

## ***Materials and Methods***

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### **Chemicals used and their sources**

#### **Biochemicals**

AMPA ( $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid), (+) MK-801 [(+)-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-iminemaleate], ethylene diamine tetra acetic acid (EDTA), Tris HCl, calcium chloride, paraformaldehyde, Streptozotocin, citric acid, Tris HCl, foetal calf serum (heat inactivated), D-glucose, calcium chloride, collagenase type XI, bovine serum albumin fraction V and RPMI-1640 medium were purchased from Sigma Chemical Co., St. Louis, MO. USA). All other reagents were of analytical grade purchased locally.

#### **Radiochemicals**

(+)-[<sup>3</sup>H] MK-801 (Sp. Activity 27.5 Ci/mmol) was purchased from Perkin Elmer NEN Life and Analytical Sciences, Boston, MA, USA. [<sup>3</sup>H] AMPA (Sp. Activity 43 Ci/mmol) was purchased from American Radiolabelled Chemicals INC, St Louis, Missouri, USA, [<sup>3</sup>H] IP3 Biotrak Assay Systems was purchased from G.E Healthcare UK Limited, UK. Radioimmunoassay kit for insulin was purchased from Baba Atomic Research Centre (BARC), Mumbai, India.

#### **Molecular Biology Chemicals**

Tri-reagent kit was purchased from Sigma Chemical Co., St. Louis, USA. ABI PRISM High Capacity cDNA Archive kit, Primers and Taqman probes for Real-Time PCR were purchased from Applied Biosystems, Foster City, CA, USA. GluR4 AMPAR (Rn00568544\_m1), GluR2 AMPAR (Rn00568514\_m1), NMDAR1 (Rn\_00433800), NMDA2B (Rn00561352\_m1), GLAST (Rn00570130\_m1), Bax (Rn\_01480160\_g1), GAD (Rn00562748\_m1), Akt 1

(Rn00583646\_m1), Caspase 8 (Rn00574069\_m1), GPx (Rn00577994), Pdx-1 (Rn\_00755591), NeuroD1 (Rn\_00824571) primers were used for the study.

### **Confocal Dyes**

Rat primary antibody for AMPAR (BD Pharmingen), NMDAR (BD Pharmingen), Insulin (Cell signaling) IP3 Receptor3 (BD Pharmingen) Vitamin D receptor (Pierce antibody) secondary antibody of either FITC (Chemicon), Rhodamine dye (Chemicon), Alexa Fluor 488 (Invitrogen), Alexa Fluor 594 (Invitrogen) and CY5 (Chemicon) were used for the immunohistochemistry studies using confocal microscope.

### **Animals**

Adult male Wistar rats of 180-250g body weight were purchased from Kerala Agriculture University, Mannuthy and Amrita Institute of Medical Sciences, Kochi and were used for all the experiments. They were housed in separate cages under 12 hours light and 12 hours dark periods and were maintained on standard food pellets and water *ad libitum*. Adequate measures were also taken to minimize pain and discomfort of the animals. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health guidelines and CPCSEA guidelines.

### **DIABETES INDUCTION**

Diabetes was induced in rats by intrafemoral injection of streptozotocin (Sigma chemicals Co., St. Louis, MO, USA.) freshly dissolved in citrate buffer pH 4.5 under anesthesia (Junod *et al.*, 1969). Streptozotocin was given at a dose of 55mg/Kg body weight (Arison *et al.*, 1967; Hohenegger & Rudas, 1971).

### **DETERMINATION OF BLOOD GLUCOSE**

The diabetic state of animals was assessed by measuring blood glucose concentrations at 72 hours after streptozotocin treatment. The rats with a blood sugar level above 250 mg/dl were selected as diabetic rats.

## **DETERMINATION OF ANTI-DIABETIC POTENTIAL OF CURCUMIN AND VITAMIN D<sub>3</sub>**

Animals used in this study were randomly divided into the following groups. Each group consisted of 6-8 animals.

- a) Group 1: Control (given citrate buffer injection)
- b) Group 2: Diabetic
- c) Group 3: Diabetic rats treated with insulin
- d) Group 4: Diabetic rats treated with Curcumin
- e) Group 5: Diabetic rats treated with Vitamin D<sub>3</sub>

The insulin treated diabetic group (Group 3) received subcutaneous injections (1Unit/kg body weight) of insulin daily during the entire period of the experiment. A mixture of both lente and plain insulin (Abbott India) were given for the better control of glucose (Sasaki & Bunag, 1983). The last injection was given 24 hr before sacrificing the diabetic rats. Curcumin was given orally to the 4<sup>th</sup> group of diabetic rats in the dosage of 60mg/Kg body weight suspension of curcumin orally at 24 hour intervals. Curcumin was suspended in 0.5% w/v sodium carboxymethylcellulose immediately before administration in constant volume of 5ml/kg body weight (Sharma *et al.*, 2006). Cholecalciferol was given orally to the 5<sup>th</sup> group of diabetic rats in the dosage of 12 µg/Kg body weight dissolved in 0.3 ml of coconut oil (deSouzaSantos *et al.*, 2005). Blood samples were collected from the tail vein at 0 hours (before the start of the experiment), 3<sup>rd</sup>, 8<sup>th</sup>, 12<sup>th</sup> and 16<sup>th</sup> day and the glucose levels were estimated. Blood samples were collected 3hrs after the administration of morning dose. Changes in the body weight of animals were monitored on 1<sup>st</sup> Day (before the start of the experiment), 7<sup>th</sup> and 15<sup>th</sup> day.

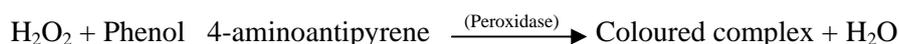
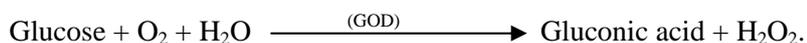
## SACRIFICE AND TISSUE PREPARATION

The animals were then sacrificed on 16<sup>th</sup> day by decapitation. The cerebral cortex, cerebellum and brain stem were dissected out quickly over ice according to the procedure of Glowinski and Iversen, (1966) and the pancreas was dissected quickly over ice. Hippocampus was dissected according to the procedure of Heffner *et al.*, (1980). The blood samples were collected and plasma was separated by centrifugation. The tissue samples and plasma were kept at -80° C until assay. All animal care and procedures were in accordance with Institutional and National Institute of Health guidelines.

## ESTIMATION OF BLOOD GLUCOSE

Blood glucose was estimated using Glucose estimation kit (Merck). The spectrophotometric method using glucose oxidase-peroxidase reactions is as follows:

Principle: Glucose oxidase (GOD) catalyses the oxidation of glucose in accordance with the following equation:



The hydrogen peroxide formed in this reaction reacts with 4-aminoantipyrine and 4-hydroxybenzoic acid in the presence of peroxidase (POD) to form N-(4-antipyryl)-p-benzo quinoneimine. The addition of mutarotase accelerates the reactions. The amount of dye formed is proportional to the glucose concentration. The absorbance was read at 510nm in (Shimadzu UV-1700 pharmaSPEC) spectrophotometer.

## **ESTIMATION OF CIRCULATING INSULIN BY RADIOIMMUNOASSAY**

### **Principle of the assay**

The assay was done according to the procedure of BARC radioimmunoassay kit. The radioimmunoassay method is based on the competition of unlabelled insulin in the standard or samples and [<sup>125</sup>I] insulin for the limited binding sites on a specific antibody. At the end of incubation, the antibody bound and free insulin were separated by the second antibody-polyethylene glycol (PEG) aided separation method. Measuring the radioactivity associated with bound fraction of sample and standards quantitates insulin concentration of samples.

### **Assay Protocol**

Standards, ranging from 0 to 200 µU/ml, insulin free serum and insulin antiserum (50µl each) were added together and the volume was made up to 250µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. They were incubated overnight at 2°C. Then [<sup>125</sup>I] insulin (50µl) was added and incubated at room temperature for 3 hours. The second antibody was added (50µl) along with 500µl of PEG. The tubes were then vortexed and incubated for 20 minutes and they were centrifuged at 1500 x g for 20 minutes. The supernatant was aspirated out and the radioactivity in the pellet was determined in a gamma counter.

A standard curve was plotted with %B/Bo on the Y-axis and insulin concentration/ml on the X-axis of a log-logit graph. %B/Bo was calculated as:

$$\frac{\text{Corrected average count of standard or sample}}{\text{Corrected average count of zero standard}} \times 100$$

Insulin concentration in the samples was determined from the standard curve plotted using MultiCalc™ software (Wallac, Finland).

### **Quantification of Glutamate**

Glutamate content in the brain regions – hippocampus, cerebral cortex, cerebellum and brain stem of control and experimental rats were quantified by displacement method using modified procedure of Enna and Snyder, (1976). Tissues were homogenized in 20 volumes of 0.32 M sucrose, 10 mM Tris/HCl and 1 mM MgCl<sub>2</sub> buffer, pH 7.4, with a polytron homogenizer. The homogenate was centrifuged twice at 27,000 x g for 15 min. The supernatant was pooled and used for the assay. The incubation mixture contained 1 nM [<sup>3</sup>H] glutamate with and without glutamate at a concentration range of 10<sup>-9</sup> M to 10<sup>-4</sup> M. The unknown concentrations were determined from the standard displacement curve using appropriate dilutions and calculated for nmoles/g wt. of the tissue.

### **Determination of SOD Activity**

The brain regions- hippocampus, cerebral cortex, cerebellum and brain stem of control and experimental rats were homogenized in 0.1M potassium phosphate buffer, pH 7.8 and centrifuged at 100,000 x g for 60 min at 4°C. The supernatant corresponds to the cytosolic fraction containing CuZn-SOD. The pellets were re-suspended in the buffer, freeze-thawed three times and centrifuged at 100,000 x g for 60 min at 4°C. The supernatant, the particulate fraction containing Mn-SOD, was mixed with the cytosolic fraction to obtain the total enzyme fraction. SOD was analyzed after inhibition by SOD of the pyrogallol autoxidation (Marklund & Marklund, 1974) at pH 8.2 in the presence of EDTA. A 3ml assay mixture contained 0.2 mM pyrogallol, 1 mM EDTA and 50 mM Tris-HCl buffer. Pyrogallol autoxidation was monitored at 420 nm for 3 min in a spectrophotometer (Shimadzu UV-1700) with or without the enzyme. The inhibition of pyrogallol oxidation was linear with the activity of the enzyme present. Fifty percent inhibition/mg protein/min was taken as one unit of the enzyme activity.

## **GLUTAMATE RECEPTOR BINDING STUDIES USING [<sup>3</sup>H] RADIOLIGANDS IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS**

### **NMDA receptor binding studies**

The membrane fractions were prepared by a modification of the method described by Hoffman *et al.*, (1996). The brain regions (cerebral cortex, hippocampus, cerebellum and brain stem) and pancreas were homogenized in a 0.32 M sucrose buffer solution containing 10 mM HEPES, 1 mM EDTA buffer, pH 7.0. The homogenate was centrifuged at  $1,000 \times g$  for 10 min and the supernatant was centrifuged at  $40,000 \times g$  for 1 h. The pellet was re-suspended and homogenized in 10 mM HEPES buffer containing 1.0 mM EDTA, pH 7.0 and centrifuged at  $40,000 \times g$  for 1 h. The final pellet was suspended in 10 mM HEPES, 1 mM EDTA buffer, pH 7.0 and stored at  $-80^{\circ}\text{C}$  until binding assays were performed. The [<sup>3</sup>H] MK-801 binding saturation assay was performed in a concentration range of 0.25 to 50 nM at  $23^{\circ}\text{C}$  in an assay medium containing 10 mM HEPES, pH 7.0, 200 - 250  $\mu\text{g}$  of protein, 100  $\mu\text{M}$  glycine and 100  $\mu\text{M}$  glutamate. After 1 h of incubation, the reaction was stopped by filtration through GF/B filters and washed thrice with HEPES buffer pH 7.0. Specific [<sup>3</sup>H] MK-801 binding was obtained by subtracting nonspecific binding in the presence of 100  $\mu\text{M}$  unlabeled MK-801 from the total binding. Bound radioactivity was counted with cocktail-T in a Perkin Elmer Tri-Carb 2810 TR liquid scintillation analyser.

### **AMPA receptor binding studies**

The brain regions (cerebral cortex, hippocampus, cerebellum and brain stem) and pancreas of control and experimental rats were homogenized in 25 volumes of cold 50 mM Tris-HCl, 10 mM EDTA, pH 7.1, buffer with a Polytron 10,000rpm for 30 s. The pellet was re-suspended in 50 volumes of 50 mM Tris-HCl pH 7.1, containing 0.04% Triton X-100. The homogenate was incubated for 30 minutes at  $37^{\circ}\text{C}$ , then washed three times with 50 mM Tris-HCl, pH 7.1,

binding buffer, and centrifuged as above. The final pellet was re-suspended in 50 volumes of binding buffer original wet weight and used as such in the assay. The final concentration of membrane in the assay was 10 mg/ml wet weight. The incubation was performed in the presence of 1, 2.5, 5, 7.5 nM [<sup>3</sup>H] AMPA respectively, specific activity 45.8 Ci/mmol]. Nonspecific binding was determined in the presence of 1 mM AMPA. After 1 h of incubation at 4°C, the suspension was filtered in Whatman GF/C and washed five times with 3 ml of cold binding buffer. The radioactivity on the filter was measured by liquid scintillation spectrometer. Specific binding was determined by subtracting non-specific binding from the total binding.

### **Protein determination**

Protein was measured by the method of Lowry *et al.*, (1951) using bovine serum albumin as standard. The intensity of the purple blue colour formed was proportional to the amount of protein which was read in Spectrophotometer at 660nm.

## **ANALYSIS OF THE RECEPTOR BINDING DATA**

### **Linear regression analysis for Scatchard plots**

The data was analysed according to Scatchard, (1949). The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding ( $B_{max}$ ) and equilibrium dissociation constant ( $K_d$ ), were derived by linear regression analysis by plotting the specific binding of the radioligand on X-axis and bound/free on Y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The  $K_d$  is inversely related to receptor affinity.

## **GENE EXPRESSION STUDIES IN DIFFERENT BRAIN REGIONS AND PANCREAS OF CONTROL AND EXPERIMENTAL RATS**

### **Isolation of RNA**

RNA was isolated from the brain regions (cerebral cortex, hippocampus, cerebellum and brain stem) and pancreas of control and experimental rats using the Tri reagent from Sigma Chemicals Co., St. Louis, MO, U.S.A). 25-50 mg tissue homogenates were made in 0.5 ml Tri Reagent. The homogenate was kept in the room temperature for 5 minutes. 100 µl of chloroform was added to the homogenate, mixed vigorously for 15 seconds kept in the RT for 10-15 minutes and was centrifuged at 12,000xg for 15 minutes at 4°C. The upper aqueous phase was transferred to a fresh tube and 250µl of isopropanol was added and the tubes were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000xg for 10 minutes at 4°C. RNA precipitated as a pellet on the sides and bottom of the tube. The supernatant was removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000xg for 5 minutes at 4°C. The pellets were semi dried and dissolved in minimum volume of DEPC-treated water. 2 µl of RNA was made up to 1 ml and absorbance was measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 was  $\geq 1.7$ . The concentration of RNA was calculated as one absorbance  $A_{260} = 42\mu\text{g}$ .

## **REAL-TIME POLYMERASE CHAIN REACTION**

### **cDNA synthesis**

Total cDNA synthesis was performed using ABI PRISM cDNA archive kit in 0.2ml microfuge tubes. The reaction mixture of 20 µl contained 0.2µg total RNA, 10 X RT buffer, 25 X dNTP mixture, 10 X random primers, MultiScribe RT (50U/µl) and RNase free water. The cDNA synthesis reactions were carried out at 25 °C for 10 minutes and 37 °C for 2 hours using an Eppendorf Personal Cycler. The primers and probes were purchased from Applied Biosystems, Foster City, CA, USA designed using Primer Express software version (3.0).

### **Real-time PCR assays**

Real Time PCR assays were performed in 96-well plates in an ABI 7300 Real Time PCR instrument (Applied Biosystems). To detect gene expression, a TaqMan quantitative 2-step reverse transcriptase “polymerase chain reaction (RT-PCR) was used to quantify mRNA levels. First-strand cDNA was synthesized with use of a TaqMan RT reagent kit, as recommended by the manufacturer. PCR analyses were conducted with gene-specific primers and fluorescently labeled TaqMan probe, designed by Applied Biosystems. All reagents were purchased from Applied Biosystems. The probes for specific gene of interest were labeled with FAM at the 5' end and a quencher (Minor Groove Binding Protein - MGB) at the 3' end. The Real-Time data were analyzed with Sequence Detection Systems software version 1.7. All reactions were performed in duplicate.

The TaqMan reaction mixture of 20 µl contained 25 ng of total RNA-derived cDNAs, 200 nM each of the forward primer, reverse primer and PCR analyses were conducted with gene-specific primers and fluorescently labelled Taqman probes of NMDAR1, NMDAR2, GluR4 AMPAR, GluR2 AMPAR, GLAST, Gpx, GAD, Akt 1, Bax and Caspases 8. Endogenous control (β-actin) was labeled with a reporter dye (VIC). 12.5 µl of TaqMan 2X Universal PCR Master Mix was taken and the volume was made up with RNase free water. Each run contained both negative (no template) and positive controls.

The thermocycling profile conditions were as follows:

50°C -- 2 minutes ---- Activation

95°C -- 10 minutes ---- Initial Denaturation

95°C -- 15 seconds ---- Denaturation                      40 cycles

50°C -- 30 seconds --- Annealing

60°C -- 1 minute --- Final Extension

Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The  $\Delta\Delta\text{CT}$  method of relative quantification was used to determine the fold change in expression. This was done by first normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control  $\beta$ -actin in the same samples ( $\Delta\text{CT} = \text{CT}_{\text{Target}} - \text{CT}_{\beta\text{-actin}}$ ). It was further normalized with the control ( $\Delta\Delta\text{CT} = \Delta\text{CT} - \text{CT}_{\text{Control}}$ ). The fold change in expression was then obtained ( $2^{-\Delta\Delta\text{CT}}$ ).

### **IP3 CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS**

The brain regions (cerebral cortex, hippocampus, cerebellum and brain stem) and pancreas were homogenised in a polytron homogeniser in 50 mM Tris-HCl buffer, pH.7.4, containing 1 mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15 min and the supernatant was transferred to fresh tubes for IP3 assay using [<sup>3</sup>H] IP3 Biotrak Assay System kit.

#### **Principle of the assay**

The assay was based on competition between [<sup>3</sup>H] IP3 and unlabelled IP3 in the standard or samples for binding to a binding protein prepared from bovine adrenal cortex. The bound IP3 was then separated from the free IP3 by centrifugation. The free IP3 in the supernatant was then discarded by simple decantation, leaving the bound fraction adhering to the tube. Measurement of the radioactivity in the tube enables the amount of unlabelled IP3 in the sample to be determined.

#### **Assay Protocol**

Standards, ranging from 0.19 to 25 pmoles/tube, [<sup>3</sup>H] IP3 and binding protein were added together and the volume was made up to 100  $\mu\text{l}$  with assay

buffer. Samples of appropriate concentration from experiments were used for the assay. The tubes were then vortexed and incubated on ice for 15 minutes and they were centrifuged at 2000 x g for 10 minutes at 4°C. The supernatant was aspirated out and the pellet was re-suspended in water and incubated at room temperature for 10 minutes. The tubes were then vortexed and decanted immediately into scintillation vials. The radioactivity in the suspension was determined using liquid scintillation counter.

A standard curve was plotted with %B/Bo on the Y-axis and IP3 concentration (pmoles/tube) on the X-axis of a semi-log graph paper. %B/Bo was calculated as:

$$\frac{(\text{Standard or sample cpm} - \text{NSB cpm})}{(\text{Bo cpm} - \text{NSB cpm})} \times 100$$

NSB- non specific binding and B<sub>0</sub> - zero binding. IP3 concentrations in the samples were determined by interpolation from the plotted standard curve.

### **NMDA R1, NMDA 2B AND AMPA GluR2 GluR4 RECEPTOR SUBUNIT EXPRESSION STUDIES IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE**

Control and experimental rats were deeply anesthetized with ether. The rats were transcardially perfused with phosphate buffered saline (PBS), pH- 7.4, followed by 4% paraformaldehyde in PBS (Chen *et al.*, 2007). After perfusion the brains were dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1 M PBS, pH- 7.0. 10 µm sections were cut using cryostat (Leica, CM1510 S). The sections were treated with PBS. To block unspecific binding the sections were incubated for 1 hour at room temperature with 5% bovine serum albumin in normal goat serum. Brain slices were incubated overnight at 4°C with either rat primary antibody for NMDA

R1 (diluted in Phosphate buffered saline Triton X- 100 (PBST) at 1: 500 dilution), NMDA 2B (diluted in PBST at 1: 500 dilution) and AMPA (GluR2) (diluted in PBST at 1: 500 dilution) and AMPA (GluR4) (diluted in PBST at 1: 500 dilution) After overnight incubation, the brain slices were rinsed with PBST and then incubated with appropriate secondary antibody of either FITC (diluted in PBST at 1: 1000 dilution) or rhodamine dye (Chemicon, diluted in PBST at 1: 1000 dilution). The sections were observed and photographed using confocal imaging system (Leica SP 5).

#### **INSULIN, AMPA GluR4, GluR2, VITAMIN D AND IP3 RECEPTOR EXPRESSION STUDIES IN THE PANCREAS OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE**

Anaesthetized rats with chloral hydrate were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (pH 7.4). After perfusion the pancreas was dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1M PBS. 10 µm sections were cut using cryostat (Leica, CM1510 S). To block unspecific binding the sections were incubated for 1 hour at room temperature with 5% bovine serum albumin in normal goat serum. Pancreatic sections were incubated overnight at 4°C with either rat primary antibody for IP3 receptor (diluted in PBST at 1: 500 dilution) and Vitamin D receptor (diluted in PBST at 1: 500 dilution). After overnight incubation, the pancreatic sections were rinsed with PBST and then incubated with appropriate secondary antibody of either FITC (diluted in PBST at 1: 1000 dilution) or rhodamine dye (Chemicon, diluted in PBST at 1: 1000 dilution).

For co-label studies after blocking with goat serum the pancreatic sections were incubated overnight at 4°C with primary antibody for AMPA (GluR4) (diluted in PBST at 1: 500 dilution) and AMPA (GluR2) receptor (diluted in PBST at 1: 500 dilution) subunits. After overnight incubation, the pancreatic sections were rinsed with PBST and then incubated with Alexa flour 594 secondary antibody (diluted in PBST at 1: 1000 dilution) for 2 h at room

temperature. At the end of incubation period, sections were washed three times using PBST and incubated overnight with rat primary antibody for Insulin. The slides were washed with PBST after the incubation time and secondary antibody Alexa Fluor 488(diluted in PBST at 1: 1000 dilution) was added and incubated for 2 hours at. The sections were observed and photographed using confocal imaging system (Leica SP 5). After incubation pancreatic slices were thoroughly washed, mounted, observed and photographed using confocal imaging system (Leica SP 5).

### **ISOLATION OF PANCREATIC ISLETS**

Pancreatic islets were isolated from male Wistar rats by standard collagenase digestion procedures using aseptic techniques (Howell & Taylor, 1968). The islets were isolated in HEPES-buffered sodium free Hanks Balanced Salt Solution (HBSS) (Pipeleers *et al.*, 1985) with the following composition: 137 mM Choline chloride, 5.4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 14.3 mM KHCO<sub>3</sub> and 10 mM HEPES. Autoclaved triple distilled water was used in the preparation of the buffer.

The pancreas from the rats were aseptically dissected out into a sterile petridish containing ice cold HBSS and excess fat and blood vessels were removed. The pancreas was cut into small pieces and transferred to a sterile glass vial containing 2 ml collagenase type XI solution (1.5 mg/ml in HBSS, pH 7.4). The collagenase digestion was carried out for 15 minutes at 37°C in an environmental shaker with vigorous shaking (300 rpm/minute). The tissue digest was filtered through 500 µm nylon screen and the filtrate was washed with three successive centrifugations and re-suspensions in cold HBSS. The pancreatic islet preparation having a viability of >90% as assessed by Trypan Blue exclusion which was chosen for experiments.

### **CALCIUM IMAGING STUDIES USING CONFOCAL MICROSCOPE**

Pancreatic islets were prepared from adult rats by collagenase digestion method as mentioned earlier. The isolated islets were incubated for 4 hours at room temperature in 1 ml of calcium free RPMI medium containing 5  $\mu\text{M}$  of  $\text{Ca}^{2+}$  fluorescent dye, fluo 4-AM (Molecular Probes, Eugene, OR) to monitor the changes in the intracellular  $\text{Ca}^{2+}$ . Following experimental conditions were given; Control: Pancreatic islet in 4mM glucose Diabetic: Pancreatic islet in 20mM glucose, D+I: Pancreatic islet in 20mM glucose, insulin and D+C Pancreatic islet in 20mM glucose, curcumin (Abdel Aziz *et al.*, 2010) and, D+V Pancreatic islet in 20mM glucose,  $10^{-12}$  M Vitamin  $\text{D}_3$  (Bourlon *et al.*, 1999). After incubation cells were washed twice in indicator free RPMI medium to remove excess dye that was non-specifically associated with the cell surface and then incubated for further 30 minutes to allow complete de-esterification of intra- cellular AM esters. The 35 mm plates, containing pancreatic islet cells were placed on the stage of a Leica TCS SP5 laser scanning confocal microscope equipped with a HC PL FLUOTAR 20.0x 0.50 dry objective (NA 0.5). Fluo 4-AM was excited with 514 nm laser lines from an argon laser, with laser intensity set at 38% of available power. For visualization of Fluo 4-AM, the emission window was set at 508.4 nm – 571.5 nm. The images were continuously acquired before and after addition of  $10^{-5}$  M AMPA (Bertrand *et al.*, 1992) at time intervals of 26.35, 104.1 seconds. Time series experiments were performed collecting 512x512 pixel images at 400 Hz. Fluorescence intensity was analysed using the quantitation mode in LAS-AF software from Leica Microsystems, Germany. A region of interest (ROI) was drawn within a field of view. For each ROI, the pixel intensity was calculated for each image in the 600 seconds sequence to analyse the intracellular  $\text{Ca}^{2+}$  release from the pancreatic islet cells in experimental conditions.

## **Statistics**

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using SIGMA PLOT (Ver 2.03). Sigma Plot software (version 2.0, Jandel GmbH, Erkrath, Germany). Competitive binding data were analysed using non-linear regression curve fitting procedure (GraphPad PRISM™, San Diego, USA). Empower software were used for HPLC analysis. Relative Quantification Software was used for analyzing Real-Time PCR results.