Evaluation of mitochondria-linked PI3K/Akt/GLUT-4 signaling pathway in rat brain tissues in chronic model of encephalopathy induced by T2DM

Introduction

The type-2 diabetes mellitus (T2DM) is considered as a metabolic epidemic with neuropsychiatric complications (Garabadu and Krishnamurthy, 2013). Epidemiological studies demonstrate that the untreated T2DM causes several complications (Cohen et al., 2007; Rolo and Palmeira, 2006) including central nervous system disorder (Ott et al., 1999; Stewart and Liolitsa, 1999; Alvarez et al., 2009; Janson et al., 2004), a condition that may be referred to as “diabetic encephalopathy” (Brands et al., 2003). In the previous chapter, we have reported that there exist anxiety-like behavioral manifestations in the T2DM animals. Further, it has been proposed that the derailment of mitochondria-linked PI3K/Akt/GLUT-4 signaling in several brain regions of these animals. These observations suggest that there is an early dysregulation in the brain activity in the course of T2DM. It has been documented that in most of the T2DM cases the encephalopathic condition is common probably due to insufficient therapeutic management (Ott et al., 1999; Stewart and Liolitsa, 1999; Alvarez et al., 2009; Brands et al., 2003; Janson et al., 2004). Therefore, it is essential to elaborate the pathophysiology of encephalopathic condition in T2DM.

The pathobiology of encephalopathy in T2DM condition involves several factors including the development of insulin resistance (IR) in the central tissues (Jurysta et al., 2013; Bazotte et al., 2014). At sub-cellular level, the derailment of the insulin receptor substrate/phopstidylionositol-3 kinase/Akt (IRS/PI3K/Akt) signaling pathway is reported as one of the plausible molecular mechanism for IR (Benomar et al., 2006). This leads to the reduction of the translocation of the glucose transporter-4 (GLUT-4) from the cytosol to the plasma membrane (Furtado et al., 2002; Watson et al., 2004; Leney and Tavaré, 2009) in brain tissues

(Jurysta et al., 2013; Blázquez et al., 2014). Moreover, IR in terms of decrease in the translocation of GLUT-4 in several anxiety-sensitive brain regions such as hippocampus (HIP) and hypothalamus (HYP) has been reported in post-traumatic stress disorder subjected animals (Cohen et al., 2009). Hence, the PI3K/Akt/GLUT-4 signaling pathway was evaluated as a molecular basis to IR in discrete brain regions in T2DM-induced encephalopathic condition.

Previously, we report the dysfunction of mitochondria in several brain regions in terms of increase in the activity of respiratory complex enzymes, increase in oxidative stress and compromised activities of antioxidant enzymes in T2DM rodents (Garabadu and Krishnamurthy, 2013; Garabadu and Krishnamurthy, 2014). Mitochondrial dysfunction is also associated with an increase in apoptosis in several brain regions of diabetic as well as post-traumatic stress disorder exposed animals (Yang et al., 2009; Zarros et al., 2009; Garabadu et al., 2015) suggesting the fact that mitochondrial dysfunction can induce apoptosis. Thus, it is speculated that mitochondrial dysfunction is strongly associated with the derailment of PI3K/Akt/GLUT-4 signaling pathway in T2DM-induced encephalopathic condition. However, till date there is no report of any mitochondrial drug on the PI3K/Akt/GLUT-4 signaling pathway in this T2DM-induced encephalopathic condition. Metformin exhibits neuroprotective effect in several neurological disorders (Zhu et al., 2014; Patil et al., 2014) and in addition shows anxiolytic activity in the comorbid condition of T2DM and stress (Garabadu and Krishnamurthy, 2014).

Therefore, the present study was undertaken to evaluate the effect of mitochondrial complex-I inhibitor, metformin, on T2DM-induced changes in the PI3K/Akt/GLUT-4 signaling pathway in HIP, HYP, pre-frontal cortex (PFC) and amygdale (AMY) of rats. The indices such as glucose homeostasis, β-cell function, hyperinsulinemia, IR, hypercorticosteronemia and dyslipidemia were estimated in T2DM-induced encephalopathic animals to evaluate the anti-

diabetic activity of metformin. The anxiolytic-like activity of metformin was estimated in several experimental animal models such as open field (OF), hole-board (HB) and elevated plus maze (EPM) tests. Further, the overt effect of metformin on mitochondrial dysfunction in terms of mitochondrial function (respiratory complex enzyme activity), integrity (mitochondrial membrane potential) and mitochondrial-linked apoptosis (levels of expression of cytochrome-C and percentage of apoptotic cells in flow cytometry) was evaluated in these discrete regions.

Experimental procedures

Animals

Male Charles Foster strain albino rats (200–250 g) were purchased from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University (BHU) and were housed in polypropylene cages under controlled environmental conditions (25 ± 1 °C, 45-55% relative humidity and 12:12 h light/dark cycle). The experiments were conducted in accordance with the principles of laboratory animal care (National Research Council US Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). Experiments on animals were approved by the Institutional Animal Ethics Committee of BHU, Varanasi, India (Protocol No: Dean/11-12/CAEC/328). The animals had free access to commercial rat feed (Doodh dhara Pashu Ahar, India) and water *ad libitum* unless stated otherwise during the experiment. Animals were acclimatized for at least one week before using them for experiments and exposed only once to every experiment.

Chemicals

Streptozotocine, thiobarbituric acid (TBA), tetra methyl rhodamine methylester (TMRM) and dexamethasone were procured from Sigma (St. Louis, MO, USA). Antibodies such as phosphor-Akt^{ser473} (p-Akt), total Akt, GLUT-4, cytochrome-C and beta-actin were purchased from Abcam

Plc., Cambridge, USA. All other chemicals and reagents were available commercially from local suppliers and were of analytical grade.

Induction of T2DM-induced encephalopathy in animals

The T2DM was induced in overnight fasted rats by a single injection of streptozotocine (45 mg/kg, i.p.), 15 min after nicotinamide (110 mg/kg, i.p.) administration. Streptozotocine was dissolved in 0.1M citrate buffer (pH 4.5) and nicotinamide was dissolved in physiological saline (Masiello et al., 1998; Garabadu and Krishnamurthy, 2013). Rats with fasting serum glucose level ≥ 13.89 mmol/l were considered diabetic and were continued in the experimental protocol of 31 days since the day of injection of streptozotocine as the commencement of the experimental design. Further, the diabetic animals with anxiety-like manifestations were considered as encephalopathic on last day of the experimental protocol (Liapi et al., 2010).

Experimental design

The whole study protocol consisted of three independent sets of experiments. The animals were acclimatized for seven days and were randomly divided into four groups of six animals each namely control, metformin treated control (MET), type 2 diabetic (T2DM) and metformin administered T2DM (DMET) in each of the experiment. The experimental protocol was followed for 31 days for all experiments. The day animals received the streptozotocine and nicotinamide injection was considered as day-1 (D-1). On D-7, after 1 hr to blood collection, either metformin (25 mg/kg, *p.o.*; Yanardag et al., 2005) or vehicle was administrated to the respective group animals. This treatment schedule was continued for 25 consecutive days i.e., from D-7 to D-31 of the experimental design. The experiment 1 and 2 were performed for the oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) respectively after 1 hr to last dose on D-31. In experiment 3, after 1 hr to last dose on D-31, all the animals were subjected to

access the level of anxiety in open field (OF), hole-board (HB) and elevated plus maze (EPM) test paradigms with a time lag of 15 min between each performance. All the behavioral observations were recorded and quantified with ANY-maze™ (Version-3.72, USA) video-tracking system. Thereafter, all the animals were killed by decapitation. The blood was collected and stored immediately at -80 °C till further study. The brains were removed and microdissected (Palkovits and Brownstein, 1988) into hippocampus (HIP), hypothalamus (HYP), pre-frontal cortex (PFC) and amygdala (AMY) and stored immediately at -80 °C till further study.

Oral glucose tolerance test (OGTT)

Oral glucose tolerance test (OGTT) is considered as a classical and model-based estimate of beta-cell function (Rijkelijkhuizen et al., 2009). The OGTT was performed on overnight fasted rats on 31st day of the experimental schedule. Metformin and vehicle were given 60 min prior to glucose administration (2 g/kg, i.g.). The blood samples were collected through retro-orbital puncture just before glucose load (0 min) and at 30, 60 and 120 min after glucose administration. Plasma glucose concentrations were determined with glucose GOD PAP kit (Priman Instrument Pvt. Ltd., India) based on glucose oxidase method (Wang et al., 2013).

Insulin tolerance test (ITT)

Insulin tolerance test (ITT) is a simple and reliable method of estimating insulin sensitivity (Duseja et al., 2007; Muniyappa et al., 2008). The ITT was performed on overnight fasted rats on 31st day of the experimental schedule. Metformin and vehicle were given 60 min prior to insulin administration (0.4 IU/kg, s.c.). The blood samples were collected through retro-orbital puncture just before glucose load (0 min) and at 30, 60 and 90 min after insulin administration. Plasma glucose concentrations were determined with glucose GOD PAP kit (Priman Instrument Pvt. Ltd., India) based on glucose oxidase method (Wang et al., 2013).

Assessment of anxiety in different behavioral models

Open field (OF) test

An open field apparatus, made of plywood and consisting of a square (61 × 61 cm) with high walls (61 × 61 cm), similar to that of Bronstein (1972) was used to study both anxiety-like behavior and locomotor activity in rats. The entire apparatus was painted black except for 6 mm white lines that divided the floor into 16 squares. Each animal was placed in the periphery of the test apparatus and the total distance travelled was recorded for 5 min. The ambulation (the number of squares crossed by the animal), rearing (number of times the animal stood on the hind limbs) and percentage in time spent in the center of the apparatus was considered as a measure of anxiety-like behaviors and the total distance travelled in the OFT was recorded as an index of locomotor activity.

Hole-board (HB) test

The HB apparatus consisted of Perspex box (60 × 60 × 35 cm) with four equidistant holes 4 cm in diameter in the floor. The floor of the box was positioned 12 cm above the ground and divided into nine (20 × 20 cm) squares. For the HB experiments, each animal was placed in the center of the apparatus and allowed to freely explore the apparatus for 5 min. Total number of head-dipping, sniffing and squares crossed were recorded. The ratio of head-dipping/sniffing was also calculated as a measure of anxiety (Garabadu and Krishnamurthy, 2014).

Elevated plus maze (EPM) test

The EPM test was used to assess anxiety-like behaviors in a fabricated apparatus (Garabadu and Krishnamurthy, 2014). The fabricated EPM consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 × 40 cm) with an open roof elevated to a height of 50 cm from the floor in a dimly lit room. The rats were placed individually on the central square of the plus maze facing

an enclosed arm. The percentage of entries and time spent into open arm were observed as indices of anxiety-like behaviors. The total arm entries and the speed in whole maze were recorded as an index of locomotor activity. An arm entry was defined when all four limbs of the rat were on the arm (Pellow et al., 1985).

Estimation of plasma glucose and lipid profile

On D-31 of the experimental protocol, 1 ml of blood was collected through retro-orbital puncture and centrifuged at 3000 × *g* for 5 min at 4 °C to obtain plasma for measuring the glucose, insulin, triglyceride, high density lipoprotein (HDL), low density lipoprotein (LDL) and corticosterone levels. The insulin was estimated by ELISA kit following manufacturer's instructions. The plasma glucose, triglyceride, HDL and LDL were determined spectrophotometrically (BioTek Instruments Inc., Epoch®, USA) in triplicate using commercial available kits.

Insulin resistance and β-cell function

Homoeostasis model assessment (HOMA) of IR (HOMA-IR) and HOMA of β-cell function (HOMA-B) were calculated by the HOMA method using the following equations (Matthews et al., 1985): IR (HOMA-IR) ¼ (fasting glucose (mmol/l) × fasting insulin (mIU/ml))/22·5, and β-cell function (HOMA-B) ¼ (20 × fasting insulin (mIU/ml))/(fasting glucose (mmol/l) – 3·5).

Estimation of plasma corticosterone level

The plasma corticosterone was quantified in a HPLC with Ultraviolet (UV) detector system (Waters, USA), according to Woodward and Emery (1987) with minor modifications using dexamethasone as an internal standard (Garabadu et al., 2011). Briefly, 500 µL of plasma containing known quantity of dexamethasone was extracted with 5 mL of dichloromethane. The dichloromethane extract was evaporated to dryness and dissolved in 100 µL of mobile phase. Twenty microliter of extract was injected into HPLC system for quantification. Mobile phase

consisted of methanol:water (70:30) at a flow rate of 1.2 ml/min and corticosterone was detected at 250 nm using UV detector (Model 2487, Waters, USA). The chromatogram was recorded and analyzed with empower software (Version 2).

Assessment of mitochondrial function, integrity and oxidative stress

Isolation of mitochondria from rat tissue

The mitochondria were isolated from HIP, HYP, PFC and AMY tissues by following standard protocol (Pedersen et al., 1978). The mitochondrial protein content was estimated using the method of Lowry et al. (1951).

Estimation of mitochondrial respiratory complex-I, II, IV and V activity

The activity of NADH dehydrogenase (complex-I) was measured by catalytic oxidation of NADH with potassium ferricyanide as an artificial electron acceptor at excitation and emission wavelengths for NADH were 350 nm and 470 nm, respectively (Shapiro et al., 1979). Activity of NADH dehydrogenase was expressed as nmole NADH oxidised/min/mg protein. The mitochondrial succinate dehydrogenase (SDH; complex-II) was determined by the progressive reduction of nitro blue tetrazolium (NBT) to an insoluble colored compound, diformazan at 570 nm (Sally and Margaret, 1989). The SDH activity was expressed as micromole formazan produced/min/mg protein. The activity of cytochrome oxidase (complex-IV) was measured in mitochondrial fraction in presence of reduced cytochrome c at 550 nm for 3 min (Storrie and Madden, 1990). Results were expressed as nmole cytochrome c oxidized/min/mg protein ($\varepsilon_{550} = 19.6\ mmol^{-1}cm^{-1}$). The F1-F0 synthase (complex-V) was measured by incubating mitochondrial suspension in ATPase buffer (Griffiths and Houghton, 1974) and the phosphate content was measured (Fiske and Subbarao, 1925). Results were expressed as nmole ATP hydrolyzed/min/mg protein.

Evaluation of MMP in discrete brain regions

The rhodamine dye taken up by mitochondria was measured in spectrofluorometer (Hitachi, F-2500) at an excitation λ of 535 ± 10 nm and emission λ of 580 ± 10 nm (Huang, 2002). The results were expressed as fluorescence intensity/mg protein.

Estimation of lipid peroxidation (LPO) and nitric oxide (NO) level

Mitochondrial malondialdehyde (MDA) content was measured as a marker of LPO at 532 nm (Ohkawa et al., 1979). The extent of LPO was expressed as micromoles of MDA/mg protein. The NO level was estimated as a marker for nitrosative stress (Green et al., 1982) and expressed as nmoles of NO/mg protein.

Assessment of superoxide dismutase (SOD) and catalase (CAT) activity

Superoxide dismutase (SOD) activity was determined by the reduction of NBT in presence of phenazine-methosulphate and NADH at 560 nm using n-butanol as blank (Kakkar et al., 1984). A single unit of the enzyme was expressed as 50% inhibition of NBT reduction/minute/mg protein. Decomposition of hydrogen peroxide in presence of CAT was followed at 240 nm (Beers and Sizer, 1952). The results were expressed as units (U) of CAT activity/min/mg of protein.

Immunoblotting

The preparation of cellular membrane fraction was performed as described previously (Nishiumi and Ashida, 2007). In brief, after washed three times with PBS, tissues were lysed with buffer A [Tris, pH 8.0, 50 mM; dithiothreitol, 0.5 mM; NP-40, 0.1% (v/v); protease inhibitors (phenylmethylsulphonyl fluoride, 1 mM; leupeptin, 5 $mg \cdot mL^{-1}$; and aprotinin, 5 $mg \cdot mL^{-1}$) and phosphatase inhibitors (NaF, 10 mM and Na_3VO_4, 1 mM)]. The lysates were then centrifuged at 1000 × *g* for 10 min at 4 °C. Pellets were resuspended in NP-40 free buffer A in ice for another

10 min with occasional vortex, and re-centrifuged at 1000 × *g* for 10 min at 4 °C. The pellets were re-suspended in buffer A and placed in ice for 1 h with occasional vortexing, and centrifuged at 16 000 × *g* for 20 min at 4 °C. The supernatant was collected as the plasma membrane fraction and stored at -80 °C until use. The supernatants from the first and second spins at 1000 × *g* were combined and centrifuged at 16 000 × *g* for 20 min at 4 °C. The resultant supernatant was collected and used as the cytosol fraction. Protein concentrations were determined according to Bradford (1976) in each fraction. A standard plot was generated using bovine serum albumin. An aliquot of each sample were electrophoresed in 10% SDS-PAGE gels for Akt, p-Akt, GLUT-4 and cytochrome-C proteins, transferred to polyvinylidene fluoride membranes and probed with specific antibodies. The membrane was incubated overnight with rabbit anti-Akt (Abcam Plc., Cambridge, USA), anti-p-Akt (Abcam Plc., Cambridge, USA), anti-GLUT-4 (Abcam Plc., Cambridge, USA), anti-cytochrome-C (Abcam Plc., Cambridge, USA) polyclonal primary antibody at a dilution of 1:1000, 1:1000, 1:1000 and 1:500 respectively. After detection with the desired antibodies against the proteins of interest the membrane was stripped with stripping buffer (25 mM Glycine pH 2.0, 2% SDS for 30 min at room temperature) and reprobed overnight with rabbit anti β-actin (Santa Cruz Biotechnology Inc.; Santa Cruz, California, USA) polyclonal primary antibody at a dilution of 1:500 to confirm equal loading of protein. Further, membrane was probed with corresponding secondary antibodies. Immunoreactive band of proteins were detected by chemiluminescence using enhanced chemiluminescence (ECL) reagents (Amersham Bioscience, USA). Quantification of the results was performed by densitometric scan of films. The immunoreactive area was determined by densitometric analysis using Biovis gel documentation software.

Flow cytometry measurement of cell death using annexin-V/PI

The assay was performed using the Annexin-V-Fluos Staining Kit (Roche Diagnostics, Penzberg, Germany; Liu et al., 2012). Briefly, single cell suspensions were then generated in cold PBS buffer from the isolated tissues, and the final concentrations were adjusted to 5×10^5/ml. 1 ml of suspensions were centrifuged at $300 \times g$ for 5 min at 4 °C and washed with 1 ml cold PBS three times. The pellet was re-suspended in 200 µl of Annexin-V-Fluos labeling solution and then incubated with 10 µl fluorescein isothiocyanate (FITC) conjugated Annexin-V and 5 µl of propidium iodide (PI) for 15 min at room temperature in the dark. Samples were kept on ice and analyzed on a BD LSRFortessa SORP flow cytometer equipped with five lasers. Emission fluorescence was measured with a 525/50 filter for FITC and with a 610/20 filter for red PI. FITC and PI were excited with two different lasers of 488 nm for the first and 561 nm for the second, thus avoiding signal compensation. Data were acquired and analyzed using BD FACSDIVA™ software (BD Biosciences, San Jose, CA). A minimum of 10,000 events were collected for each sample. The apoptosis rate was calculated with the following formula: (number of apoptotic cells /total cells) × 100%.

Data Analysis

All the data were mean ± standard error of the mean (SEM). The statistical significance for time-course effect on the plasma glucose level in OGTT and ITT, and body weight in T2DM study were evaluated by using repeated measure two-way analysis of variance (ANOVA) with Bonferroni post hoc test. All other statistical analyses of data were done using one-way ANOVA with Newman–Keuls post hoc analysis to monitor significance among groups. $p<0.05$ was considered as significant.

Results

Metformin improves glucose tolerance and insulin sensitivity in OGTT and ITT respectively in T2DM-induced encephalopathic rats

Table-13 and 14 depicts the effect of metformin on plasma glucose level of T2DM rats at different time points after administration of glucose and insulin respectively. Repeated measures of two-way ANOVA revealed that there were significant differences for plasma glucose level in OGTT and ITT among groups ([$F (3, 80) = 727.4$; $p<0.05$] and [$F (3, 80) = 1353.0$; $p<0.05$] respectively), time ([$F (3, 80) = 28.1$; $p<0.05$] and [$F (3, 80) = 24.6$; $p<0.05$] respectively) and there was a significant interaction between group and time ([$F (9, 80) = 4.1$; $p<0.05$] and [$F (9, 80) = 5.5$; $p<0.05$] respectively). Post-hoc test showed that before loading of either glucose or insulin to the animals (0 min), metformin treatment to normal animals did not cause any change in the level of plasma glucose compared to the vehicle treated rats. However, metformin showed a significant decrease in the plasma glucose level in T2DM rats. Metformin did not cause any change in the level of plasma glucose of normal rats at any point of time after administration of either glucose or insulin. The level of plasma glucose was increased at all time points after administration of either glucose or insulin to the animals compared to control and MET group rats. Metformin significantly decreased the plasma glucose level in T2DM rats at all time points after administration of either glucose or insulin.

Effect of metformin on change in body weight in T2DM-induced encephalopathic rats

The effect of metformin on change in the body weight of normal and T2DM rats at D-1 and D-31 of the experimental design is illustrated in Table-15. Repeated measures of two-way ANOVA revealed that there were significant differences in the body weight among groups [$F (3, 40) = 7.2$; $p<0.05$], time [$F (1, 40) = 9.8$; $p<0.05$] and there was a significant interaction between group and time [$F (3, 40) = 6.8$; $p<0.05$]. Post-hoc test revealed that metformin treatment to the normal

rats did not cause any change in the body weight. There was a significant decrease in the body weight of T2DM rats compared to vehicle and metformin treated normal rats. Metformin treatment reversed the T2DM-induced decrease in the body weight in the animals.

Metformin attenuates fasting blood glucose, insulin and IR, and improved β-cell function in T2DM-induced encephalopathic rats

Table-15 demonstrates the effect of metformin on the level of fasting blood glucose and insulin, and the extent of IR, and the function of pancreatic β-cell in normal as well as T2DM rats. Statistical analysis revealed that there were significant differences among groups for the level of fasting blood glucose [$F (3, 20) = 808.4$; $p<0.05$], insulin [$F (3, 20) = 53.2$; $p<0.05$], HOMA-IR [$F (3, 20) = 1400.0$; $p<0.05$] and HOMA-B [$F (3, 20) = 61.6$; $p<0.05$]. Post-hoc test showed that metformin treatment to normal rats did not cause any significant change in the levels of fasting blood glucose, insulin, HOMA-IR and HOMA-B. The levels of fasting blood glucose, insulin and HOMA-IR were significantly increased and the level of HOMA-B was significantly reduced in T2DM rats compared to control and MET group rats. Metformin attenuated the changes in the levels of fasting blood glucose, insulin, HOMA-IR and HOMA-B in T2DM rats.

Metformin exhibits anxiolytic activity in OF, HB and EPM tests

The effect of metformin on ambulation (A), rearing (B), percentage of time spent in the center (C) and total distance travelled (D) in OF test for control and T2DM rats is illustrated in Fig-13. Statistical analysis showed that there were significant differences among groups in ambulation [$F (3, 20) = 23.5$; $p<0.05$], rearing [$F (3, 20) = 18.2$; $p<0.05$] and percentage of time spent in the center [$F (3, 20) = 60.7$; $p<0.05$] during OF test. However, there was no significant difference among groups in total distance travelled [$F (3, 20) = 0.1$; $p>0.05$] during OF test. Post-hoc test revealed that metformin treatment did not cause any significant change in the ambulation, rearing

and percentage of time spent in the center of control animals. The number of ambulation and rearing, and the percentage of time spent in the center were decreased in T2DM animals compared to control and MET group rats. However, metformin significantly mitigated the T2DM-induced decrease in the ambulation, rearing and percentage of time spent in the center in rats indicating its anxiolytic-like activity.

Fig-14 depicts the effect of metformin on number of head-dip (A) and edge-sniff (B), ratio of head-dip/edge sniff (C), and number of squares crossed (D) of both control and T2DM rats in HB test paradigm. One-way ANOVA showed that there were significant differences in head-dip [F (3, 20) = 8.9; $p<0.05$] and edge-sniff [F (3, 20) = 7.3; $p<0.05$], and ratio of head-dip/edge sniff [F (3, 20) = 41.4; $p<0.05$] among groups. However, there was no significant difference in number of squares crossed [F (3, 20) = 0.05; $p>0.05$] among groups. Post-hoc test revealed that metformin treatment did not cause any significant change in either number of head-dip or edge-sniff, or ratio of head-dip/edge sniff in vehicle administered rats. The number of head-dip and the ratio of head-dip/edge sniff were significantly decreased and the number of edge-sniff was significantly increased in T2DM rats compared to control and MET group animals. Metformin treatment exhibited anxiolytic activity in rats in terms of increase in the T2DM-induced decrease in the number of head-dip and ratio of head-dip/edge sniff. Moreover, metformin attenuated the T2DM-induced increase in the number of edge-sniff in rats.

The effect of metformin on percentage entries (A) and time spent (B) into open arm, total arm entries (C) and speed in the whole maze (D) of control and T2DM rats in EPM test paradigm is illustrated in Fig-15. One-way ANOVA revealed that there were significant differences in percentage entries [F (3, 20) = 27.0; $p<0.05$] and time spent [F (3, 20) = 33.3; $p<0.05$] into open arm among groups. However, there were no significant differences among groups in total arm

entries [F (3, 20) = 0.1; p>0.05] and speed in the whole maze [F (3, 20) = 0.01; p>0.05]. Post-hoc test showed that metformin did not cause any change in the percentage entries and time spent into open arm in control animals. T2DM induction caused significant decrease in the percentage entries and time spent into the open arm in rats compared to control and MET group animals. Metformin treatment attenuated the T2DM-induced decrease in the percentage entries and time spent into open arm in the animals.

Metformin reverses T2DM-induced changes in the plasma level of TC, TG, HDL, LDL and corticosterone

Table-16 documents the effect of metformin on the level of TC, TG, HDL, LDL and corticosterone in plasma of control as well as T2DM rats. Statistical analysis showed that there were significant differences in the plasma level of TC [F (3, 20) = 122.5; p<0.05], TG [F (3, 20) = 56.5; p<0.05], HDL [F (3, 20) = 36.8; p<0.05], LDL [F (3, 20) = 63.0; p<0.05] and corticosterone [F (3, 20) = 123.5; p<0.05] in animals. Post-hoc test revealed that metformin treatment to the control animals did not cause any change in the level of all these biochemical parameters. T2DM caused significant increase in the level of TC, TG, LDL and corticosterone in rats compared to control and MET group animals. However, the level of HDL was significantly decreased in the plasma of T2DM animals compared to control and MET group rats. Metformin significantly attenuated the T2DM-induced changes in all these biochemical parameters in plasma of rats.

Metformin attenuates T2DM-induced decrease in the level of expression of p-Akt in HIP, HYP, PFC and AMY

Fig-16 illustrates the effect of metformin on the level of expression of Akt and p-Akt in HIP, HYP, PFC and AMY of control and T2DM rats. Statistical analysis showed that there were

significant differences in the level of expression of p-Akt and the ratio of p-Akt/Akt in HIP ([$F (3, 8) = 25.1$; $p<0.05$] and [$F (3, 8) = 55.3$; $p<0.05$] respectively), HYP ([$F (3, 8) = 19.1$; $p<0.05$] and [$F (3, 8) = 46.3$; $p<0.05$] respectively), PFC ([$F (3, 8) = 25.2$; $p<0.05$] and [$F (3, 8) = 32.9$; $p<0.05$] respectively) and AMY ([$F (3, 8) = 31.6$; $p<0.05$] and [$F (3, 8) = 69.7$; $p<0.05$] respectively) among groups. However, there were no significant differences in the level of expression of Akt in HIP [$F (3, 8) = 0.1$; $p>0.05$], HYP [$F (3, 8) = 0.4$; $p>0.05$], PFC [$F (3, 8) = 0.2$; $p>0.05$] and AMY [$F (3, 8) = 0.1$; $p>0.05$] among groups. Post-hoc test revealed that metformin treatment did not cause any change in either the level of expression of p-Akt or the ratio of p-Akt/Akt in any tissue of animals compared to vehicle administered rats. The level of expression of p-Akt and the ratio of p-Akt/Akt were significantly decreased in all the tissues in the T2DM group rats compared to control and MET group animals. Metformin treatment significantly reversed the T2DM-induced decrease in level of expression of p-Akt and the ratio of p-Akt/Akt in all the tissues.

Metformin attenuates changes in the translocation of GLUT-4 from cytoplasm to plasma membrane in HIP, HYP, PFC and AMY in T2DM-induced encephalopathic rats

The effect of metformin on the level of expression of cytoplasmic and membranous GLUT-4 in HIP, HYP, PFC and AMY of control and T2DM rats is depicted in Fig-17. Statistical analysis showed that there were significant differences in the level of expression of cytoplasmic and membranous GLUT-4, and the ratio of membranous/cytoplasmic GLUT-4 in HIP ([$F (3, 8) = 17.5$; $p<0.05$], [$F (3, 8) = 62.0$; $p<0.05$] and [$F (3, 8) = 44.9$; $p<0.05$] respectively), HYP ([$F (3, 8) = 40.4$; $p<0.05$], [$F (3, 8) = 84.1$; $p<0.05$] and [$F (3, 8) = 45.9$; $p<0.05$] respectively), PFC ([$F (3, 8) = 17.4$; $p<0.05$], [$F (3, 8) = 44.2$; $p<0.05$] and [$F (3, 8) = 20.8$; $p<0.05$] respectively) and AMY ([$F (3, 8) = 13.6$; $p<0.05$], [$F (3, 8) = 39.3$; $p<0.05$] and [$F (3, 8) = 22.8$; $p<0.05$]

respectively) among groups. Post-hoc test showed that metformin treatment did not cause any change in the level of expression of cytoplasmic and membranous GLUT-4, and the ratio of membranous/cytoplasmic GLUT-4 in any tissue in rats compared to control group animals. T2DM induction caused significant increase and decrease in the level of expression of cytoplasmic and membranous GLUT-4 respectively in all tissues compared to control and MET group rats. Moreover, there was a significant decrease in the ratio of membranous/cytoplasmic GLUT-4 in all tissues in T2DM animals compared to control and MET group rats. Metformin treatment significantly attenuated the T2DM-induced changes in the level of expression of cytoplasmic and membranous GLUT-4, and the ratio of membranous/cytoplasmic GLUT-4 in all the tissues.

Metformin ameliorates changes in the activity of mitochondrial complex-I, II, IV and V, and level of MMP in HIP, HYP, PFC and AMY in T2DM-induced encephalopathic rats

Table-17 illustrates the effect of metformin on the activity of mitochondrial complex-I, II, IV and V, and level of MMP in HIP, HYP, PFC and AMY in control and T2DM rodents. Statistical analysis showed that there were significant differences in the activity of mitochondrial complex-I, II, IV and V, and level of MMP in HIP ([$F(3, 20) = 20.3$; $p<0.05$], [$F(3, 20) = 63.1$; $p<0.05$], [$F(3, 20) = 11.8$; $p<0.05$], [$F(3, 20) = 26.8$; $p<0.05$] and [$F(3, 20) = 22.4$; $p<0.05$] respectively); HYP ([$F(3, 20) = 9.5$; $p<0.05$], [$F(3, 20) = 42.2$; $p<0.05$], [$F(3, 20) = 20.6$; $p<0.05$], [$F(3, 20) = 21.6$; $p<0.05$] and [$F(3, 20) = 25.3$; $p<0.05$] respectively); PFC ([$F(3, 20) = 10.2$; $p<0.05$], [$F(3, 20) = 60.2$; $p<0.05$], [$F(3, 20) = 38.6$; $p<0.05$], [$F(3, 20) = 87.6$; $p<0.05$] and [$F(3, 20) = 44.1$; $p<0.05$] respectively) and AMY ([$F(3, 20) = 9.9$; $p<0.05$], [$F(3, 20) = 8.5$; $p<0.05$], [$F(3, 20) = 45.7$; $p<0.05$], [$F(3, 20) = 37.3$; $p<0.05$] and [$F(3, 20) = 8.8$; $p<0.05$] respectively) among groups. Post-hoc test revealed that metformin treatment did not cause any

change in the activity of mitochondrial complex-I, II, IV and V, and level of MMP in all the tissues of animals compared to control rats. T2DM significantly increased the activities of all mitochondrial complex enzymes in animals compared to control and MET group rats. Moreover, the level of MMP was significantly decreased in all the tissues of T2DM group rats compared to control and MET group rodents. Metformin treatment mitigated the T2DM-induced changes in all these parameters except mitochondrial complex-II activity in all the tissues.

Metformin mitigates changes in the level of mitochondrial NO and LPO, and activity of SOD and catalase in HIP, HYP, PFC and AMY in T2DM-induced encephalopathic rats

The effect of metformin on the level of mitochondrial NO and LPO, and activity of SOD and catalase in HIP, HYP, PFC and AMY in control and T2DM rodents is illustrated in Table-18. Statistical analysis showed that there were significant differences in the level of mitochondrial NO and LPO, and activity of SOD and catalase in HIP ([$F (3, 20) = 7.8$; $p<0.05$], [$F (3, 20) = 14.8$; $p<0.05$], [$F (3, 20) = 6.7$; $p<0.05$] and [$F (3, 20) = 11.8$; $p<0.05$] respectively); HYP ([$F (3, 20) = 29.4$; $p<0.05$], [$F (3, 20) = 6.1$; $p<0.05$], [$F (3, 20) = 26.7$; $p<0.05$] and [$F (3, 20) = 8.0$; $p<0.05$] respectively); PFC ([$F (3, 20) = 49.0$; $p<0.05$], [$F (3, 20) = 22.8$; $p<0.05$], [$F (3, 20) = 57.1$; $p<0.05$] and [$F (3, 20) = 62.6$; $p<0.05$] respectively) and AMY ([$F (3, 20) = 14.3$; $p<0.05$], [$F (3, 20) = 12.2$; $p<0.05$], [$F (3, 20) = 9.0$; $p<0.05$] and [$F (3, 20) = 15.1$; $p<0.05$] respectively) among groups. Post-hoc test revealed that metformin treatment did not cause any change in the level of mitochondrial NO and LPO and, activity of SOD and catalase in any tissue of the rats compared to control group rodents. The level of mitochondrial NO and LPO were significantly increased and activity of SOD and catalase were significantly decreased in T2DM-induced rats compared to control and MET group animals. Metformin treatment significantly attenuated the

T2DM-induced changes in the oxidative stress markers except NO in all the tissues compared to control and MET group rats.

Metformin attenuates increase in the mitochondrial-linked apoptosis in HIP, HYP, PFC and AMY in T2DM-induced encephalopathic rats

Fig-18 depicts the effect of metformin on the level of expression of cytochrome-C in HIP, HYP, PFC and AMY in control and T2DM rats. Statistical analysis showed that there were significant differences in the level of expression of cytochrome-C in HIP [$F(3, 8) = 21.7$; $p<0.05$]; HYP [$F(3, 8) = 18.1$; $p<0.05$]; PFC [$F(3, 8) = 16.5$; $p<0.05$] and AMY [$F(3, 8) = 21.6$; $p<0.05$] among groups. Post-hoc test showed that there was no significant change in the level of expression of cytochrome-C in all the tissues of metformin treated rats compared control group rodents. T2DM significantly increased the level of expression of cytochrome-C in all the tissues in rats compared to control and MET group animals. Metformin treatment attenuated the T2DM-induced increase in the level of expression of cytochrome-C in all the tissues.

The flow cytogram and the extent of apoptosis in terms of percentage of apoptotic cell death of HIP, HYP, PFC and AMY tissue for each group have been illustrated in Fig-19 and 20 respectively. Statistical analysis showed that there were significant differences in the percentage of apoptotic cell death in HIP [$F(3, 8) = 57.1$; $p<0.05$]; HYP [$F(3, 8) = 34.8$; $p<0.05$]; PFC [$F(3, 8) = 64.0$; $p<0.05$] and AMY [$F(3, 8) = 140.1$; $p<0.05$] among groups. Post-hoc test revealed that metformin administration to the normal rats did not cause any significant change in the percentage of apoptotic cell death compared to the vehicle treated animals. However, metformin treatment attenuated T2DM-induced increase in the percentage of apoptotic cell death in the animals.

Table-13: Effect of metformin on the plasma glucose level of T2DM-induced encephalopathic rats in OGTT.

	Plasma glucose level (mmol/L)			
Groups	0 min	30 min	60 min	120 min
Control	3.9± 0.13	6.9 ± 0.09	6.7 ± 0.14	5.1 ± 0.13
MET	3.7 ± 0.12	7.3 ± 0.17	6.8 ± 0.20	5.1 ± 0.12
T2DM	26.7 ± 1.19[a,b]	29.1 ± 1.33 [a,b]	29.3 ± 1.31 [a,b]	26.9 ± 0.59 [a,b]
DMET	9.3 ± 0.23 [a,b,c]	18.5 ± 1.21 [a,b,c]	17.8 ± 1.36 [a,b,c]	17.1 ± 1.05 [a,b,c]

All values are mean ± SEM (n = 6). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to metformin control group (MET) and [c]$p<0.05$ compared to T2DM (repeated measures of two-way ANOVA followed by Bonferroni post hoc test).

Table-14: Effect of metformin on the plasma glucose level of T2DM-induced encephalopathic rats in ITT.

	Plasma glucose level (mmol/L)			
Groups	0 min	30 min	60 min	90 min
Control	4.3 ± 0.13	2.3 ± 0.25	3.3 ± 0.37	2.9 ± 0.32
MET	4.1 ± 0.23	2.3 ± 0.26	3.1 ± 0.24	2.8 ± 0.32
T2DM	26.5 ± 0.63 [a,b]	19.2 ± 0.73 [a,b]	24.3 ± 0.95 [a,b]	24.5 ± 0.98 [a,b]
DMET	15.7 ± 0.67 [a,b,c]	14.1 ± 0.40 [a,b,c]	16.8 ± 0.51 [a,b,c]	16.4 ± 0.74 [a,b,c]

All values are mean ± SEM (n = 6). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to metformin control group (MET) and [c]$p<0.05$ compared to T2DM (repeated measures of two-way ANOVA followed by Bonferroni post hoc test).

Table-15: Effect of metformin on body weight, fasting blood glucose and insulin level, HOMA-IR and HOMA-B indices of normal and T2DM rats.

Parameters	Control	MET	T2DM	DMET
Body weight on day-1 (g)	237.1 ± 10.90	233.9 ± 6.11	234.8 ± 9.11	235.8 ± 4.21
Body weight on day-31 (g)	231.7 ± 3.12	234.0 ± 4.74	185.8 ± 5.03 [a,b]	233.8 ± 2.85[c]
Fasting blood glucose (mmol/L)	4.5 ± 0.12	4.3 ± 0.11	27.3 ± 0.72 [a,b]	7.9 ± 0.23 [a,b,c]
Fasting blood Insulin (pmol/L)	79.2 ± 3.33	78.7 ± 3.13	123.3 ± 2.74 [a,b]	93.0 ± 2.11 [a,b,c]
HOMA-IR	2.5 ± 0.12	2.4 ± 0.17	21.3 ± 0.41 [a,b]	4.2 ± 0.17 [a,b,c]
HOMA-B	189.1 ± 10.11	188.5 ± 20.12	16.1 ± 0.91 [a,b]	60.1 ± 2.14 [a,b,c]

All values are mean ± SEM (n = 6). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to MET and [c]$p<0.05$ compared to T2DM (repeated measures of two-way ANOVA followed by Bonferroni post hoc test for body weight and one-way ANOVA followed by Student–Newman–Keuls test for other analysis).

Table-16: Effect of metformin on the levels of TC, TG, HDL, LDL and CORT in T2DM-induced encephalopathic rats.

Groups	TC (mmol/l)	TG (mmol/l)	HDL (mmol/l)	LDL (mmol/l)	CORT (µg/dl)
Control	1.5 ± 0.03	0.9 ± 0.04	1.6 ± 0.07	0.9 ± 0.05	16.9 ± 1.27
MET	1.4 ± 0.04	0.8 ± 0.07	1.6 ± 0.03	0.8 ± 0.04	16.8 ± 1.23
T2DM	2.4 ± 0.06 [a,b]	1.7 ± 0.05 [a,b]	1.0 ± 0.02 [a,b]	1.6 ± 0.05 [a,b]	43.8 ± 0.86 [a,b]
DMET	1.4 ± 0.04 [a,b,c]	1.1 ± 0.05 [a,b,c]	1.4 ± 0.05 [a,b,c]	1.0 ± 0.04 [a,b,c]	23.3 ± 1.19 [a,b,c]

All values are mean ± SEM (n = 6). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to MET and [c]$p<0.05$ compared to T2DM (one-way ANOVA followed by Student–Newman–Keuls test).

Table-17: Effect of metformin on mitochondrial complex-I, II, IV, V and MMP in HIP, HYP, PFC and AMY of T2DM-induced encephalopathic rats.

Tissue	Control	MET	T2DM	DMET
Complex-I(nmol NADHoxidized/min/mg protein)				
HIP	6.1 ± 0.53	6.1 ± 0.77	11.9 ± 0.53 [a,b]	9.5 ± 0.65 [a,b,c]
HYP	6.3 ± 0.43	6.2 ± 0.85	10.3 ± 0.65 [a,b]	6.6 ± 0.56 [c]
PFC	4.2 ± 0.13	4.1 ± 0.32	6.3 ± 0.22 [a,b]	4.2 ± 0.53 [c]
AMY	6.5 ± 0.45	5.9 ± 0.83	10.7 ± 0.87 [a,b]	7.5 ± 0.45 [c]
Complex-II(µmol formazan produced/min/mg protein)				
HIP	0.5 ± 0.02	0.5 ± 0.03	1.0 ± 0.04 [a,b]	1.1 ± 0.06 [a,b]
HYP	0.5 ± 0.10	0.5 ± 0.14	1.5 ± 0.02 [a,b]	1.5 ± 0.04 [a,b]
PFC	0.5 ± 0.05	0.5 ± 0.04	1.5 ± 0.04 [a,b]	1.4 ± 0.12 [a,b]
AMY	0.4 ± 0.05	0.4 ± 0.05	0.6 ± 0.02 [a,b]	0.6 ± 0.03 [a,b]
Complex-IV(nmol cytochrome coxidized/min/mg protein)				
HIP	0.9 ± 0.14	1.1 ± 0.15	1.9 ± 0.13 [a,b]	1.6 ± 0.11 [a,b,c]
HYP	1.2 ± 0.07	1.1 ± 0.08	2.1 ± 0.05 [a,b]	1.5 ± 0.16 [a,b,c]
PFC	1.5 ± 0.13	1.4 ± 0.12	3.0 ± 0.11 [a,b]	2.4 ± 0.13 [a,b,c]
AMY	1.4 ± 0.06	1.3 ± 0.06	2.1 ± 0.05 [a,b]	1.7 ± 0.04 [a,b,c]
Complex-V(nmol ATPhydrolyzed/mg protein)				
HIP	8.2 ± 0.61	8.5 ± 0.63	17.7 ± 1.4 [a,b]	12.3 ± 0.45 [a,b,c]
HYP	17.4 ± 1.41	18.2 ± 1.29	35.5 ± 2.37 [a,b]	27.1 ± 2.05 [a,b,c]
PFC	13.4 ± 1.01	14.5 ± 0.99	39.2 ± 1.70 [a,b]	28.1 ± 1.40 [a,b,c]
AMY	13.4 ± 1.13	13.2 ± 1.04	30.3 ± 1.98 [a,b]	23.3 ± 1.05 [a,b,c]
MMP				
HIP	554.4 ± 8.15	544.5 ± 12.83	391.7 ± 21.66 [a,b]	475.9 ± 17.64 [a,b,c]
HYP	549.0 ± 14.41	549.7 ± 15.82	393.1 ± 8.44 [a,b]	497.8 ± 18.10 [c]
PFC	544.0 ± 12.33	540.9 ± 18.96	345.3 ± 15.52 [a,b]	448.4 ± 7.02 [a,b,c]
AMY	542.7 ± 23.22	542.2 ± 40.67	357.4 ± 18.70 [a,b]	484.8 ± 30.04 [c]

All values are mean ± SEM (n = 6). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to MET and [c]$p<0.05$ compared to T2DM (one-way ANOVA followed by Student–Newman–Keuls test).

Table-18: Effect of metformin on mitochondrial level of NO and LPO, and activity of SOD and CAT in HIP, HYP, PFC and AMY of T2DM-induced encephalopathic rats.

Tissue	Control	MET	T2DM	DMET
NO(µM MDA/mg of protein)				
HIP	1.3 ± 0.04	1.4 ± 0.14	2.1 ± 0.15 [a,b]	1.9 ± 0.18 [a,b]
HYP	1.0 ± 0.03	1.1 ± 0.13	2.0 ± 0.13 [a,b]	2.0 ± 0.08 [a,b]
PFC	1.0 ± 0.03	1.0 ± 0.07	2.2 ± 0.14 [a,b]	2.0 ± 0.09 [a,b]
AMY	1.1 ± 0.03	1.1 ± 0.04	2.0 ± 0.21 [a,b]	2.0 ± 0.17 [a,b]
LPO (nmol of MDA/mg of protein)				
HIP	0.5 ± 0.03	0.5 ± 0.04	0.8 ± 0.05 [a,b]	0.6 ± 0.02 [c]
HYP	0.6 ± 0.04	0.6 ± 0.03	0.8 ± 0.05 [a,b]	0.6 ± 0.04 [c]
PFC	0.9 ± 0.03	1.0 ± 0.09	1.9 ± 0.11 [a,b]	1.3 ± 0.12 [a,b,c]
AMY	0.6 ± 0.23	0.7 ± 0.13	1.7 ± 0.13 [a,b]	0.7 ± 0.04 [a,b,c]
SOD (Units/min/mg of protein)				
HIP	0.4 ± 0.03	0.4 ± 0.02	0.3 ± 0.01 [a,b]	0.4 ± 0.01 [c]
HYP	0.4 ± 0.03	0.4 ± 0.02	0.2 ± 0.01 [a,b]	0.4 ± 0.01 [c]
PFC	0.3 ± 0.01	0.3 ± 0.01	0.1 ± 0.02 [a,b]	0.3 ± 0.01 [c]
AMY	0.7 ± 0.02	0.7 ± 0.01	0.3 ± 0.02 [a,b]	0.7 ± 0.13 [c]
CAT (Units/min/mg of protein)				
HIP	2.2 ± 0.14	2.2 ± 0.17	1.3 ± 0.12 [a,b]	2.1 ± 0.04 [c]
HYP	3.5 ± 0.05	3.5 ± 0.06	2.3 ± 0.31 [a,b]	3.3 ± 0.25 [c]
PFC	2.6 ± 0.03	2.6 ± 0.10	0.7 ± 0.16 [a,b]	1.6 ± 0.13 [a,b,c]
AMY	2.5 ± 0.19	2.4 ± 0.14	1.3 ± 0.10 [a,b]	1.9 ± 0.12 [a,b,c]

All values are mean ± SEM (n = 6). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to MET and [c]$p<0.05$ compared to T2DM (one-way ANOVA followed by Student–Newman–Keuls test).

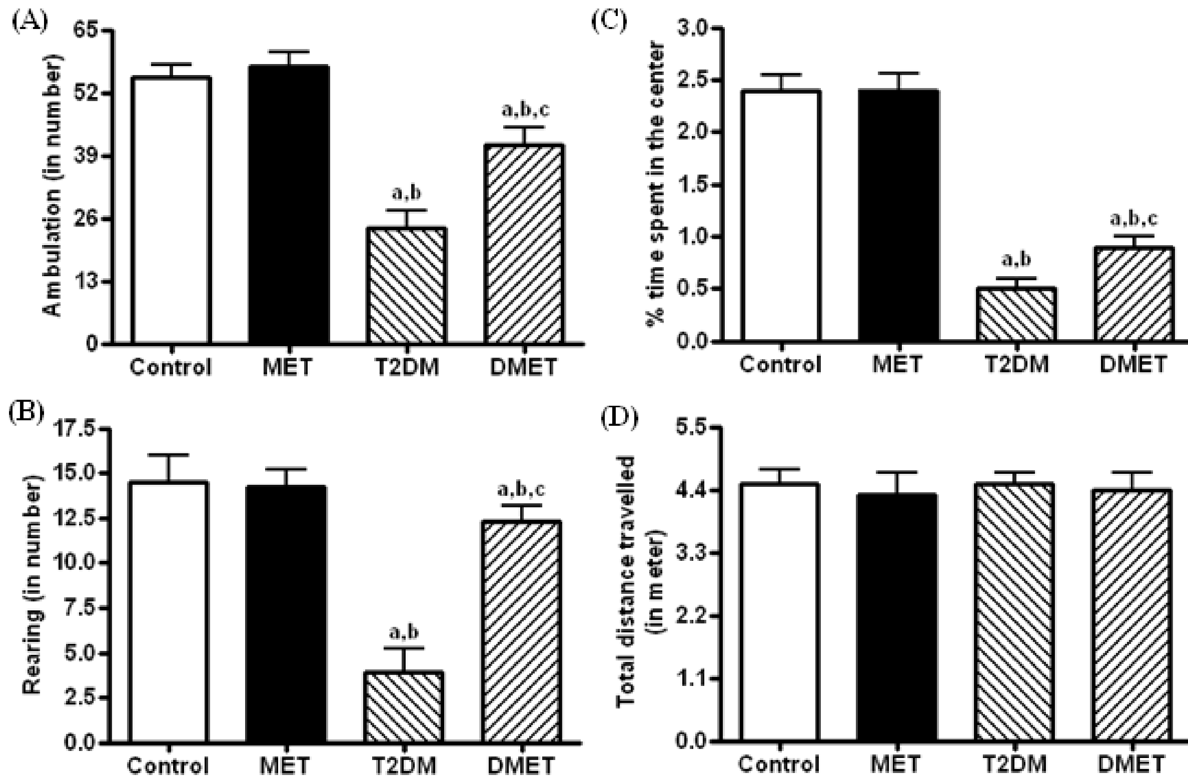

Figure 13: The effect of metformin on ambulation (A), rearing (B), percentage of time spent in the center (C) and total distance travelled (D) in control and T2DM-induced encephalopathic rats during open field test on day-31. All values are mean ± SEM (n = 6). [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to metformin control group (MET) and [c]$p<0.05$ compared to T2DM [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

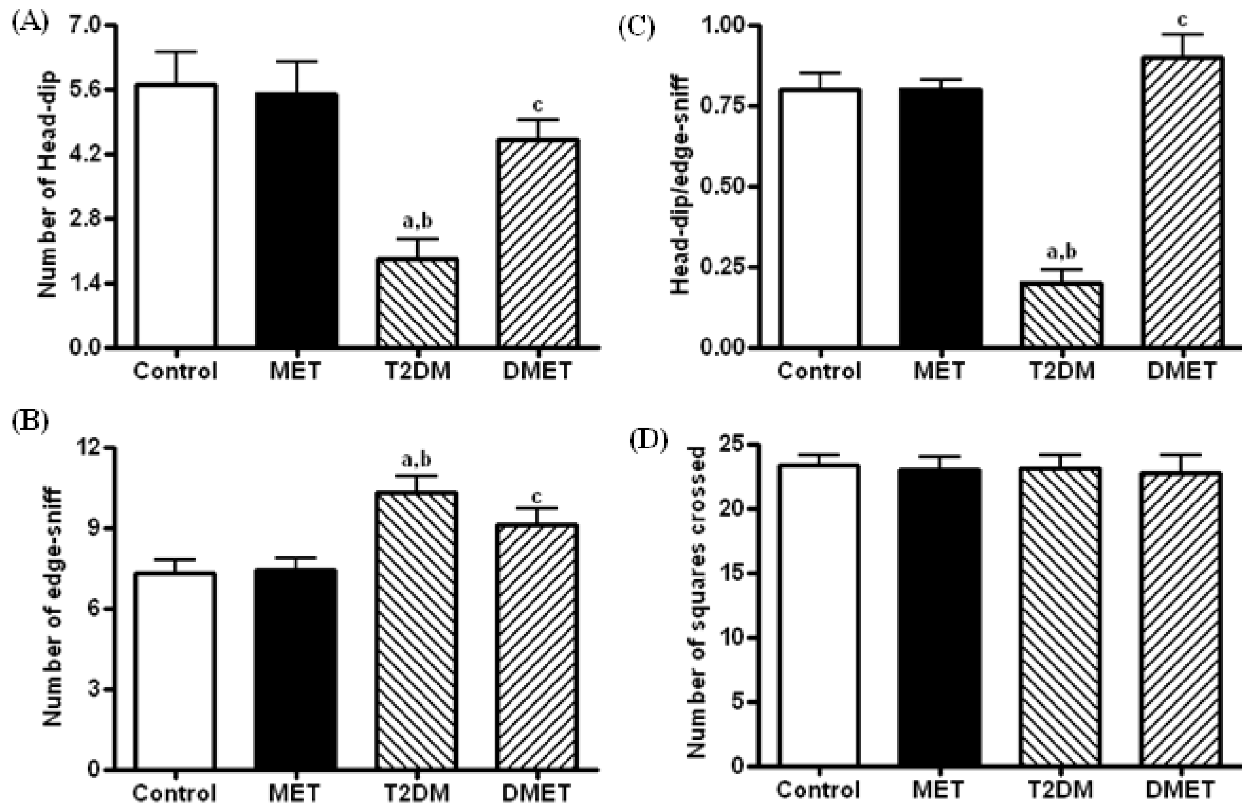

Figure 14: The effect of metformin on number of head-dips (A) and edge-sniffs (B), ratio of head-dip/edge sniff (C), and number of squares crossed (D) in control and T2DM-induced encephalopathic rats during hole-board test. All values are mean ± SEM (n = 6). $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to MET and $^{c}p<0.05$ compared to T2DM [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

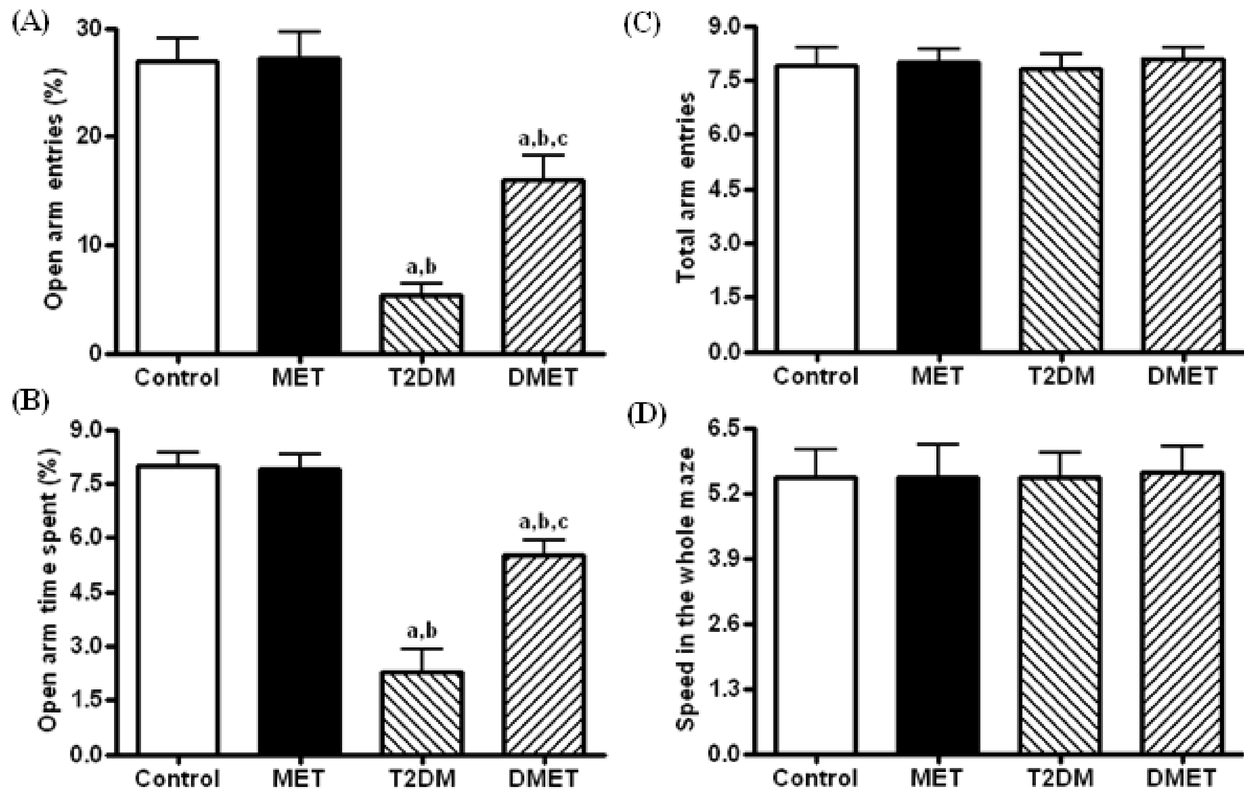

Figure 15: The effect of metformin on percentage entries (A) and time spent (B) into open arm, total arm entries (C) and speed in the whole maze (D) in control and T2DM-induced encephalopathic rats during elevated plus maze test. All values are mean ± SEM (n = 6). $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to MET and $^{c}p<0.05$ compared to T2DM [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

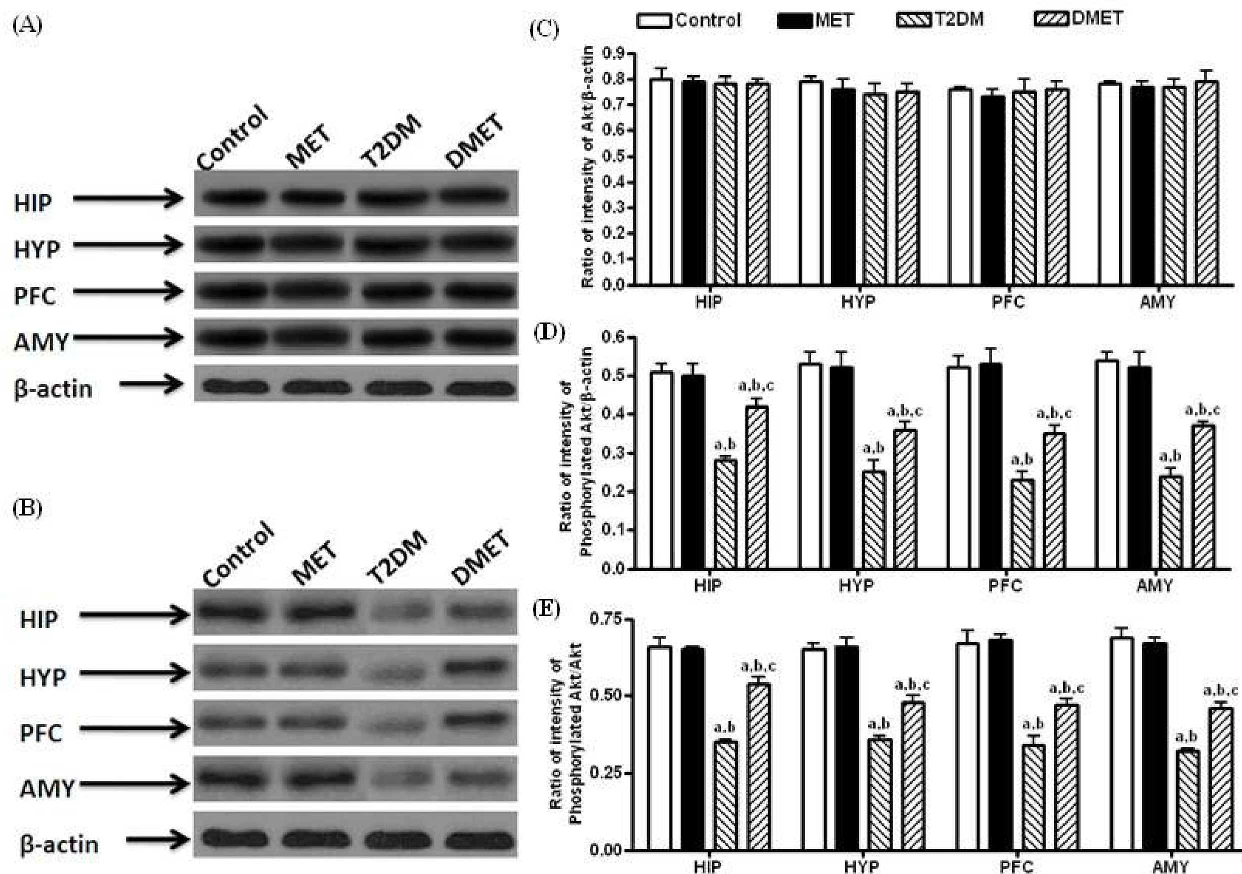

Figure 16: The effect of metformin on the level of expression of Akt (B) and p-Akt (D), and ratio of p-Akt to Akt (E) in hippocampus (HIP), hypothalamus (HYP), pre-frontal cortex (PFC) and amygdale (AMY)of control and T2DM-induced encephalopathic rats. The blots are representative of Akt (A) and p-Akt (C) in HIP, HYP, PFC and AMY. The results in the histogram are expressed as the ratio of relative intensity of levels of expression of Akt or p-Akt to β-actin. All values are mean ± SEM of three separate sets of independent experiments. $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to MET and $^{c}p<0.05$ compared to T2DM [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

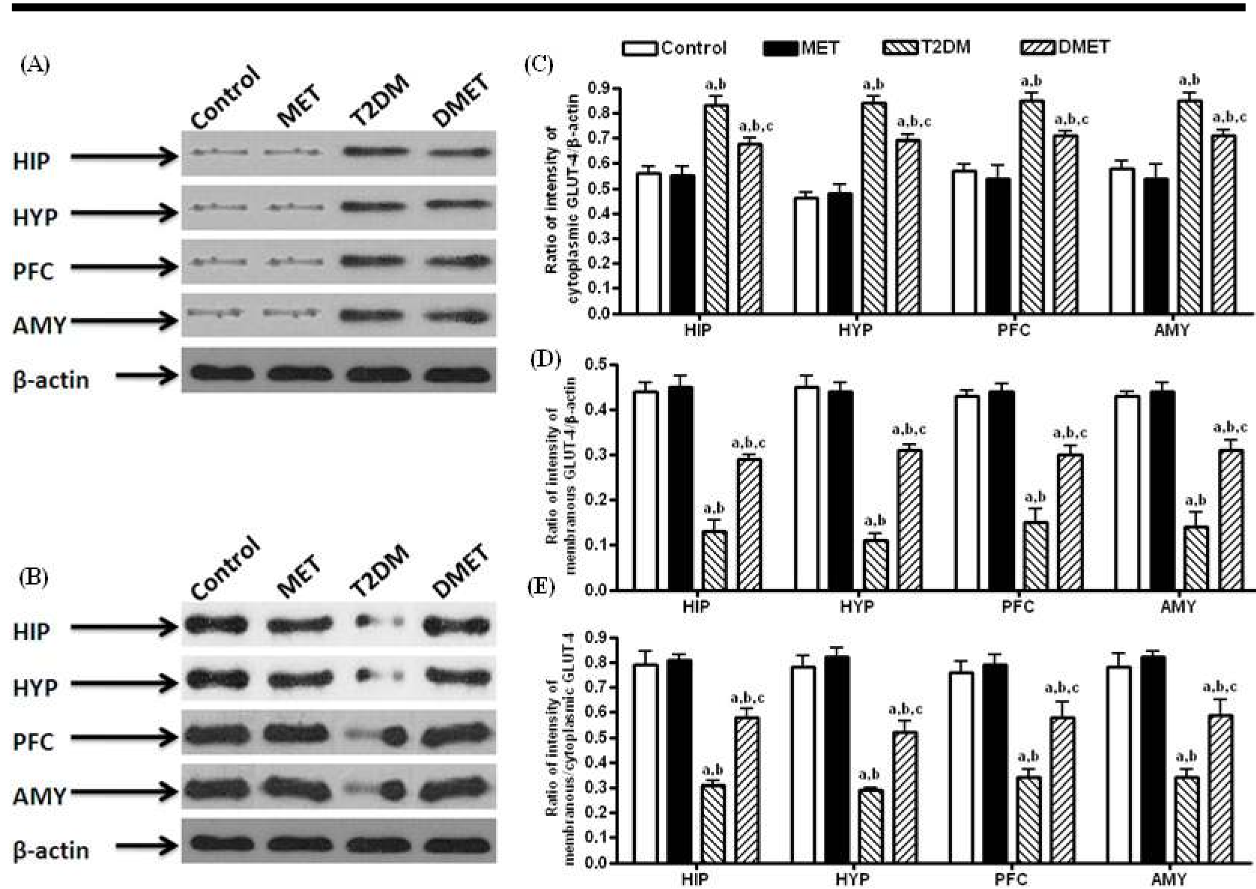

Figure 17: The effect of metformin on the level of expression of cytoplasmic (B) and membranous (D), and ratio of p-membranous to cytoplasmic (E) GLUT-4 in HIP, HYP, PFC and AMY of control and T2DM-induced encephalopathic rats. The blots are representative of cytoplasmic (A) and membranous (C) GLUT-4 in HIP, HYP, PFC and AMY. The results in the histogram are expressed as the ratio of relative intensity of levels of expression of cytoplasmic or membranous GLUT-4 to β-actin. All values are mean ± SEM of three separate sets of independent experiments. $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to MET and $^{c}p<0.05$ compared to T2DM [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

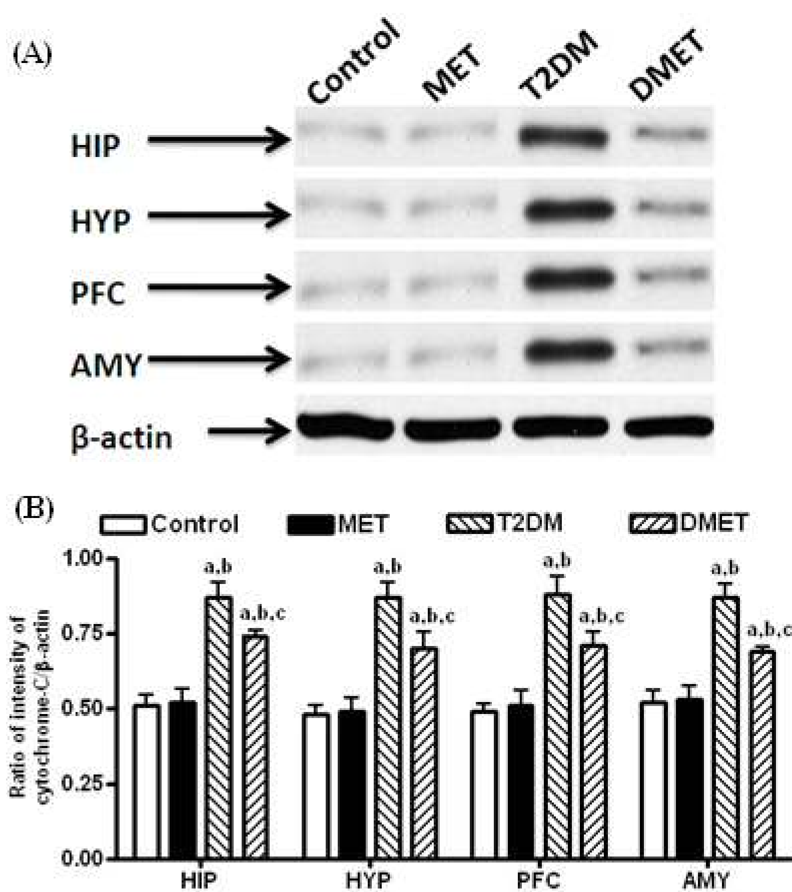

Figure 18: The effect of metformin on the level of expression of cytochrome-C (B) in HIP, HYP, PFC and AMY of control and T2DM-induced encephalopathic rats. The blots are representative of cytochrome-C (A) in HIP, HYP, PFC and AMY. The results in the histogram are expressed as the ratio of relative intensity of levels of expression of cytochrome-C to β-actin. All values are mean ± SEM of three separate sets of independent experiments. $^{a}p<0.05$ compared to control, $^{b}p<0.05$ compared to MET and $^{c}p<0.05$ compared to T2DM [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

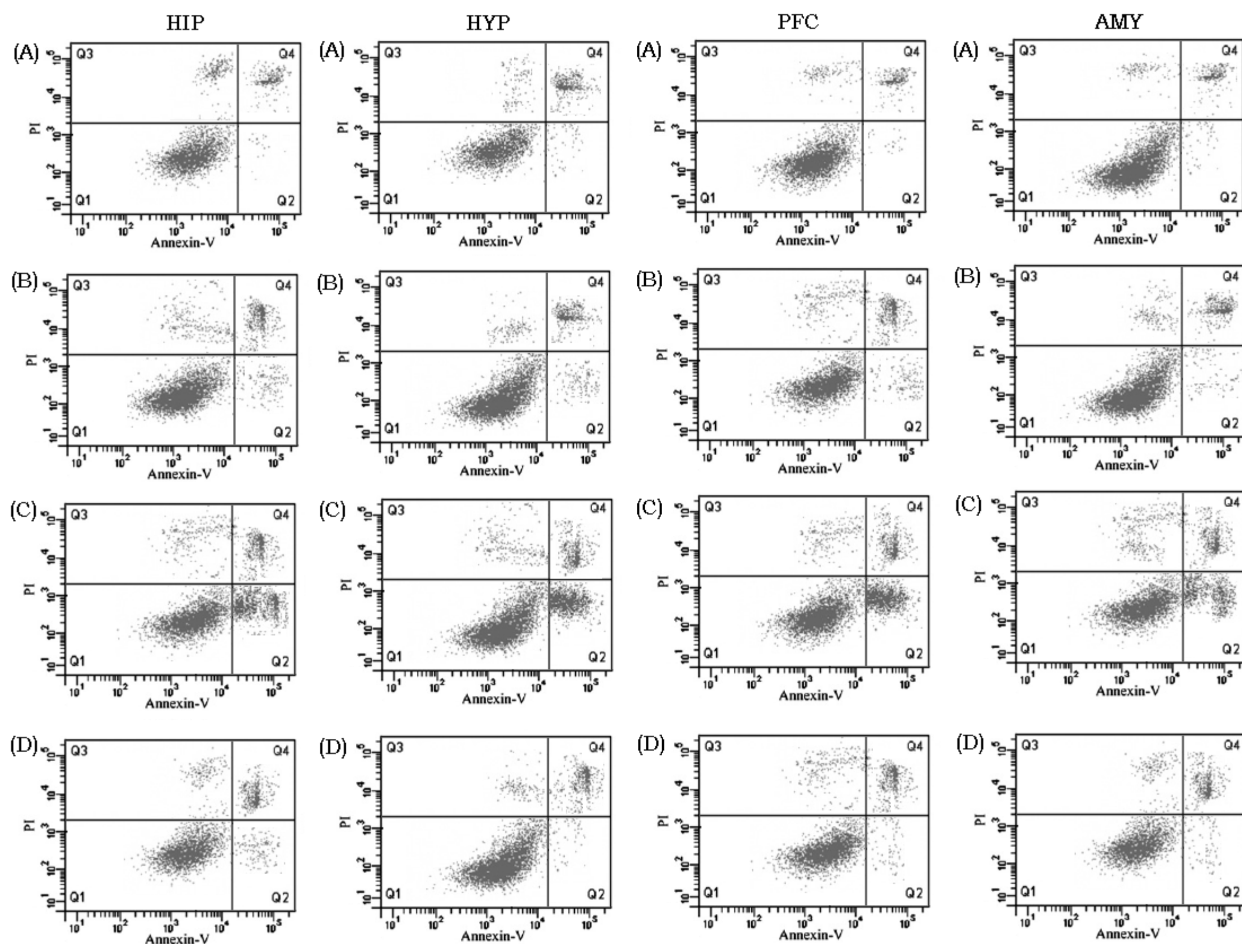

Figure 19: Flow cytogram showing cell death assessed by cytometric analysis using annexin-V/PI. Dot plots are representative for HIP, HYP, PFC and AMY of control (A), MET (B), T2DM (C) and DMET (D). Each cytogram has been divided into four quadrants; **Lower left quadrant (Q1):** It represents cells that were negative for both annexin-V and PI and thus regarded as live cells, **Lower right quadrant (Q2):** It represents those cells that were positive for annexin-V and negative for PI. These cells assumed to be undergoing early stages of apoptosis, in which the plasma membrane is still intact, and exclude PI, **Upper left region (Q3):** It represents the population of annexin-V-negative and PI-positive cells. They are regarded as necrotic cells and **Upper right region (Q4):** It displays both annexin-V-positive and PI-positive cells. It represents the late stages of apoptosis and necrosis when dying cells can no longer exclude PI. Intensity of red fluorescence by PI-stained cells is indicated on the y-axis, whereas intensity of green fluorescence emerging from cell-bound annexin-VFITC is indicated on the x-axis.

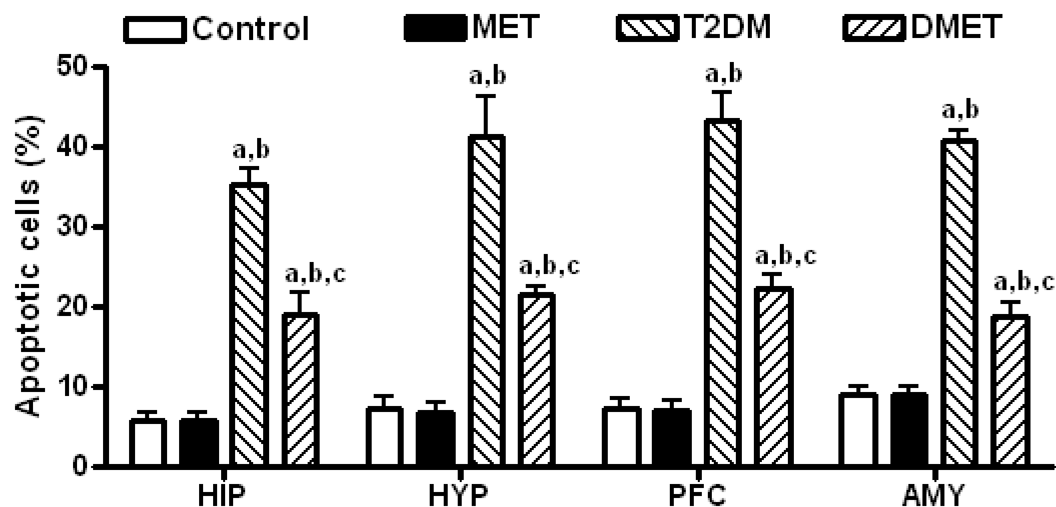

Figure 20: The effect of metformin on the percentage in apoptotic cells in HIP, HYP, PFC and AMY of control and T2DM-induced encephalopathic rats. All values are mean ± SEM of three separate sets of independent experiments. [a]$p<0.05$ compared to control, [b]$p<0.05$ compared to MET and [c]$p<0.05$ compared to T2DM [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

Discussion

In the present study, we for the first time report the beneficial effect of mitochondrial complex-I inhibitor on derailed PI3K/Akt/GLUT-4 signaling pathway and mitochondria-dependent apoptosis in T2DM-induced encephalopathic rat brain. Metformin also attenuated T2DM and anxiety-like manifestations in these animals. These observations suggest that mitochondria targeted drugs could be potential alternative options in the pharmacotherapy of T2DM-induced encephalopathic condition.

The T2DM-induced encephalopathic condition is attributed with several behavioral manifestations including anxiety. Clinical and pharmacoeconomic studies suggest a clear co-occurring condition between T2DM and anxiety (Roy-Byrne et al., 2008). Similar to earlier reports, in the present study metformin exhibited anxiolytic-like activity in different animal models without alteration in locomotor activity (Sakandelidze et al., 2011). Interestingly, the presence of anxiety is reported as a contributor for an additional time of hospitalization of diabetic patients (Ball et al., 2002). Therefore, it is prerequisite to investigate the underlying molecular mechanism in the central tissues of T2DM-induced encephalopathic animals.

The concept of β-cell dysfunction is considered as a major event in the development of IR in T2DM-induced encephalopathy (Tahara et al., 2008). In the present study, metformin attenuated hyperglycemia, hyperinsulinemia and IR in these animals. Our findings as well as those shown by other authors have demonstrated that animals displayed several attributes of IR including hyperglycaemia, hyperinsulinaemia and decreased β-cell function (HOMA-B; Tahara et al., 2008; Garabadu and Krishnamurthy, 2014). It has been reported that the state of hypercortisolaemia may cause IR in brain tissues (Haas and Biddinger, 2009; van Donkelaar et al., 2014). Similar to our earlier result, the present study showed hypercortisolaemia and

dyslipidemia in T2DM-induced rats with IR and these changes were ameliorated with metformin treatment (Garabadu and Krishnamurthy, 2014). Moreover, metformin attenuated T2DM-induced reduction in body weight in these animals (Tahara et al., 2008) indicating that the presence of poor glycemic control and dyslipidemia may contribute towards the loss in body weight in T2DM rodents. In support to our results, another study has reported that increased corticosterone level as a consequence of hyperactivity of hypothalamic-pituitary-adrenal cortex-axis causes conversion of glycerol to glucose through a substrate push mechanism, lipolysis and conversion of pyruvate to glucose through greater acetyl-CoA allosteric activation of pyruvate carboxylase flux (Perry et al., 2014). These results suggest that hypercortisolaemia and dyslipidemia may form a vicious cycle that perhaps induces tissues-specific IR in this condition.

We report for the first time that metformin at the sub-cellular level attenuated T2DM-induced derailment in the PI3K/Akt/GLUT-4 signaling pathway in terms of increase in the phosphorylation of Akt and translocation of GLUT-4 in all brain tissues. The effect of metformin is also observed in other tissues similar to that of our findings (Jurysta et al., 2013; Blázquez et al., 2014). This suggests that metformin exhibited similar tissue specific effect on IR in central tissues in these T2DM-induced encephalopathic rats. It has also been reported that the selected brain regions of animals subjected to predator scent stress paradigm exhibits IR (Cohen et al., 2009), suggesting the fact that derailment in the PI3K/Akt/GLUT-4 signaling may be the contributing factor for the pathogenesis of tissue-specific IR in T2DM-induced encephalopathic condition.

Mitochondrial dysfunction is considered as one of the predisposing factor for IR (Petersen et al., 2003; Lowell and Shulman, 2005; Johannsen and Ravussin, 2009). Therefore, in the present study, we explored the mitochondrial function and integrity, and mitochondrial-

linked apoptosis in central tissues of T2DM-induced encephalopathic rats. We report that there was a significant increase in the activity of all the mitochondrial respiratory complex enzyme activities in all the selected tissues similar to that of earlier studies (Garabadu and Krishnamurthy, 2013; Garabadu and Krishnamurthy, 2014). These observations indicate that there was mitochondria dysfunction in most of the rat tissues subjected to T2DM-induced encephalopathy. This effect could be attributed due to hypercortisolaemia (Perry et al., 2014). Therefore, it can be presumed that tissue-specific IR could be due to mitochondrial dysfunction caused by hypercortisolaemia. Our present study demonstrated that metformin treatment attenuated T2DM-induced over activation of respiratory enzymes except mitochondrial complex-II in all of the tissues. In support to our study, it has already been reported that metformin is considered as a mitochondrial complex-I inhibitor (Pryor et al., 2000; Kumar and Dey, 2002; Yuan et al., 2003; Sonntag 2005; Kukidome et al., 2006; Chakraborty et al., 2011; Qiu et al., 2012) and probably this inhibitory potential may improve the overall mitochondrial function in such tissues in this condition.

Metformin ameliorated the extent of lipid peroxidation and failure in antioxidant defense system (decreased activity of SOD and catalase) in central tissues of T2DM-induced encephalopathic rats similar to that of earlier findings (Chakraborty et al., 2011). However, it did not mitigate the T2DM-induced increase in the level of NO in any of the tissues as observed in other report (Chakraborty et al., 2011). There was a loss in the mitochondrial integrity in terms of decrease in the MMP in all the tissues in T2DM subjected animals similar to that of previous studies (Asensio-Lopez et al., 2014; Chung et al., 2015). Additionally, metformin mitigated the increase in the extent of mitochondria-linked apoptosis as evidenced from its ability to decrease the elevated level of expression of cytochrome-C, considered to be the marker of mitochondria-

linked apoptosis, and the increased percentage of apoptotic cell death in all the tissues similar to that of earlier studies (Asensio-Lopez et al., 2014; Chung et al., 2015). Moreover, there exist a bidirectional relationship between mitochondria function and insulin signaling pathway in tissues (Wen et al., 2015). Hence, based on these observations, it can be assumed that the beneficial effect of mitochondrial complex-I inhibitor on tissue-specific IR may involve the improvement of the PI3K/Akt/GLUT-4 signaling pathway in T2DM-induced encephalopathic animals.

In conclusion, mitochondrial complex-I inhibitor exhibited anti-diabetic and anxiolytic activity against T2DM-induced encephalopathic animals. It improved PI3K/Akt/GLUT-4 signaling pathway and also restored mitochondrial function in all the brain tissues. Additionally, at sub-cellular level, it ameliorated the tissue-specific IR, mitochondrial dysfunction, mitochondrial oxidative stress and mitochondria-linked apoptosis in all of these tissues of T2DM-induced encephalopathic rodents. These results emphasize the fact that mitochondrial complex-I inhibitor could be a potential candidate in the management of T2DM-induced central complications. Additionally, it can be postulated that mitochondria targeted drugs would be alternative option in the pharmacotherapy of T2DM-induced encephalopathic condition.