3. MATERIALS AND METHODS

3.1. Materials

3.1.1 Assay kits, primers, antibodies, chemicals, reagents and solvents

Assay kits for glucose and HDL-C were obtained from Agappe Diagnostics Pvt. Ltd., Kerala, India and for insulin from Monobind Microwells Inc., CA, USA. cTnI and cTnT immunoassay kits were procured from Roche Diagnostics, Basel, Switzerland and VITROS Immunodiagnostic products, NY, USA respectively. cDNA conversion kit was purchased from GeNei, Bangalore, India. Enhanced chemiluminescence (ECL, Super Signal West Pico Chemiluminescent Substrate) assay kit was obtained from Thermo Scientific, PA, USA. TriZol reagent was purchased from Genei, Bangalore, India and cDNA conversion kit and Taq DNA polymerase were purchased from Thermo Scientific, PA, USA respectively.

The primers used in this study were purchased from Sigma-Aldrich Pvt. Ltd., MO, USA. The list of primers employed for qRT-PCR is given in Table 1.

Antibodies against IR-8, IRS-1, phosphotyrosine, phospho Thr308 Akt, total Akt, AMPK and phospho Thr172 AMPK and GLUT-4 antibodies were purchased from Cell Signaling Technology, MA, USA and antibodies for PI3K, Na\(^+\)K\(^+\)ATPase, caspases -9 and -3, BAX, BCL-2, and cytochrome c were purchased from Santa Cruz Biotechnology CA, USA. Antibodies against MMPs -9 and -2 were purchased from Abcam, Cambridge, UK. TGF-81 antibody (Santa Cruz Biotechnology, CA, USA), and TIMP-2 (Santa Cruz Biotechnology, CA, USA) were given as gift by Dr. Ram Sharma, Administrative Officer for Research, Kansas City, VA
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Medical Center, MO, USA, Dr. Farabegoli, Department of Experimental Pathology, University of Bologna, Bologna, Italy respectively. Antibodies against α-SMA (IA4 Clone) and TIMP-1(R and D systems, MN, USA) were obtained from Dr. Christine Chaponnier, Department of Pathology and Immunology, Faculty of Medicine, Centre Médical Universitaire, Genève 4, Switzerland and Dr. Ningjun Li, Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA respectively. p22 phox NADPH oxidase subunit antibody was provided by Dr Serfozo Hungarian Academy of Science, Tihany, Hungary. Anti-UCP-2 and anti-UCP-3 antibodies were purchased from Sigma-Aldrich Pvt. Ltd., MO, USA. Anti-rabbit, anti-goat and anti-mouse secondary antibodies were purchased from Genei, Bangalore, India.

TX (C₃₃H₄₂O₁₉), 2’, 7’-dichloro dihydro fluoresein diacetate (DCFH-DA), rotenone, coenzyme Q1 and Q2, antimycin A, dodecyl-β-D-maltoside, cytochrome c, cardiolipin (bovine heart) and gelatin were purchased from the Sigma Chemical Company, St. Louis, MO, USA.

Fine chemicals, reagents, solvents of analytical grade, stains for histology and other chemical consumables were purchased from Himedia Laboratories, Pvt. Ltd., Mumbai, India or Sisco Research Laboratories Pvt. Ltd., Mumbai, India.

3.1.2. Animals

Adult male Mus musculus albino mice of Swiss strain weighing 25-30 g were used for the study. Animals were obtained and maintained in the Central Animal House, Rajah Muthiah Medical College and Hospital (RMMC and H), Annamalai Nagar, Tamil Nadu, India. The animals were housed under hygienic conditions (22–24°C) in polypropylene cages and maintained on a 12 h light/ 12 h dark cycle. Animals had free access to standard pellet diet and water. The animals
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were cared according to the guidelines of the Institutional Animal Ethical Committee (IAEC) in accordance with the Indian National Law on Animal Care and Use and this study protocol was approved by the IAEC, RMMC and H (Reg. No. 160/1999/CPCSEA-888).

3.1.3. Diet preparation

The HFFD prepared in the laboratory had the following ingredients (g/100 g): fructose 45.0, groundnut oil 10.0, beef tallow 10.0, casein 22.5, DL-methionine 0.3, wheat bran 5.5, vitamin mixture 1.2 and mineral mixture 5.5. The composition of mineral mix (g/kg)- MgSO$_4$ 7H$_2$O-30.5; NaCl-65.2; KCl-105.7; KH$_2$PO$_4$-200.2; 3MgCO$_3$-3.65, Mg (OH)$_2$3H$_2$O-38.8; FeC$_6$H$_5$O$_7$5H$_2$O-40.0; CaCO$_3$-512.4; KI-0.8; NaF-0.9; CuSO$_4$.5H$_2$O-1.4; MnSO$_4$.0.4 and CONH$_3$.0.05. One kg of vitamin mix contained thiamine mono nitrate, 3 g; riboflavin, 3 g; pyridoxine HCl, 3.5 g; nicotinamide, 15 g; d-calcium pantothenate, 8 g; folic acid, 1 g; d-biotin, 0.1 g; cyanocobalamin, 5mg; vitamin A acetate, 0.6 g; $\alpha$-tocopherol acetate, 25 g and choline chloride, 10 g.

The standard diet, commercially obtained from Sai Enterprise, Chennai contained 60% (w/w) starch, 22.08% (w/w) protein and 4.38% (w/w) fat. The standard diet provided 382.61 cal/100 g while HFFD provided 471.25 cal/100 g.

3.2. Methods

3.2.1. Study design 1-Dose fixation study

In this study protocol, 36 mice were randomly divided into six groups with 6 mice in each group. The experimental period was 60 days. The experimental design is shown in Fig 7.
Fig. 7 Schematic representation of dose fixation study

At the end of 15 days, plasma glucose and insulin levels were estimated in all the groups (data not shown). The plasma glucose and insulin values indicated the development of insulin resistance in HFFD-fed mice. Then the mice were subjected to oral administration of different concentrations of TX (50, 100, 150 and 200 mg/kg bw) for the next 45 days. At the end of 60 days, animals were sacrificed by cervical dislocation. Blood was collected in fresh vials containing EDTA. Among the four doses used, 150 and 200 mg/kg bw of TX was effective in maintaining body weight, plasma glucose and insulin levels (data given in Table 2). Thus, 150 mg/kg bw (lower dose) was fixed as the optimum dose for further investigations.
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