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4.1 In Vivo Studies

4.1.1 Induction Of Diabetes

4.1.1.1 Induction of IDDM

Male Wistar rats weighing 200-225 g were used for the study. The animals were housed in a group of 3 rats per cage under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12h/12h light-dark cycle. Animals had free access to conventional laboratory diet and tap water ad libitum. Diabetes was induced with Streptozotocin (STZ) (Sigma Ltd., USA) 40mg/kg dissolved in 0.9% NaCl, administered as a single intravenous (i.v.) tail-vein injection under light ether anesthesia. Control animals were injected with an equivalent volume of 0.9% NaCl. Animals were checked for the extent of glucosuria 48h after the injection of STZ using Diastix (Bayer Diagnostics, India). Animals showing glucosuria (>2%) were considered as diabetic. Control rats were randomly divided into two groups, namely control and control treated with test drug. Similarly, diabetic rats were divided in two groups, namely IDDM control and IDDM treated with test drug.

4.1.1.2 Induction of NIDDM

Wistar rats from an inbred colony were bred under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12h/12h light-dark cycle. Conventional laboratory diet and tap water were provided ad libitum. Two-day-old male Wistar neonates were injected intraperitoneally (i.p.) with 90 mg/kg STZ (Sigma Ltd., USA) in 0.9% sodium chloride solution. Control neonates received equivalent amount of isotonic saline alone. The neonates were left with their own mothers and weaned at four weeks of age. Twelve weeks after the injection of STZ, animals were checked for fasting glucose levels. The animals showing fasting glucose levels >140 mg/dl were considered as diabetic. Control rats were randomly divided into two groups, namely control and control treated with test drug. Similarly, diabetic rats were divided into two groups, namely NIDDM control and NIDDM treated with test drug.
4.1.1.3 **Treatment Protocol**

Animals were maintained for six week treatment period with free access to conventional dietary feed and water *ad libitum*. BMOV, VUR1, chromium chloride and chromium picolinate were dissolved in water and administered at a concentration of 0.75 mg/ml, 0.75 mg/ml, 10 µg/ml and 8 µg/ml respectively *ad libitum* in the drinking water for six weeks. The untreated control and untreated diabetic groups received tap water to drink, whereas the treated control and treated diabetic groups received test drugs in drinking water. Experimental animals were monitored regularly for changes in body weight, food and water consumption and mortality throughout the course of the study.

4.1.2 **Blood Sample Collection And Analysis**

Blood samples were collected in clean dry centrifuge tubes at the end of six weeks of treatment after 8h fast by nicking the tip of tail under light ether anesthesia and were allowed to clot for 30min at room temperature. Serum was separated by centrifugation at 3000rpm for 25min and stored at -20°C until the analysis was carried out. Serum samples were analyzed for glucose, cholesterol, triglyceride, creatinine, urea, serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) using diagnostic kits (Bayer Diagnostics, India) colorimetrically using UV-Visible spectrophotometer (Shimadzu UV-1601, Japan). Serum insulin was estimated by radioimmunoassay technique using kits obtained from Board of Radiation and Isotope Technology, Mumbai in a five well gamma counter (Riastar, Packard, USA).

4.1.2.1 **Oral Glucose Tolerance Test (OGTT)**

At the end of six weeks of treatment, oral glucose tolerance test was performed after an overnight fast (Olefsky, 1981). The animals were orally administered with 1.5 g/kg of glucose and blood samples were collected from the tail vein under light ether anesthesia before i.e. 0 min and 15, 30, 60 and 120 min after oral glucose administration. Samples were allowed to clot for 30min at room temperature. Serum was
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separated by centrifugation at 3000 rpm for 25 min and analyzed for glucose and insulin as explained earlier. Plotting the glucose or insulin concentration versus time gives a curve showing rise and fall in glucose and insulin levels with time after an oral glucose load. Comparison of such curves gives only a vague idea about alterations in insulin-mediated glucose disposal and insulin release in response to oral glucose load. Therefore, results were expressed as integrated area under the curve (AUC) for glucose and insulin which was calculated by after trapezoid rule \[\text{AUC} = (C_1 + C_2)/2 \times (t_2 - t_1)\] and changes in glucose and insulin concentrations over 120 min during OGTT were expressed as AUCg (mg/dl.120min) and AUCi (µU/ml.120min) respectively.

4.1.2.2 Insulin Tolerance Test

Insulin tolerance test (Alford et al., 1971) is used to access peripheral insulin resistance. This test measures insulin sensitivity using \(K_{ITT}\) as an index of insulin-mediated glucose metabolism. The animals were fasted overnight and 0.2U/100g bodyweight of purified porcine insulin (Actrapid, Novo Nordisk Pharma India Ltd.) was injected by slow intravenous injection through tail vein. Neutral insulin injection was diluted with 0.9% saline to get the final concentration of 0.2U/0.1ml. Blood samples were collected from the tail vein before i.e. 0 min and 5, 10, 20 and 30 min after insulin administration under light ether anesthesia. Serum was separated as described earlier and analyzed for glucose. The \(K_{ITT}\), an index of insulin-mediated glucose metabolism, was determined from the slope of a linear portion of the regression line of natural log of glucose versus time (Alford et al., 1971) and calculated using the formula given by Lundbaek (1962):

\[
K_{ITT} = \frac{0.693}{t_{1/2}} \times 100
\]

where \(t_{1/2}\) represents the half life of plasma glucose decay, obtained by plotting plasma glucose concentration versus time on semilogarithmic graph paper.
4.1.2.3 Estimation of Biochemical Parameters

(A) Glucose (GOD/POD method)

Principle

Glucose is oxidized by the enzyme glucose oxidase (GOD) into gluconic acid and hydrogen peroxide. Hydrogen peroxide in the presence of enzyme peroxidase (POD) oxidizes the chromogen 4-aminoantipyrine/phenolic compound to a red coloured compound. The intensity of the colour produced is proportional to glucose concentration in the sample and is measured at 505nm. This final colour is stable for two hours.

\[
\text{GOD} \\
\text{Glucose} + \text{O}_2 \rightarrow \text{gluconic acid} + \text{H}_2\text{O}_2
\]

\[
\text{POD} \\
\text{H}_2\text{O}_2 + \text{Phenolic} + 4\text{-amino} \rightarrow \text{Red compound} + 2\text{H}_2\text{O}
\]

Preparation of working solution

One buffer/enzyme/chromogen tablet was gently dissolved in 20 ml of distilled water in a clean beaker, with continuous stirring.

Procedure

One ml of the working solution was added to test tube containing 10 µl of serum sample. Similarly standard and blank were prepared by using 10 µl of glucose standard (provided in the kit) and distilled water respectively. They were then mixed and incubated at room temperature for 30 min (end point reaction). Absorbance of test and standard was measured against blank at 505nm using UV-Visible spectrophotometer (UV-1601 Shimadzu, Japan).

The concentration of glucose in test samples was calculated using following formula.

\[
\text{Serum Glucose (mg/dl)} = \frac{\text{O.D. of test}}{\text{O.D. of std}} \times 100
\]
(B) Insulin (Radioimmunoassay method)

Principle
The assay is based upon the competition between unlabeled insulin in the standard samples and radioiodinated (I\(^{125}\)) insulin for the limited binding sites on a specific antibody. At the end of incubation, the antibody bound and free insulin are separated by the second antibody polyethylene glycol (PEG) separation method. Insulin concentration of samples is quantitated by measuring the radioactivity associated with the bound fraction of sample and standards. The amount of radiolabeled insulin bound to the antibody is inversely proportional to the amount of insulin in the serum. A standard curve with the known amounts of test substances can thus be constructed and the amount of insulin in the unknown samples can be calculated.

Procedure
1. All reagents were brought to room temperature before use and reconstituted as described in the leaflet supplied along with the kit.
2. Tubes were arranged and labeled as total, blank, standards, insulin controls and unknowns.
3. 0.3 ml of assay buffer was added to zero standard tube while 0.2 ml of assay buffer was added to insulin standard tubes and 0.4 ml of assay buffer was added to the blank tube. Assay buffer was not added to the total count tubes.
4. 100 µl of insulin standards (7.5 to 200 µU/ml) and unknown samples (serum samples) were added to the appropriate tubes.
5. 100 µl insulin free serum was added to blank, zero standard and insulin standard tubes.
6. 100 µl of anti-insulin serum was added to all the tubes except total and blank tubes.
7. The contents of the tubes were mixed gently and refrigerated at 2°C to 4°C overnight.
8. 100 µl of I\(^{125}\)-insulin reagent was added to all the tubes.
9. Contents of the tubes were mixed gently and incubated at room temperature for 3h.
10. 100 µl second antibody was added to all tubes except total count tubes.
11. One ml of precipitating reagent (PEG) was added to all tubes except total count tubes.
12. All tubes were vortexed and incubate at room temperature for 20 min.
13. Later the tubes except the total count tubes were centrifuged at 4500 rpm for 20 min.
14. After centrifugation the tubes were decanted and radioactivity in the precipitate was measured using gamma counter (Riastar, Packard, USA)

**Calculations**
1. Background counts were subtracted from all the counts to get actual counts.
2. All the duplicates were averaged.
3. Blank count was subtracted from all the other counts to give corrected average counts.
4. \[ \% \text{B/Bo} = \frac{\text{Corrected counts of sample/standard}}{\text{Corrected count of zero standard}} \times 100 \]
5. The standard curve of % B/Bo on the logit was plotted against µl/ml of insulin on the logarithmic scale using logit-log graph paper.
6. The concentration of insulin in sample was read from the standard curve by extrapolation.

(C) **Cholesterol**

*Principle*

Cholesterol esterase

\[ \text{Cholesterol Ester} + \text{O}_2 \rightarrow \text{Cholesterol} + \text{Fatty acids} \]

Cholesterol oxidase

\[ \text{Cholesterol} + \text{O}_2 \rightarrow \text{Dehydroacetone phosphate} + \text{H}_2\text{O}_2 \]

\[ 2\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-aminoantipyrine} \rightarrow \text{Red quinone} + 4\text{H}_2\text{O} \]
The intensity of the red complex (red quinone) formed during the reaction is directly proportional to the cholesterol concentration in the sample and is measured at 500nm.

Procedure

Reagents are reconstituted as described in the leaflet supplied along with the kit. 10 μl of serum samples, distilled water serving as control and standard triglyceride (200 mg/dl) serving as standard were mixed well with 1.0 ml reconstituted reagent i.e. enzyme/chromogen mixture. They were incubated at 37°C for min and absorbance was read against blank at 500nm.

Calculations

\[
\text{Serum cholesterol (mg/dl)} = \frac{\text{O.D. of test}}{\text{O.D. of std}} \times 200
\]

(D) Triglyceride

Principle

Triglycerides are enzymatically hydrolysed to glycerol according to the following reactions

\[
\begin{align*}
\text{Triglycerides} + \text{H}_2\text{O} & \xrightarrow{\text{lipoprotein lipase}} \text{Glycerol} + \text{free fatty acids} \\
\text{Glycerol} + \text{ATP} & \xrightarrow{\text{glycerol kinase}} \text{Glycerol-3-Phosphate} + \text{ADP} \\
\text{Glycerol-3-Phosphate} + \text{O}_2 & \xrightarrow{\text{peroxidase}} \text{Dehydroacetone phosphate} + \text{H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} + \text{ADPS} & \xrightarrow{\text{peroxidase}} \text{Red quinone} + 4\text{H}_2\text{O}
\end{align*}
\]

GPO = Glycerol-3-Phosphate Oxidase

ADPS = N-Ethyl-N-Sulfopropyl-n-anisidine
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The intensity of the red complex (red quinone) complex formed during the reaction is directly proportional to the triglyceride concentration in the sample and is measured at 546nm. The final colour is stable for at least 30 min.

Procedure

Reagents are reconstituted as described in the leaflet supplied along with the kit. 10 μl of serum samples, distilled water serving as control and standard triglyceride (200 mg/dl) serving as standard were mixed well with 1.0 ml reconstituted reagent 1 i.e. enzyme/chromogen mixture. They were incubated at 37°C for min and absorbance was read against blank at 546nm.

Calculations

\[
\text{Serum triglyceride (mg/dl)} = \frac{\text{O.D. of test}}{\text{O.D. of std}} \times 200
\]

(E) Creatinine

Principle

Creatinine in a protein free solution reacts with alkaline picrate and produces a red coloured complex, which is measured colorimetrically at 520nm.

Procedure

Deproteinization of test sample

0.5 ml of serum sample was mixed well with 0.5 ml distilled water and 3 ml picric acid (Reagent 1). It was kept in boiling water bath exactly for one minute and cooled immediately under running tap water and centrifuged.

2.0 ml of the supernatant from the above step is mixed with 1.0 ml sodium hydroxide solution (Reagent 2). 0.5 ml of distilled water and working creatinine standard mixed with 1.5 ml picric acid and 0.5 ml sodium hydroxide solution served as blank and standard respectively.
All the tubes were allowed to stand at room temperature after thorough mixing for 20 min. The absorbance of blank, standard and samples were measured immediately against distilled water at 520nm.

Calculations

Serum creatinine concentration was calculated using following formula

\[
\text{Serum Creatinine (mg/dl)} = \frac{\text{O.D. test} - \text{O.D. blank}}{\text{O.D. std} - \text{O.D. blank}}
\]

(F) Urea

Principle

The test is based on the Berthelot’s reaction. Urease splits urea into ammonia and carbon dioxide. The ammonia reacts with phenol in the presence of hypochlorite to form an indophenol which with alkali gives a blue coloured compound. The intensity of the colour is proportional to the concentration of urea in the sample and is measured at 546 nm. The colour of the reaction is stable for 8h.

Procedure

Reagents are reconstituted as described in the leaflet supplied along with the kit. 10 µl of serum sample, urea standard (40 mg/dl) and distilled water as blank were mixed with 100 µl of urease solution (Reagent 1). Contents are mixed and incubated at 37°C for 10 min. 1.5 ml of phenol (Reagent 2) and sodium hypochlorite solution (Reagent 3) were added to all test tubes and mixed well. The absorbance was read at 546nm UV-Visible Spectrophotometer (UV-1601 Shimadzu, Japan). The final colour developed is stable for at least 30 min.

The concentration of urea was calculated by using following formula

\[
\text{Serum Urea (mg/dl)} = \frac{\text{O.D. of test}}{\text{O.D. of std}} \times 40
\]
(G) Serum Glutamate Pyruvate Transaminase (SGPT)
(Reitman & Frankel's Method)

**Principle**

SGPT catalyses transfer of amino group from L-alanine to α-ketoglutarate with formation of pyruvate and glutamate. The pyruvate so formed, is allowed to react with 2,4 DNPH to produce 2,4-dinitrophenylhydrazone derivative which is brown coloured in alkaline medium. The absorbance of this hydrazone derivative is correlated to SGPT activity by plotting a calibration curve using pyruvate standard.

\[
\text{L-alanine} + \text{SGPT} \rightarrow \text{Pyruvate} + \alpha\text{-ketoglutarate} \rightarrow \text{Pyruvate} + \text{L-glutamate} \]

\[
\text{Pyruvate} \quad \text{Alkaline} \rightarrow 2,4\text{-dinitrophenylhydrazone} \quad \text{(Brown coloured)}
\]

**Procedure**

**Calibration Curve**

In five clean test tubes buffered substrate, pyruvate standard distilled water and DNPH colour reagent were added as per mentioned in the leaflet supplied in with the kit. Contents were mixed well and incubated at room temperature for 20 min. 5 ml of working sodium hydroxide was added to all the tubes, mixed and allowed to stand at room temperature for 10 min. Absorbance of tubes 2 to 5 was measured against tube 1 as reagent blank at 505nm. A graph of absorbance was plotted against enzyme activity (supplied in leaflet).

**Assay**

0.5ml buffered substrate was incubated at 37°C for 3 min. 0.1 ml serum sample was added to buffered substrate and incubated at 37°C for 60 min. To this DNPH colour reagent was added and allowed to stand at room temperature for 20 min. Finally 5.0 ml working sodium hydroxide solution was added to all the tubes. After mixing well the tubes were allowed to stand at room temperature for 10 min. In case on blank similar procedure was followed except that instead of serum 0.1 ml
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distilled water was added. Absorbance of test samples was measured against reagent blank at 505nm and was read on calibration curve to find out enzyme activity.

(H) Serum glutamate oxaloacetate transaminase (SGOT)
(Reitman & Frankel's Method)

Principle

SGOT catalyses transfer of amino group from L-aspartate to α-ketoglutarate with formation of oxaloacetate and glutamate. The oxaloacetate so formed, is allowed to react with 2,4-DNPH to produce 2,4-dinitrophenylhydrazone derivative which is brown coloured in alkaline medium. The absorbance of this hydrazone derivative is correlated to SGOT activity by plotting a calibration curve using pyruvate standard.

\[
\begin{align*}
\text{L-aspartate} & \quad \text{SGOT} & \quad \text{Oxaloacetate} \\
\alpha \text{-ketoglutarate} & \quad \text{pH 7.4} & \quad \text{L-glutamate} \\
\text{Oxaloacetate} & \quad \text{Alkaline} & \quad 2,4\text{-dinitrophenyl hydrazone} \\
2,4\text{-DNPH} & \quad \text{medium} & \quad \text{(Brown coloured)}
\end{align*}
\]

Procedure

Calibration Curve

In five clean test tubes buffered substrate, pyruvate standard distilled water and DNPH colour reagent were added as per mentioned in the leaflet supplied with the kit. Contents were mixed well and incubated at room temperature for 20 min. 5 ml of working sodium hydroxide was added to all the tubes, mixed and allowed to stand at room temperature for 10 min. Absorbance of tubes 2 to 5 was measured against tube 1 as reagent blank at 505nm. A graph of absorbance was plotted against enzyme activity (supplied in leaflet).

Assay

0.5 ml buffered substrate was incubated at 37°C for 3min. 0.1 ml serum sample was added to buffered substrate and incubated at 37°C for 60 min. To this DNPH colour reagent was added and allowed to stand.
at room temperature for 20 min. Finally 5.0 ml working sodium hydroxide solution was added to all the tubes. After mixing well the tubes were allowed to stand at room temperature for 10 min. In case on blank similar procedure was followed except that instead of serum 0.1 ml distilled water was added. Absorbance of test samples was measured against reagent blank at 505nm and was read on calibration curve to find out enzyme activity.

4.1.3 HISTOPATHOLOGICAL STUDY

Histopathological study of kidney and liver was carried out to study the effects of chronic treatment with vanadium and chromium compounds on degenerative changes induced by diabetes and also to assess the nephrotoxic and hepatotoxic potential of these heavy metals under the conditions of the present investigation.

Fixation of the tissues

Dissected kidney and liver were washed with normal saline and then kept in 10% formal saline. The tissues were then kept in Bouin's fixative for 18 to 24h. Tissues were then washed twice with distilled water and kept in 70% alcohol. A pinch of lithium carbonate was added to remove excessive stain. The tissues were washed and kept in 70% alcohol again. After that tissues were transferred to 90% alcohol and kept in it overnight. Next morning all tissues were transferred into 100% alcohol and kept for 3h. Then tissues were transferred to xylene and kept till they become transparent.

Microtomy

Tissues were fixed in melted paraffin in wooden blocks, so that sectioning can be performed. Several sections of 3 μm thickness were taken from each tissue and sections with uniform shape and size were selected for histology. Selected sections were fixed on the clear glass slide with the help of egg albumin.
Staining

Tissues were stained using Hematoxylin and Eosin (H&E) stain.

- Slides → Xylene → 100% alcohol → 90% alcohol
  - 20 min → 2-3 dips → 1-2 min

- Distilled Water → 30% alcohol → 50% alcohol → 70% alcohol
  - 2-5 min → 2-5 min → 2-5 min

- Hematoxylin stain → running tap water → 1% acid alcohol
  - 2-4 min → 5-10 min → 2-4 drops

- Acetone → Eosin stain → Running tap water
  - 2-3 min → 5 min

- Dry → Xylene → DPX mounting
  - 48 hrs

- Distilled water
  - 2 min
4.2  In Vitro Studies

4.2.1 Effect Of Vanadium And Chromium Synthesis On 3T3-L1 Preadipocyte Differentiation.

The 3T3-L1 fibroblasts (American Type Culture Collection, USA) were maintained in RPMI 1640 containing 5% fetal calf serum (FCS) (Gibco BRL, USA), 100 U/ml penicillin (Gibco BRL, USA) and 100 μg/ml streptomycin (Gibco BRL, USA), 0.5 μg/ml fungizone (Gibco BRL, USA) in a 75 cm² flask (Costar, USA) at 37°C in a humidified 95% air, 5% CO₂ atmosphere (Shibata et al., 1999). Preadipocytes (1 X 10⁵ cells/well) were cultured to confluency in 6 well plates (Costar, USA) for 2 days, then adipocyte differentiation was initiated by treating confluent preadipocytes with 1μM dexamethasone and 0.5 mM isobutylmethylxanthine (IBMX, Sigma Ltd, USA). After 2 days, the cells were given fresh medium containing the test compound (0-10 μM) in the presence and absence of 1 μg/ml insulin (Sigma Ltd, USA) and allowed to differentiate for additional 4 days. The triglyceride content of the cells was used as the principal end point of preadipocyte differentiation. Cells were washed once with 0.9% NaCl solution and 500 μl water was then added to each culture. The cells were scraped from the dishes using rubber policeman and transferred to 1.5 ml microcentrifuge tubes. The cell suspension was disrupted by sonication at maximum output for 15 seconds with a microtip. Triglycerides were determined using Ponte Scientific colorimetric estimation kit, USA. Cell layer protein content was estimated according to the method of Lowry et al. (1951). Triglycerides were expressed as μg/mg protein. Cellular triglyceride accumulation normalized by protein levels was measured as an index of the differentiated adipocyte phenotype.