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4.1. Chemicals

Chrysin (purity 97%), STZ and NA were procured from Sigma Chemicals Co., (St. Louis, MO, USA). IRS-1 and IRS-2 antibody were purchased from Cell Signalling Technology, Inc. GLUT-2 and GLUT-4 was purchased from Santa-Cruz Biotechnology, Inc, USA. All other chemicals, reagents and solvents used for the experiments were of analytical grade of highest purity and were obtained from standard commercial suppliers.

4.2. Experimental animals

Healthy adult male albino rats weighing 150-180g obtained from Sri Venkateshwara Enterprises, Bangalore were used for the experiment. Animals were acclimated to laboratory housing conditions (25 ± 2°C) with 12 h light and dark cycle, fed on standard pellet diet supplied by Sai Durga Feeds and Foods, Chennai, India. Animals were housed in polypropylene cages with stainless steel grill top, bedded with rice husk and free access to diet and water ad libitum. The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and the experimental protocols were performed after the approval of the Institutional Animal Ethical Committee (IAEC) of Bharathidasan University (Reg. No. BDU/IAEC/11/2013/09.04.2013).
4.3. Experimental induction of type 2 diabetes in rats

STZ (45 mg/kg b.w.) was dissolved in cold citrate buffer (0.1 M, pH 4.5) and NA (110 mg/kg b.w.) was dissolved in normal physiological saline. NIDDM was induced in 12 h fasted experimental rats by single intraperitoneal (i.p.) injection of freshly prepared STZ followed by i.p. administration of NA 15 min later (Masiello et al., 1998). STZ-NA injected animals were given 10% glucose solution for 24 h to overcome initial drug-induced hypoglycemia. Animals exhibited hyperglycemia within few days. Diabetic rats were confirmed by measuring the elevated glucose level in blood and exhibited glycosuria, 7 days after injection with STZ. The animals with glucose above 280 mg/dl were selected for the experiment.

4.4. Preparation of chrysin for oral administration

The vehicle solution was prepared as 0.5% dimethyl sulphoxide (DMSO). Chrysin (50 mg/kg) was suspended in vehicle solution and administered to rats by force feeding through oral gavage tube. The animals were fasted 30 min before and after the treatment to ensure maximum bioavailability (Sridhar et al., 2005).

4.5. Preliminary study- Dose determination for chrysin

Animals were divided into five groups, with 6 rats in each group. Group I served as Normal control, group II served as diabetic control, group III, IV, V served as diabetic rats treated with chrysin at the dose of 25 mg/kg, 50 mg/kg and 100 mg/kg respectively each day once for 28 days. The blood glucose was determined at the end of 28th day. Chrysin at the dose of 50 mg/kg and 100 mg/kg exhibited a maximum glucose lowering effect. Hence, optimum dose of 50 mg/kg was confirmed as effective dose for the whole study.

4.6. Experimental design

The experimental animals were divided into five groups, each group comprising 6 rats as given below.
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**Group 1:** Normal control rats with vehicle alone (0.5% DMSO).

**Group 2:** Normal rats treated with chrysin (50 mg/kg/day) in 0.5% DMSO orally administered for 28 days.

**Group 3:** STZ–NA induced diabetic rats without treatment.

**Group 4:** STZ–NA induced diabetic rats treated with chrysin (50 mg/kg/day) in 0.5% DMSO, administered orally for 28 days.

**Group 5:** STZ–NA induced diabetic rats treated with glibenclamide (5 mg/kg/day) in aqueous suspension, orally administered for 28 days.

According to the changes in body weight, the dosages were adjusted every week to maintain similar dose per kg b.w of rats over the entire period of study for each group. No irritation or restlessness was observed after each drug or vehicle administration. No noticeable adverse effect (i.e., respiratory distress, abnormal locomotion and catalepsy) was observed in any animal after the drug administration. Fasting blood glucose level was estimated every week to ascertain the status of diabetes in different groups of rats.

4.7. Preparation of the samples

After 28 days of treatment, the rats were deprived of food overnight, anaesthetized with ketamine (90 mg/kg b.w. i.m.) and sacrificed by cervical decapitation. Blood was collected in two different tubes i.e., one with whole blood for serum separation and another with anticoagulant-heparin for plasma separation centrifuged at 4,000 rpm for 20 min using refrigerated centrifuge at 4°C. Serum and plasma were assayed either immediately or stored at −80°C. A portion of pancreas, liver, kidney, skeletal muscle and adipose tissue were excised immediately washed with
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ice-cold saline, cleared off the adherent lipids, patted dry by pressed gently between the folds of blotting paper and were stored in liquid nitrogen container to avoid protein degradation for molecular studies. For biochemical analysis 1 gm of appropriate tissue from each rat was weighed, cut in to fragments and homogenized 10% (w/v) in 0.1M Tris–HCl buffer (pH 7.4) using Potter-Elvehjem homogeniser with a teflon pestle and centrifuged at 7,000 rpm for 10 min at 4°C. Remove the debris and the supernatant was used as enzyme source for assays. For histopathological examination, small pieces of pancreas, liver and kidney tissues from all the animals were immediately fixed in 10% formalin solution.

4.8. Hypoglycemic effect of chrysin on STZ-NA induced experimental T2DM rats

4.8.1. Body weight, food and water intake

Body weight, food and water intake of all groups of animals were monitored on a daily basis for 28 days at a fixed time. Fixed amount of rat chow and fluid was given to each rat and replenished the next day.

4.8.2. Oral Glucose Tolerance Test

Diabetic rats were administered with chrysin 50 mg/kg up to 28 days and after treatment fasting blood sample was taken from all groups. After 30 min of the final drug administration all the rats were orally administered with 2mg/kg of glucose solution. Blood samples were collected at 30, 60, 90 and 120 min intervals after the administration of glucose. Blood glucose was analysed by using reactive strips and glucometer (Bayer Contour TS Glucometer, Bayer health care, USA).
4.8.3. Estimation of serum glucose

Principle

Glucose was oxidized by the enzyme glucose oxidase (Diagnostic Kit–Reddy’s Laboratories, India) to give D-Gluconic acid and hydrogen peroxide. Hydrogen peroxide, in the presence of the enzyme peroxidase oxidized phenol, combine with 4-aminoantipyrine to produce a pink-coloured quinoneimine dye. The intensity of the colour produced was proportional to glucose concentration in the sample.

Glucose oxidase

\[
\text{D-Glucose} + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{D-Gluconic acid} + \text{H}_2\text{O}_2
\]

Peroxidase

\[
\text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine} + \text{Phenol} \rightarrow \text{Quinoneimine dye} + \text{Phenol} + \text{H}_2\text{O}
\]

Procedure

For the estimation of glucose, 10 μl each of serum and working standard were incubated with 1 ml of the reagent for 15 min at 37 °C and the absorbance at 505 nm was measured against a reagent blank. For reagent blank, 10 μl of distilled water was added to 1 ml of the reagent. The concentration of glucose in serum samples was calculated as:

\[
\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100 = \text{mg/dl}
\]

4.8.4. Determination of Plasma Insulin

Plasma insulin levels were assayed by INSU – Fully Automated Chemiluminescent Immuno Assay- C.L.I.A (Flier et al., 1979) based on the sandwich principle using commercial kit. The microtiter wells are coated with monoclonal antibody directed toward a unique antigenic site on insulin molecule. Then anti-insulin antibody labelled with conjugate-horseradish peroxidase (HRP) was added. The mount
of bound HRP complex is proportional to the concentration of insulin in the sample. Absorbance was measured at 450 nm and results were expressed as µU/ml.

4.8.5. **Determination of haemoglobin**

Haemoglobin in the blood was estimated after 28 days by the method of Drabkin and Austin (1932). To 0.02 ml of blood, 5.0 ml of Drabkin’s reagent was added, mixed well and allowed to stand for 10 min. The absorbance of test and standard solution were read at 540 nm against a reagent blank. Values were expressed as g/dl of blood.

4.8.6. **Determination of glycosylated haemoglobin**

Glycosylated haemoglobin was assayed after 28 days in the whole blood by ion-exchange high-performance liquid chromatography (HPLC) using Bio-Rad D-10 Hemoglobin A1c. The samples are automatically diluted on the D-10 and injected into the analytical cartridge. The D-10 delivers a programmed buffer gradient of increasing ionic strength to the cartridge, where the haemoglobins are separated based on their ionic interactions with the cartridge material. The separated haemoglobins then pass through the flow cell of the filter photometer, where changes in the absorbance at 415 nm are measured and expressed as gms %.

4.8.7. **Urine Sugar and other reducing substances**

Urine samples were collected from the animals of all the five groups after housing them in metabolic cages and urine sugar was determined by Benedict’s method (Benedict, 1911) on day 1 and 28th day of the experiment. Free aldehyde (or) keto groups present in sugar reduces copper sulphate of Benedict’s solution to cuprous oxide on boiling forming a green, yellow, orange red and brick red precipitate depending on the concentration of the sugar present in the urine. The change in colour was observed and the approximate urine sugar was determined as follows:
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Green+
Yellow++
Orange+++ 
Reddish brown++++

In the animals, which survived the STZ injection became diabetic was confirmed by weight loss, presence of glycosuria (+++ and hyperglycemia.

4.8.8. Histopathological Studies

For histopathological study, a portion of pancreas, liver and kidney were excised immediately, perfused with ice cold saline and the liver, pancreas and kidney tissues were separated and placed in formalin immediately. Fixation was performed at room temperature for 24 hrs. Later, the tissues were sectioned using microtome, dehydrated in graded alcohol, embedded in paraffin and stained with Haematoxylin and Eosin (H & E) staining. The stained slides were examined under low power objective of the light microscope to get the microscopic image of the section (Bancroft and Gamble, 2008).

4.8.8.1. Tissues sectioning (microtomy)

The block was trimmed by removing the excess paraffin all around. Sections were cut in a (4-5 mm thickness) rotary microtome (Leica, Germany). Sections were transferred from the cutting edge of the microtome knife with the help of a spatula to a tissue floatation bath having warm water (40-45°C). Sections were spread out uniformly and then taken on clean glass slides coated with Meyer's albumin-glycerine mixture. The glass slides were then placed in a warm oven for about 15 min to help the sections adhere to the slides.
4.8.8.2. Tissues staining

H&E method of staining was employed for the histopathological examination of pancreas, liver and kidney tissues. The sections were deparaffinised by passing xylol for 5-10 minutes. After removing xylol using absolute alcohol, the sections were washed in tap water. Then the preparations were stained (primary) with haematoxylin for 3-4 minutes, excess stain was removed by washing in tap water for 5-10 minutes. The sections were counter stained (secondary) with eosin (0.5%) until the sections appear light pink (15-30 seconds), then excess stain was removed by washing in tap water and blot dried. The stained sections were dehydrated by alcohol and cleared with xylol (15-30 seconds). After mounting in D.P.X mount and cover slipping, the slides were kept for drying and removal air bubbles. Finally, the stained slides were examined under low power objects of the light microscope (Olympus 1x71) to obtain the microscopic image of the sections.

4.2. The effect of chrysin on hyperglycemia mediated oxidative stress in STZ-NA induced experimental T2DM rats

4.2.1. Biochemical estimations

4.2.1.1. Determination of serum hepatic biomarker enzymes

The activities of serum hepatic biomarkers aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were assayed spectrophotometrically according to the standard procedures using commercially available diagnostic kits (DiaSys Diagnostic Systems, Germany). Enzyme activities were expressed as units/litre.

4.2.1.2. Determination of serum renal biomarkers

Renal biomarkers creatinine, urea, and uric acid were estimated from serum using standard diagnostic kits (SPAN Diagnostics, India). Activities were expressed as mg/dl.
4.2.1.3. **Determination of serum lipid profile**

Serum lipid profile, including the levels of total cholesterol (TC), triglycerides (TGs) and high density lipoprotein (HDL) cholesterol content in serum was estimated by using standard assay kits (DiaSys Diagnostic Systems, Germany). Low density lipoprotein (LDL) cholesterol and very low density lipoprotein (VLDL) was calculated using the formula.

\[ \text{LDL cholesterol} = \text{Total cholesterol} - \text{HDL} + (\text{TG} / 5) \]

VLDL cholesterol was calculated using the formula (TG/5) respectively. The units of each parameter were expressed as mg/dl

4.2.2. **Preparation of tissue homogenate**

The liver and pancreatic tissues were dissected immediately following decapitation and rinsed in ice-cold saline. For biochemical analyses, 1 g of tissues was weighed, cut into fragments and homogenized with 10 ml of 0.1 M Tris-HCl buffer, pH 7.4 using a Potter-Elvehjem homogeniser with a Teflon pestle and centrifuged at 7,000 rpm for 10 min at 4 °C; supernatant was collected and used for enzymatic and non-enzymatic assays.

4.2.2.1. **Estimation of protein**

Protein content was estimated by the method of Lowry *et al.*, 1951 using bovine serum albumin (BSA) as standard. The blue colour developed in this reaction is due to the reaction of the peptide bond with copper sulphate in alkaline medium and to the reaction of tyrosine and tryptophan with phenol reagent. 0.1 ml of 10% tissue homogenate was diluted to 1 ml with distilled water. The reaction mixture consisted of 0.1 ml liver or pancreatic tissue homogenate made up to 1.0 ml with distilled water. To all the tubes 4.5 ml of alkaline copper reagent was added and kept at room temperature for 10 min. Then 0.5 ml of Folin’s phenol reagent was added and the colour developed was read after 20 mins at 640 nm against reagent blank using spectrophotometer. The concentration of protein was expressed in mg per gram of tissue.
4.2.3. Determination of activities of antioxidant enzymes and other antioxidants

The activities of enzymatic antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST); non enzymatic antioxidants such as reduced glutathione (GSH) and vitamin C in liver and pancreatic tissue of experimental rats were determined by standard methods.

4.2.3.1. Assay of superoxide dismutase

SOD activity was assayed by the method of Marklund and Marklund (1974) based on inhibition of the pyrogallol auto-oxidation by the supernatant of tissue homogenate. 0.5 ml of the hepatic or pancreatic tissues supernatant was added to an assay mixture containing 0.25 ml of absolute ethanol and 0.15 ml of chloroform. After shaking for 15 min in a mechanical shaker, the suspension was centrifuged and the supernatant was used for the assay. The reaction mixture for autooxidation consisted of 2.0 ml of 0.1 M Tris buffer pH 8.2, 0.5 ml of pyrogallol (2 mM) and 2.0 ml of distilled water. The rate of auto-oxidation of pyrogallol was noted every minute for three minutes. This was considered as 100% autooxidation. The assay mixture for the enzyme contained 2.0 ml of Tris-HCL buffer pH 8.2, 1.5 ml of distilled water, 0.5 ml of the supernatant of each tissue homogenate and 0.5 ml of 2 mM of pyrogallol. The change in absorbance was read at 470 nm against blank every min for 3 min on spectrophotometer. The SOD activity was expressed as Units/mg of protein (one unit was the amount of SOD utilized to inhibit 50% of autooxidation of pyrogallol/min/mg of protein).

4.2.3.2. Assay of catalase

CAT activity in liver and pancreatic tissue was assayed by the method (Sinha, 1971) using dichromate-acetic acid reagent. Dichromate acetic acid was reduced to chromic acetate on heating in the presence of H$_2$O$_2$ with the formation of perchloric
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Acid. The reaction mixture (1.5 ml) contained 0.5 ml of 0.2 M H$_2$O$_2$, 1.0 ml of 0.01 M (pH 7) sodium phosphate buffer and 0.4 ml distilled water. Following this, 0.1 ml of tissue homogenate (supernatant of hepatic and pancreatic tissues) was added to initiate the reaction. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). The tubes were then heated for 10 min and allowed to cool. Green colour developed was read at 570 nm against blank on spectrophotometer. The activity of CAT was expressed in terms of μmol of H$_2$O$_2$ consumed/ min/mg protein.

4.2.3.3. Assay of glutathione S-transferase

The glutathione-S-transferase (GST) activity was determined spectrophotometrically by the method of Habig et al., (1974). The reaction mixture 3.0 ml contained 1.0 ml of 0.3 mM phosphate buffer (pH 6.5), 0.1 ml of 30 mM 1-chloro- 2, 4-dinitrobenzene (CDNB) and 1.7 ml of double distilled water. After preincubating the reaction mixture at 37°C for 10 min in a water bath, the reaction was started by the addition of 0.1 ml of tissue homogenate and 0.1 ml of reduced glutathione (GSH) as substrate. The conjugation of GSH with CDNB, a hydrophilic substrate was formed to measure the activity of GST. The absorbance was followed for 5 minutes at 340 nm. Reaction mixture without the enzyme was used as blank. The activity of GST is expressed as μmoles of GSH-CDNB conjugate formed per minute/mg of protein.

4.2.3.4. Assay of glutathione peroxidase

The activity of GPx was assayed in tissue homogenate by the method of Rotruck et al., (1973). The principle of this method was the reaction between glutathione remaining after the action of GPx and 5, 5’-dithiobis-2-nitro benzoic acid (DTNB) to form a complex. GPx activity was assayed by taking 0.2 ml of Tris HCl buffer (0.4 M), 0.2 ml of 0.4 mM ethylenediaminetetraacetic acid (EDTA) along with
0.1 ml of sodium azide, 0.2 ml of 4 mM GSH and 0.1 ml of 2.5 mM H\textsubscript{2}O\textsubscript{2} was added and mixed well. To this, 0.1 ml of liver or pancreatic tissue homogenate was added and the total volume was made upto 2.0 ml with distilled water. This mixture was incubated for 5 min at 37 °C. The overall reaction was arrested by adding 0.5 ml of 10% trichloroacetic acid (TCA). The precipitate was removed by centrifugation at 3000 rpm for 10 minutes and the supernatant was obtained. To this 4.0 ml of 0.3 M disodium hydrogen phosphate solution and DTNB were added. The absorbance was read at 412 nm against reagent blank. GPx activity was expressed as nmoles of GSH oxidized per minute per mg of protein.

4.2.3.5. Assay of reduced glutathione

Reduced glutathione in the liver and pancreatic tissue homogenates was estimated by the method of Moren et al., (1979). This method was based on the development of yellow colour when DTNB was added to compounds containing sulfhydryl groups. 1.0 ml of tissue homogenate was added to equal volume of ice cold 10% TCA. The precipitate was removed by centrifugation at 3000 rpm for 10 minutes. To the protein free supernatant 1.0 ml aliquot, 4.0 ml of 0.3 M phosphate buffer (pH 8.0) and 0.5 ml of DTNB (0.6 mM in 0.2 M phosphate buffer, pH 8.0) was added and mixed well. The absorbance of the resulting yellow colour was read at 412 nm using spectrophotometer. Results were expressed as μg of GSH content per mg of protein.

4.2.3.6. Assay of Ascorbic acid (Vitamin C)

Ascorbic acid was measured by the method of Omaye et al., (1979). Ascorbic acid is oxidized by copper to form dehydro ascorbic acid which reacts with 2, 4-dinitrophenyl hydrazine (DNPH) to form bis-2, 4-dinitrophenyl hydrazine. This undergoes further rearrangement to form a product with an absorption maximum at 520 nm. Thiourea provides a reducing medium which helps to prevent interference from non-ascorbic acid chromogens. For this assay, 0.5 ml of hepatic and pancreatic tissue
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homogenate, 0.5 ml of distilled H₂O and 1.0 ml of 5% TCA were added, mixed thoroughly and centrifuged at 12,000 g for 20 min. To 1.0 ml of the resulting supernatant, 0.2 ml of 2,4-dinitrophenyl hydrazine-thiourea-copper sulphate reagent (DTC) was added and incubated at 37 °C for 3 h. Then, 1.5 ml of 65% sulphuric acid (H₂SO₄) were added and mixed well, and the solutions were allowed to stand at room temperature for another 30 min. A graded amount of ascorbic acid standard was treated similarly. The results were expressed as μg/mg of protein.

4.2.4. Determination of lipid peroxidation (LPO)

The concentration of malondialdehyde (MDA), a measure of LPO was assayed in the form of thiobarbituric acid reactive substances (TBARS) by the method of Ohkawa et al., (1979). TBARS were measured by their reactivity with thiobarbituric acid (TBA) in acidic conditions, which upon reaction generates a pink coloured chromophore of which the absorbance, was read at 532 nm. Briefly, 0.2 ml of homogenate and 1.5 ml of TBA was added and placed in a boiling water bath for 20 min. The tubes were cooled to room temperature and centrifuged at 3000 rpm for 10 min and the precipitate obtained was removed. Then the absorbance of the supernatant was determined at 532 nm using spectrophotometer (SHIMADZU-UV1800). Results were expressed as nmoles of TBARS formed/mg tissue protein.

4.3. The role of chrysin on glucose homeostasis and insulin signaling in STZ-NA induced experimental T2DM rats

4.3.1. Determination of carbohydrate-metabolizing enzymes

The activities of key enzymes in glycolysis including hexokinase, pyruvate kinase and lactate dehydrogenase, activities of gluconeogenic enzymes including glucose-6-phosphatase and fructose-1, 6-bisphosphatase and activities of hexose monophosphate shunt (HMP shunt) enzyme glucose-6-phosphate dehydrogenase in liver and kidney tissue of experimental rats were determined by standard methods.
4.3.1. Assay of hexokinase (HK)

Hexokinase (HK) activity in the tissues was assayed by the method of Brandstrup et al., (1957). The reaction mixture contained 1 ml of glucose, 0.5 ml adenosine triphosphate (ATP) solution, 0.1 ml of magnesium chloride (MgCl₂), 0.4 ml dipotassium hydrogen phosphate (K₂HPO₄), 0.4 ml potassium chloride (KCl), 0.1 ml sodium fluoride (NaF) and 2.5 ml of Tris-HCl buffer (pH 8.0). This was pre-incubated at 37°C for 5 min. The reaction was initiated by the addition of 2.0 ml of tissue homogenate. From that, an aliquot 1.0 ml of the reaction mixture was immediately transferred (zero time) to a tube containing 1 ml of 10% TCA and served as control. A second aliquot of the reaction mixture was removed and deproteinised after 30 min of incubation at 37°C likewise transferred to tubes containing 10% TCA. The protein precipitate was removed by centrifugation and the residual glucose in the supernatant was estimated in all the tubes by the O-toluidine method. Reagent blank was run with each test. The difference between the two values (zero time aliquot and 30 min aliquot) indicated the quantum of glucose phosphorylated. Hexokinase enzyme activity was expressed as μmoles of glucose-6-phosphate formed/h/mg of protein.

4.3.1.2. Assay of pyruvate kinase (PK)

Pyruvate kinase (PK) activity was assayed by the method of Pogson and Denton (1967). The reaction mixture containing 0.1 ml of Tris-HCl buffer, 240 μl of KCl, 0.05 ml of MgCl₂, 0.01 ml of phospho enol pyruvate (PEP), 0.002 ml of adenosine diphosphate (ADP), 0.5 μl of nicotinamide adenine dinucleotide (NADH), 0.1 ml of lactate dehydrogenase (LDH) and 20 μl of enzyme was incubated at 25 °C for 5 min. The color developed was read at 340 nm. The enzyme activity was expressed as mU of pyruvate formed/mg of protein.
4.3.1.3. Assay of lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH) was assayed by the method of King (1959). LDH level in liver was assayed based on the conversion of lactate to pyruvate in the presence of coenzyme NAD\(^+\). The pyruvate formed was made to react with DNPH in HCl, resulting in the formation of hydrazone (an orange colored complex in alkaline medium), which was measured at 420 nm. The activity of LDH was expressed as µmoles of pyruvate formed/h/mg of protein.

4.3.1.4. Assay of glucose-6-phosphatase

Glucose-6-phosphatase in the tissues was assayed by the method of Koide and Oda (1959) based on inorganic phosphate liberated from glucose-6-phosphate. The reaction mixture contained 0.7 ml of citrate buffer 0.1 M, pH 6.5 and 0.3 ml of substrate 0.01 M and 0.3 ml of tissue homogenate. The reaction mixture was incubated at 37°C for 1 h. Addition of 1 ml of 10% TCA to the tubes terminated the reaction of the enzyme. The suspension was centrifuged and the phosphorus content of the supernatant of tissue homogenate was estimated by the method of Fiske and Subbarow, 1925. The supernatant was made up to a known volume. To this, 1 ml of ammonium molybdate was added followed by 0.4 ml of 1-amino-2-naphthol-4-sulfonic acid (ANSA). The blue color developed was read at 640 nm after 20 min. The enzyme activity was expressed as µmol of Pi liberated/h/mg protein.

4.3.1.5. Assay of Fructose-1, 6-bisphosphatase

Fructose-1, 6-bisphosphatase was assayed by the method of Gancedo and Gancedo, 1971. The assay medium in a final volume of 2.0 ml contained 1.0 ml of Tris-HCl buffer (0.1 M, pH 7), 0.4 ml of substrate 0.05 M, 0.1 ml of MgCl\(_2\) (0.1M), 0.2 ml of KCl (0.1M), 0.1 ml of EDTA (0.001M) solution and 0.2 ml of enzyme source. The incubation was carried out at 37°C for 15 min. The reaction was terminated by the addition of 1.0 ml of 10% TCA. The suspension was centrifuged and the phosphorus
content of the supernatant was estimated according to the method described by Fiske and Subbarow, 1925 as described above. The activity of fructose-1, 6-bisphosphatase was expressed as μmol of Pi liberated/h/mg protein.

4.3.1.6. Assay of glucose-6-phosphate dehydrogenase (G6PDH)

Glucose-6-phosphate dehydrogenase was assayed by the method of Ellis and Kirkman, 1961. The incubation mixture contained 1.0 ml of Tris-HCl buffer (0.05 M, pH 7.5), 0.1 ml of MgCl₂ (0.1 M), 0.1 ml of NADP⁺ (0.1 M), 0.5 ml of phenazine methosulphate (PMS), 0.4 ml of the dye solution and 0.2 ml of the enzyme extract. The mixture was allowed to stand at room temperature for 10 min to permit the oxidation of endogenous materials. The reaction was initiated by the addition of 0.5 ml of glucose-6-phosphate. The absorbance was read at 640nm against water blank at 1 min intervals for 3-5 min in a UV spectrophotometer. The activity of the enzyme was calculated in units by multiplying the change in OD/min by the factor 6/17.6, which is the molar extinction coefficient of the reduced coenzyme. The activity of enzyme was expressed as μmoles of NADPH/min/mg protein.

4.3.2. Determination of glycogen metabolism

Levels of glycogen synthase, glycogen phosphorylase in liver and glycogen content in liver and skeletal muscle were determined by standard methods.

4.3.2.1. Assay of glycogen synthase

The activity of glycogen synthase was measured by coupling with pyruvate kinase and the amount of uridine diphosphate (UDP) formed from UDP-glucose in the presence of glycogen and glucose-6-phosphate. Pyruvate kinase catalyzes the transfer of phosphate from PEP to UDP and the pyruvate was liberated. Hepatic glycogen synthase was assayed by the method of Leloir and Goldmberg (1960). The reaction mixture containing 0.09 ml of glycogen, glucose-6 phosphate solution, 0.015 ml of
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cysteine hydrochloride solution and 0.5 ml of enzyme. The reaction was started by the addition of 0.03 ml of UDP glucose and the incubation was performed at 37º C for 10 min at room temperature. The tubes were then heated in a boiling water bath for 5 min. A blank in which UDP glucose was added after incubation was run at the same time with the UDP standards (10-60 μg). To estimate the UDP formed in the reaction, 0.075 ml each of PEP solution and pyruvate kinase were added. The tubes were incubated for 15 min at 37º C and 0.45 ml of DNPH was then added. After 10 min, 0.60 ml of 10N NaOH solution and 1.1 ml of ethanol were added, the contents were mixed well and centrifuged. The supernatant was read at 530 nm. The enzyme activity was expressed as μmoles of UDP formed/h/mg of protein.

4.3.2.2. Assay of glycogen phosphorylase

Hepatic glycogen phosphorylase was assayed by the method Cornblath et al., 1963. The property of synthesizing glycogen from glucose-1-phosphate liberating inorganic phosphate is made use in the assay of glycogen phosphorylase activity. The reaction mixture was started by the addition of 1 ml of substrate, 0.1 ml of 5’ Adenosine Mono Phosphate (AMP), 0.2 ml of NaF and 0.2 ml of enzyme. 0.5 ml of aliquot was taken from the reaction mixture at zero time and after 10 min of incubation at 37ºC, 1.0 ml of 10% TCA solution was added to arrest the reaction. The contents were mixed well and centrifuged. The liberated phosphorous in the supernatant was estimated by the method of Fiske and Subbarow, 1925. The activity of glycogen phosphorylase enzyme was expressed as μmoles of Pi liberated/h/mg protein.

4.3.2.3. Estimation of glycogen content

Estimation of glycogen was performed by the method of Morales et al., 1973. Glycogen in liver and skeletal muscle was liberated when heated with strong alkali. Released glycogen was precipitated by the addition 3 ml 100% of ethanol and 0.2 ml of sodium sulphate (saturated) to give a quantitative yield of glycogen. The
polysaccharide was then hydrolysed in dil H$_2$SO$_4$ and the glucose released was estimated. 1.0 g each of liver and skeletal muscle was weighed and 1:3 volume of 95% ethanol and a drop of 1 M ammonium acetate were added. The tubes were kept in the boiling water bath for 20 min with occasional shaking. The tubes were cooled, shaken well and placed in freezer over night. Glycogen was precipitated by adding 5 ml of ethanol (95% v/v) and the precipitate was collected after centrifugation at 3000 g for 30 min. The precipitate was redissolved in water and ethanol and centrifuged again. The final precipitate was dissolved in 3.0 ml of water and heated for 5 min in boiling water bath and this extract was used for the estimation of glycogen.

0.5 ml of sample was made up to 1 ml with water. A setoff standard glucose solutions (25-100 μg) and blank containing water alone were set up. All the tubes were cooled in an ice-bath and 4 ml of anthrone reagent was added. The reaction mixture was mixed well. All the tubes were covered with glass marbles and heated for 20 min in a boiling water bath. The tubes were cooled and the green colour developed was read at 640 nm using spectrophotometer. Glycogen content was expressed as mg of glucose/g wet tissue.

4.3.3. Assessment of Mitochondrial enzymes

Mitochondrial enzymes Isocitrate dehydrogenase (ICDH), Succinate Dehydrogenase (SDH) and nicotinamide adenine dinucleotide dehydrogenase were assayed using standard methods.

4.3.4. Isolation of liver mitochondria

The liver mitochondria were isolated from cell debris, nuclei, microsomes, soluble components and contaminant RBC using differential centrifugation by the method of Johnson and Lardy, 1967. A 20% (w/v) homogenate was prepared in 0.25 M sucrose containing 0.05 M Tris–HCl buffer and 5 mM EDTA. To remove cell debris,
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tissue fragments and cell nuclei (nuclei pellet), the homogenate was centrifuged at 600g for 10 min. The supernatant fraction was centrifuged in a refrigerated centrifuge at 10000g for 5 min at 4°C to bring down the mitochondrial pellet. After using a Pasteur pipette to remove the last bit of liquid, the remaining mitochondrial pellet was resuspended in KCl and used for the estimation of various parameters.

4.3.4.1. Assay of Isocitrate dehydrogenase

The enzyme activity of isocitrate dehydrogenase was assayed by the method of Bell and Baron, 1960. The incubation mixture contained 0.4 ml of Tris–HCl, 0.2 ml of trisodium isocitrate, 0.2 ml of NADP and the required amount of enzyme. NADP was replaced by 0.2 ml saline in control. After 60 min of incubation at 37°C, 1.0 ml of colouring reagent was added followed by 0.5 ml of EDTA. The contents were mixed well and kept at room temperature for 20 min, then 10 ml of NaOH was added and the colour developed was read at 420 nm after 10 min. A calibration curve was established with α-ketoglutarate as standard. The isocitrate dehydrogenase activity was expressed as nmol of α-ketoglutarate formed/h/mg protein.

4.3.4.2. Assay of Succinate dehydrogenase

The activity of succinate dehydrogenase was assayed according to the method of Slater and Bonner, 1952. The reaction mixture containing 1.0 ml of phosphate buffer, 0.1 ml of EDTA, 0.1 ml of BSA, 0.3 ml of sodium succinate and 0.2 ml of potassium ferricyanide was made up to 2.8 ml with double-distilled water. The reaction was started by the addition of 0.2 ml of mitochondrial suspension. The change in OD was recorded at 15-sec intervals for 5 min at 420 nm. The succinate dehydrogenase activity was expressed as nmol of succinate oxidised/min/mg protein.
4.3.4.3. Assay of NADH dehydrogenase

The enzyme activity of NADH dehydrogenase was assayed according to the method of Minakami et al., 1962. The reaction mixture contained 1.0 ml of phosphate buffer, 0.1 ml of potassium ferricyanide, 0.1 ml of NADH and 0.2 ml of mitochondrial suspension. The total volume was made up to 3.0 ml with distilled water. NADH was added just before the addition of the enzyme source (mitochondrial suspension). A control was also treated similarly without NADH. The change in OD was measured at 420 nm for 3 min at 15-sec intervals. The activity of NADH dehydrogenase was expressed as nmol of NADH oxidised/min/mg protein.

4.3.5. Immunoblot Analysis

Immunoblot analysis was performed to determine the relative concentrations of IRS-1, IRS-2 and GLUT-2 in liver; GLUT-4 in skeletal muscles and adipose tissue of control and experimental groups. Liver, skeletal muscle and adipose tissue were homogenised and total amount of protein was estimated by Bradford protein estimation kit. All steps were carried out on ice or at 4°C. Proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Equal amounts of total proteins (40 µg) were subjected to 12% SDS–PAGE gels and transferred onto poly vinylidene fluoride (PVDF) membranes using semi-dry blotting apparatus. Blotting was done in 24 V for 1 h and was stained by ponceau S solution to check for the efficiency of transfer and blocking was done by 5 % w/v non-fat dry milk in 1 X Tris buffer saline (TBS) pH 7.5 and 0.1% Tween 20 at 4°C with gentle shaking overnight. Antibodies against IRS-1 & 2 (1:1000 dilutions) [purchased from Cell Signaling Technology Inc.,] and antibodies against GLUT-2 & 4 (1:200 dilutions) [purchased from Santa Cruz Biotechnology, Inc.,] were used. Incubate with Anti-biotin, HRP-linked antibody, to detect biotinylated protein markers, in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature. The membranes were then probed with
the indicated antibodies, and the proteins were visualized using an ECL detection system. The blotted protein was quantified using Quantity one software system (Bio-Rad, Hercules, CA, USA).

4.4. The protective role of chrysin on inflammatory biomarkers in STZ-NA induced experimental T2DM rats

4.4.1. Biochemical Estimation

4.4.1.1. Determination of protein carbonyl content (PCC)

Carbonyl content was measured by the method of Levine et al., (1990). Briefly, 0.5ml of liver and pancreatic tissue homogenate from all groups was taken in two tubes labelled as “test” and “control.” Equal volume of 10 mM of DNPH prepared in 2.5 M HCl was added to the test tube and equal volume of 2.5 M HCl alone was added to the control tube. The contents were mixed thoroughly and incubated in the dark (room temperature) for 1 h, with intermittent shaking every 15 min. After 1 h, equal volume of 10% TCA w/v was added to both the tubes and centrifuged at 3000 rpm for 20 min to obtain the protein pellet. The supernatant was carefully aspirated and discarded. This was followed by a second wash with 10% TCA as described above. Further, the precipitates were washed three times with of ethanol/ethyl acetate (1:1 v/v) to remove unreacted DNPH and lipid remnants. The final protein pellet was dissolved in 2 ml of 6 M guanidine hydrochloride and incubated at 37 °C for 10 min. The insoluble materials were removed by centrifugation and the carbonyl content was determined by taking the absorbance of the representative samples at 370 nm. The test sample was read against the corresponding control sample and the carbonyl content was calculated using an absorption coefficient of 22 000 M⁻¹ N cm⁻¹. The PCC was expressed as nmoles/mg protein.
4.4.1.2. Determination of Nitric oxide (NO)

Total nitrite and nitrate concentrations were used as an indicator of NO synthesis (Sastry et al., 2002). This assay determines NO on the basis of the enzymatic conversion of nitrate to nitrite by nitrate reductase. To determine the nitrate concentration in each sample, the endogenous nitrite concentration was subtracted from the total nitrite concentration. Copper cadmium (Cu-Cd) alloy reduces nitrate to nitrite which reacts with Griess reagent (sulfanilamide and N-naphthylethylenediamine) in acidic medium to give pink color. This assay is sensitive to 1 μM nitrate. Cu-Cd alloy can completely reduce nitrate to nitrite in 1 h. To 0.1 μl of liver and pancreatic tissue homogenate, 0.4 μl of carbonate buffer and 0.15 g of cadmium filings (washed in same buffer and dried on filter paper) were added and incubated at room temperature for 1 h with thorough shaking. Cu-Cd fillings were not added for nitrite concentration. The reaction was stopped by adding 0.1 μl of 0.35 M NaOH and 0.4 μl of 120 mM zinc sulphate (ZnSO₄) and incubated at room temperature for 10 min and centrifuged at 4000 rpm for 10 min. 0.1 μl of clear supernatant was aliquoted into microtiter plate wells and 0.05 μl of 1% sulphanilamide and 0.05 μl of 0.1% N-naphthylethylenediamine were added. The contents were mixed well and the color was read at 545 nm after 10 min.

4.4.1.3. Determination of Reactive Oxygen Species (ROS)

ROS generation was determined in fresh liver and pancreatic tissue homogenate by using dichlorofluoroscein (DCF) as a probe, according to Lebel and Bondy 1990 as modified by Kim et al., 1996. The 2', 7' Dichlorofluoroscein acetate (DCFDA) stock solution was made freshly by dissolving it in 1.25 mM methanol and kept in a dark room at 0 °C. 0.05 μl of homogenate were added to 2.988 μl of 0.1 M phosphate buffer (pH 7.4) and 12 μl of 1.25 mM DCFDA (total volume = 3 ml). DCFDA was dissolved in methanol to aid in the transport across membranes. The assay mixture was incubated
Materials and Methods

for 15 mins at 37 °C to allow the DCFDA probe to cross any membranes and for nonspecific esterases to cleave diacetate groups. Dichlorofluorosin (DCF) formation was determined spectrofluorometrically with fluorescence spectrophotometer at excitation and emission wavelength of 488 and 525 nm at 37 °C. Measurements were made every 15 min for 60 nm and a linear DCF production rate was determined relative to the amount protein added to the cuvette. Blank consisting of appropriate buffer and 5 μM DCFDA without homogenate were used for autooxidation rate of DCFDA. The DCF assay was carried out with in 1 h of tissue harvest. The units were expressed as nanomoles DCF formed /min/mg protein.

4.4.2. Reverse transcription (RT)-PCR analysis of mRNA transcript levels

4.4.2.1. Isolation and quantification of total RNA

Total ribonucleic acid (RNA) was extracted from hepatic and pancreatic tissues using Trizol (Sigma–Aldrich, St. Louis, USA) reagent (1 ml/100 mg tissue). Liver and pancreatic tissue were homogenized (100mg/ml) in Trizol reagent and the homogenate was transferred to a micro centrifuge tube and kept at 4° C for 5 min to permit the complete dissociation of nucleoprotein complexes. Then 0.2 ml of chloroform was added, vortexed vigorously for 15 sec and incubated at 4° C for 10 min. After incubation, the mixture was centrifuged at 12,000 g for 15 min at 4° C. The upper aqueous phase (600 μl) was carefully transferred to a fresh micro centrifuge tube and equal volume of isopropanol was added, followed by through vortexing. The solution was again incubated at 4° C for 10 min. The incubated mixture was then centrifuged at 12,000 g for 10 min and the supernatant was removed and the pellet was washed twice with 75% ethanol by vortexing. Subsequently tubes were centrifuged at 7,500 g for 5 min at 4° C. After centrifugation the ethanol was removed carefully and the pelleted RNA were determined by absorbance at 260/280 nm Sambrook et al., 1989. UV-spectrophotometer and RNA integrity was checked by agarose gel electrophoresis.
Agarose gel electrophoresis is simple and highly effective method for separation, identification and purification of deoxyribonucleic acid (DNA).

### 4.4.2.2. Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

Total RNA sample was used for the synthesis of cDNA by one-step RT-PCR kit (Qiagen, Germany) per manufacturer’s instructions with the gene-specific primers for TNF-α, IL-1β, IL-6, iNOS, NF-κB and β-actin. Amplification was done in Eppendorf thermal cycler. After completion of PCR 10 µl of each PCR product were analyzed by gel electrophoresis on a 2% Agarose gel. The ethidium bromide-stained gel was subjected to densitometric scanning and the band intensity of the cDNA fragments of the genes was normalized against the band intensity of the cDNA fragment of the internal control (β-actin) gene, using Quantity One Software (BIO RAD, Hercules, CA).

**Table 3: Primer sequences and expected product size of the genes amplified**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Primer</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>NM_000600</td>
<td>Forward</td>
<td>5’-TGCAATAACCACCCCTGACC-3’</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-GTGCCCATGCTACATTTGCC-3’</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>NM_000576</td>
<td>Forward</td>
<td>5’-ACCAAAACCTCTCTCGAGGCAC-3’</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-AGCCATCATTTCTACTGGCGA-3’</td>
<td></td>
</tr>
<tr>
<td>NF-κB</td>
<td>NM_002502</td>
<td>Forward</td>
<td>5’-ACTTCTCTTGGGTTTGGATGCGG-3’</td>
<td>286</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-AGGAAGAGGTTTGGATGCGG-3’</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>NM_000594</td>
<td>Forward</td>
<td>5’-CTGGGCAGGTCTACTTTTGG-3’</td>
<td>272</td>
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<td></td>
<td>Reverse</td>
<td>5’-CTGGAGGGCCAGTTTGGAT-3’</td>
<td></td>
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<tr>
<td>iNOS</td>
<td>NM_000625</td>
<td>Forward</td>
<td>5’-TGCCGGAAGGGCGCTACTTTGG-3’</td>
<td>798</td>
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<td></td>
<td>Reverse</td>
<td>5’-CGCAGTGCCGGTGCAGATTC-3’</td>
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<tr>
<td>β-actin</td>
<td>NM_031144</td>
<td>Forward</td>
<td>5’-ATCGCTGAGAGATGCAGAA-3’</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-AGAGCCACCAATCCACACAG-3’</td>
<td></td>
</tr>
</tbody>
</table>
4.4.3. Statistical Analysis

The results were expressed as mean ± SD, (n = 6) where ‘n’ represents the number of samples. Statistical analyses were carried out using GraphPad Prism 4.0 (GraphPad Software, San Diego; CA; USA). One-way ANOVA, followed by post hoc analysis using Tukey’s was adapted to all the parameters under study to test the level of statistical significance. The difference was considered significant if p<0.05.