage of oil

Edible, refined palm oil and rice bran oil, used in this study was purchased from the local market in Delhi, stored in amber colored glass bottles, and used by the "best before date".

4. Extraction of tocotrienol rich fraction (TRF) from palm oil (PO) and rice bran oil (RBO)

4.1. Palm oil TRF

TRF used in the feeding regimes was isolated from refined edible grade PO (Ruchi Gold, Ruchi Soya Industries Ltd., Indore, India) by extraction in ethanol (Ng et al., 2004). Briefly, 4.0 ml of ethanol was added per gram of PO and stirred for 1 h. After 1 h, the ethanol layer containing the TRF was separated and evaporated at 65°C under vacuum in a rotary evaporator and TRF was recovered. The percent yield of TRF isolated from PO was 5.5 ± 0.37 for ten separate preparations. The purity of TRF was determined by high performance liquid chromatography (HPLC).

4.2. Rice bran oil TRF

TRF used in the feeding regimes was isolated from refined edible grade RBO (Rice Gold, Sethia oils Ltd, Bardhaman, India) by extraction in methanol (Budin et al., 1995). Briefly, 7.0 ml of methanol was added per gram of RBO and stirred for 1 h. After 1 h, the methanol layer containing the TRF was separated and evaporated at 65 °C under vacuum in a rotary evaporator and TRF was recovered. The percent yield of TRF isolated from RBO was 4.4 ± 0.37 for 10 separate preparations. The purity of TRF was determined by HPLC.

4.3. HPLC Analysis

The purity of TRF was determined within one week of storage by normal phase HPLC at room temperature. The analysis was carried out in a Waters Alliance HPLC system (Waters, Milford, MA, USA) equipped with a 1525 binary pump and injector fitted with

a 20 µl sample loop, a 2475 multi-wavelength fluorescence detector (Waters, Milford, MA, USA) and Empower ™ 2 software for instrument control and data processing. The sample was injected into a silica column (Sunfire Prep Silica column, 5-µm particle size, 4.6 mm i.d. x 25 cm) with the solvent system of n-hexane and 1,4 dioxane (95:5 by volume) and a flow rate of 2.5 mL min^{-1}. The detector was operated using an excitation length of 296 nm and an emission wavelength of 330 nm. The results were analysed based on the area under the peak. The tocols content obtained for PO-TRF was 29 mg% α-T3, 35 mg% γ- T3, 13 mg% δ- T3 and 23 mg% α-TOC whereas the tocols content obtained for RBO-TRF was 14.5 mg% α- T3, 2.3 mg% β- T3, 6.2 mg% γ- T3, 6.2 mg% δ- T3, 39.2 mg% TOC and 31 mg% unidentified T3, TOC and other unsaponifiable matter.

5. Animals

Male Wistar rats with body weight (bw) of 175-250 g, bred in the Central Animal House of Jamia Hamdard (Hamdard University), New Delhi, India were used for the study. All animals were housed in colony cages maintained at an ambient temperature of 25 ± 2 °C on 12 h light/dark cycle and relative humidity of 45–55%. They had free access to standard rodent pelleted diet (Hindustan Lever Ltd., Bombay, India) and water *ad libitum*. The experimental protocols were approved and conducted in accordance with the Institutional Animal Ethical Committee of Hamdard University that is fully accredited by the Committee for Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Chennai, India.

6. Sample collection and tissue preparation

The urine samples were collected over a period of 24 hours and maintained under mineral oil to avoid evaporation. Post collection, the urinary volume was measured. Blood was collected retroorbitally from the inner canthus of the eye using micro hematocrit capillaries (Top-Tech Biomedicals, India) and the rats were sacrificed by cervical dislocation under light ether anesthesia. The animals were perfused transcardially through ascending aorta with normal saline. The left kidney was rapidly removed for homogenate

preparation and perfusion was continued using 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The right kidney was then immediately removed and preserved in 10% buffered formalin solution for histopathological examinations. Simultaneously, the pancreas were also dissected out and preserved in 10% buffered formalin solution for histopathological examinations. Slices of left kidney, approximately 0.3 g pieces, were homogenized with a Polytron homogenizer (Kinematica A.G.), in 3ml of ice-cold phosphate buffer (pH 7.4) to give a 10% homogenate (1g/10 ml). The homogenate was centrifuged at 800×g for 5 min at 4°C to separate the nuclear debris and was used for estimation of thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA). The supernatant was further centrifuged at 10,000×g for 20 min at 4°C to get the post-mitochondrial supernatant (PMS), which was used for antioxidant enzyme assays.

7. Biochemical Analyses

7.1. Determination of blood glucose

Blood glucose concentration was measured by a strip-operated glucometer (Ascensia ENTRUST, Bayer Polychem Ltd. Thane, India).

7.2. Determination of percentage glycosylated haemoglobin (HbA1c %)

HbA1c % was determined in EDTA-blood samples using commercial assay kit from Crest biosystems, a division of Coral clinical systems, Goa, India. The estimation was done according to the instructions of manufacturer.

7.3. Determination of alpha Amylase

The level of alpha amylase in serum was estimated using commercial assay kit from SPAN Diagnostics Ltd. (Surat, India). The estimation was done according to the instructions of manufacturer and expressed as IU (International Units).

7.4. Determination of serum insulin

Insulin was determined in serum samples using Ultra sensitive rat insulin ELISA kit from Crystal chem inc. (USA). The estimation was done according to the instructions of manufacturer and expressed as μIU/ml. Homeostasis model assessment–insulin resistance (HOMA-IR) score was calculated to estimate the insulin resistance by using the following formula: [insulin (μIU/ml) × fasting glucose (mmol/L)]/22.5 (Haffner et al., 1997).

7.5. Determination of serum lipid profile

The level of triglycerides, total cholesterol and HDL-cholesterol were estimated using commercial assay kits from SPAN Diagnostics Ltd. (Surat, India), while, LDL-C and VLDL-C were calculated using the formula of Friedewald et al (1972). The estimations were performed according to the instructions of the manufacturer and the data were expressed in mg/dl.

7.6. Evaluation of renal function

Glomerular filtration rate (GFR) was estimated through the creatinine clearance and blood urea nitrogen. Proteinuria which is a sensitive indicator of renal damage at the glomerulus or tubular epithelium was measured as total protein (mg/24 hr) in urine. Serum and urinary nitric oxide (NO) was also estimated owing to the the role of NO in the maintainence of renal perfusion and glomerular filtration as implicated in several studies.

7.6.1. Creatinine

Creatinine was measured in urine and serum by the Jaffe reaction using commercial assay kit from SPAN Diagnostics Ltd. (Surat, India). The estimation was done according to the instructions of manufacturer and expressed as mg/dl. Clearance was calculated by the standard equation as previously described by Arreola–Mendosa et al (2006).

7.6.2. Blood urea nitrogen (BUN)

BUN level was measured in serum by the diacetyl monoxime method using commercial assay kit from SPAN Diagnostics Ltd. (Surat, India). The estimation was done according to the instructions of manufacturer and expressed as mg/dl.

7.6.3. Serum and urinary nitric oxide, NO (NO_2^-+NO_3^-)

NO_2^- + NO_3^- can be taken as an index of NO production because NO has a very short half life while its oxidation products, NO_2^- and NO_3^- are relatively stable. The quantification of total NO was done by the help and instructions of colorimetric nitric oxide assay kit from Calbiochem, Merck Ltd. (Mumbai, India) which is based on assay described by Bories and Bories (1995). The method involved enzymatic conversion of nitrate to nitrite by nitrate reductase from Aspergillus species followed by the spectrophotometric quantitation of nitrite levels using Griess reagent. The NO values were expressed as nmol/min in urine samples and as µmole/lit in serum samples

7.6.4. Proteinuria

Total protein was estimated in the urine by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard. Briefly10, 20, 40, 60, 80 and 100 µl of stock BSA standard solution (1 mg/ml) was pipetted in duplicate in different testubes and the final volume was made to 100 µl with distilled water. Then 5 ml of Bradford reagent was added to each tube and mixed well avoiding formation of foam. Bradford reagent was used as the standard blank. Absorbance was recorded at 595 nm between 2 min. to 1 hour and calibration curve was drawn. To estimate the protein in sample, 20 µl of sample was taken whose final volume was made to 100 µl followed by addition of 5 ml of Bradford reagent. The absorbance at 595 nm was taken and the corresponding concentration was derived from the standard calibration curve.

7.7. Assessment of oxidative stress in kidney tissue

7.7.1. Assay for TBARS

The method of Utley et al (1967), with some modification was used to estimate the rate of lipid peroxidation (LPO). Tissue homogenate (0.25 ml) was pipetted into 15 mm×100 mm test tubes and incubated at 37°C in a metabolic shaker for 1 h. An equal volume of homogenate was pipetted into a centrifuge tube and placed at 0°C. After 1 h of incubation, 0.5 ml of 5% (w/v) chilled trichloroacetic acid (TCA), followed by 0.5 ml of 0.67% TBA (w/v) was added to each test tube and centrifuge tube, and centrifuged at 1000×g for 15 min. Thereafter, the supernatant was transferred to other test tubes and placed in a boiling water bath for 10 min. The absorbance of pink color produced was measured at 535 nm in a spectrophotometer. The TBARS content was calculated by using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nmol of TBARS formed min^{-1} mg^{-1} of protein.

7.7.2. Assay for MDA

MDA which is a measure of the end product of LPO was measured as described by Ohkawa et al (1979). Briefly, the reagents 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.8% TBA and 0.2 ml of 8.1% sodium dodecyl sulfate were added to 0.1 ml of homogenate. The mixture was heated at 100°C for 1 h and then cooled with tap water followed by addition of 5ml of n-butanol: pyridine (15:1%, v/v) and 1ml of distilled water. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was withdrawn, and absorbance was measured at 532 nm using a spectrophotometer. The amounts of MDA formed in each of the samples were expressed as nmol of MDA formed h^{-1} mg^{-1} of protein by using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

7.7.3. Assay for Superoxide dismutase (SOD)

SOD activity was measured according to the method of Marklund and Marklund (1974). The assay mixture consisted of 2.875 ml of Tris–HCl buffer (50 mM, pH 8.5), 25 µl of pyrogallol (24 mM in 10 mM HCl) and 100 µl of PMS in a total volume of 3 ml. The enzyme activity was measured at 420 nm and expressed as units mg^{-1} protein. One unit of enzyme is defined as the enzyme activity that inhibits autoxidation of pyrogallol by 50%.

7.7.4. Assay for Catalase

Catalase activity was assayed by the method of Claiborne (1985). The assay mixture consisted of 1.9 ml of phosphate-buffer (0.05 M, pH 7.0), 1 ml of hydrogen peroxide (0.019M), and 0.1 ml of 10% PMS in a total volume of 3 ml. Changes in absorbance were recorded kinetically at 240 nm. Catalase activity was expressed as nmol H_2O_2 consumed min^{-1} mg^{-1} protein using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

7.7.5. Assay for Glutathione Peroxidase (GPx)

The GPx activity was measured by the method of Mohandas et al (1984). The reaction mixture consisted of 1.49 ml phosphate buffer (0.1 M, pH 7.4), 0.1 ml of EDTA (1 mM), 0.1 ml of sodium azide (1 mM), 0.05 ml of GR (1 eu/ml), 0.05 ml of GSH (1 mM), 0.1 ml of NADPH (0.2 mM), and 0.01 ml of H_2O_2 (0.25 mM) and 0.1 ml PMS (10%) in a total volume of 2.0 ml. Disappearance of NADPH at 340 nm was recorded at 25°C. The enzyme activity was calculated as nmol NADPH oxidized min^{-1} mg^{-1} protein using molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

7.7.6. Assay for Glutathione Reductase (GR)

The GR activity was measured by the method of Carlberg and Mannervik (1975), as modified by Mohandas et al (1984). The assay system consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml GSSG (1 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml PMS (10%) in a total volume of 2.0 ml. The enzyme activity was

quantified at 25°C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized min^{-1} mg^{-1} protein using molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

7.7.7. Protein assay

Total protein was estimated in the homogenate and PMS by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

8. Western immunoblot analysis of TGF β, Fibronectin and Type IV Collagen

Kidney tissue was homogenized in RIPA(Radio Immuno Precipitation Assay) buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 20 μg/mL aprotinin, 20 μg/mL leupeptin, 0.1% SDS and 1% NP-40] for 30 minutes. Whole kidney lysates were centrifuged at 12,000×g for 20 minutes at 4°C to obtain supernatant. Protein concentration was estimated in supernatant by using Bradford reagent. For immunoblotting, equal amounts of protein (40 μg) per lane were loaded and electrophoresed under reducing conditions on SDS-PAGE gels, then transferred onto nitrocellulose membrane. After blocking in 5% skimmed milk for 1-2 h, membranes were rinsed twice with Tris-buffer saline [150 mmol/L NaCl, 10 mmol/L Tris-HCl, and 0.1% Tween 20]. This was followed by overnight incubation at 4°C with antibodies to fibronectin [1:5000], type IV collagen [1:1000], or transforming growth factor-β (TGF-β) [1:1000]. The blots were incubated with horseradish peroxidase-conjugated bovine antigoat IgG [1:2000] for 1 h at room temperature. Detection by the chemiluminescence reaction was carried out for 4–5 min using the enhanced chemiluminescence western blot detection solution (Amersham Life Science, Buckinghamshire, UK) and exposed to X-ray film. Films were scanned, and band densities were quantified with densitometric analysis using Scion Image program (Epson GT-X700, Tokyo, Japan). All values were normalized by setting the density of control samples as 1.0. The mean density values were expressed as ratios relative to that of control group. β Actin [1:3000, BD, USA] was used as a loading control.

9. Histopathological analysis of tissues

Kidney and pancreas preserved in 10% buffered formalin solution were used for histopathological examinations. After fixation in 10% buffered formalin solution, thin slices of kidney tissue with cortex and medulla and the whole pancreatic tissue were dehydrated and embedded in paraffin. At least four cross-sections of 3-4 μm thickness were taken from each kidney and pancreas and then stained with Jones periodic acid-Schiff (PAS) and hematoxylin and eosin (H and E) respectively. Following two changes of xylene washes of 2 min each, tissue sections were mounted with DPX mountant. The slides were then observed for bright field microscopic evaluation. Microphotographs were taken using an Olympus BX50 microscope system (Olympus, Japan). The PAS stains of kidney were used to assess the extent of mesangial expansion, glomerular lesions, the presence or absence of any proteinaceous casts in the tubules, tubular atrophy and lymphocytic infiltration. The H and E stains of pancreas were used to assess the extent of damage to the islet structure.

10. Statistical analysis

Data are expressed as mean ± standard deviation (S.D.) in case of biochemical analyses and mean ± standard error of mean (S.E.M) in case of western blot analysis. The statistical significance of difference between the experimental groups was calculated by one way ANOVA followed by Tukey–Kramer tests in case of more than 2 experimental groups, whereas two tailed p value was calculated by unpaired t test in case of only 2 experimental groups. Analyses were performed using the statistical software Graph Pad InStat v 3 (San Diego, CA).