Diazepam potentiates the anti-diabetic, anti-stress and anxiolytic activities of metformin in type-2 diabetes mellitus with co-occurring stress in experimental animals.

Introduction

In the earlier objective, we have shown that there was metabolic disorder in the brain of PTSD animals and the effect was ameliorated by the SER. In the current objective, we would like to evaluate the effect of co-administration of anxiolytic drug diazepam and anti-diabetic drug metformin in co-occurring conditions of stress and T2DM over its individual administration. This is the reason for which we had earlier shown (chapter-5) the $GABA_A$-mediated regulation of insulin sensitive PI3K/ AKT pathway in brain. This would help in choosing the more appropriate pharmacotherapy for co-occurring conditions of T2DM and stress.

Considerable research has confirmed biological and behavioral variables as risk factors for the development of T2DM (Magliano et al., 2008). Psychological distress has long been considered as one of the predisposing factors in the development of T2DM. Stress is an aversive stimulus which disturbs physiological homeostasis and is reported to play an important role in the genesis and pathophysiology of different psychological disorders (Southwick et al., 2005). Stress can be measured as the actual exposure to events assumed to be stressful, or as an individual's interpretation and perception of stressors and can be assessed across all contexts of life. However, the role of psychosocial risk factors, including psychological characteristics, social relationships, and stressors, in the development of T2DM has received much less attention.

Only a small number of studies have explored the role of psychosocial stress on the development of diabetes. Clinical studies suggest that there is a strong relationship between psychological stress and diabetes. One study reports that there is one measure of work stress, effort–reward imbalance (a model based on the importance of the reciprocal balance between the effort spent at work and the intrinsic and extrinsic rewards received) and diabetes incidence in

men but not in women (Kumari et al., 2004). In contrast, another measure of work stress, iso-strain (the combination of high demands, low control, and social isolation) as a predictor of diabetes incidence in women but not in men (Heraclides et al., 2009). A meta-analysis of the work stress-diabetes literature failed to show statistically significant relationships between any individual aspect of work-related stress and risk of diabetes (Cosgrove et al., 2012). Recently, a preclinical study reveals that psychological stress after T2DM induction aggravates the diabetogenesis (Garabadu and Krishnamurthy, 2013). Thus, psychological stress could be an important factor in the management of T2DM.

Insulin resistance is considered as an important pathophysiological mechanism for the progression to both T2DM and neurodegeneration conditions (Stumvoll et al., 2005; Zhao and Townsend, 2009; Donath and Shoelson, 2011). In most cases, insulin resistance is associated with a complex network of signaling pathways, including reduced insulin-stimulated tyrosine phosphorylation of insulin receptor (IR) and insulin receptor substrate (IRS) as well as Akt serine phosphorylation in the main target tissues of insulin, including the liver, skeletal muscle and adipose tissue (Hotamisligil et al., 1996; Pessin and Saltiel, 2000). Several studies have recommended that the phosphorylation levels of IRS-1 on serine residue 307 (IRS-1^{ser307}) and of Akt on serine residue 473 (Akt^{ser473}) in rodents could be used as insulin resistance markers (Aguirre et al., 2002; Shoelson et al., 2006; Donath and Shoelson, 2011). Thus, information regarding insulin resistance would be crucial in the development of novel drugs in the pharmacotherapy of co-occurring T2DM and stress condition.

Mitochondria are produced in the cell body, transported to specific neuronal locations of increased energy demands such as synapses (Li et al., 2004) and plays an important role in the neuronal activity pertinent to specific neurotransmitters (Chen et al., 2007; Chen et al., 2008).

Brain mitochondrial function has been reported to decline in both diabetes and stress conditions (Cardoso et al., 2013), indicating that mitochondrial dysfunction is common to both disorders. Recently, it has been reported that mitochondrial electron transport chain enzyme activity increased while mitochondrial integrity decreased with the co-occurring T2DM and stress condition in experimental animals (Garabadu and Krishnamurthy, 2013). Moreover, both hyperglycemia (Fiorentino et al., 2013) and stress (Volkova and Davydov, 2009) are reported to elicit an increase in reactive oxygen species production in brain. Increased mitochondrial biogenesis is part of the cellular response to oxidative stress (Rasbach and Schnellmann, 2007). Therefore, as mitochondria are the common substrate for both T2DM and stress, drugs targeted to mitochondria would be a better therapeutic option in the management of T2DM with co-occurring stress condition.

Metformin (N, N-dimethylimidodicarbonimidic diamide) is one of the most widely used anti-hyperglycemic agents as the first-line drug therapy for management of T2DM (Goodarzi and Bryer-Ash, 2005; Nathan et al., 2009). Metformin inhibits gluconeogenesis through mechanisms linked to perturbation of mitochondrial function (Foretz et al., 2010). Complex I of mitochondrial respiration chain is considered as one of the possible targets of metformin action (El-Mir et al., 2000). Metformin also inhibits mitochondrial transition pore and mitochondria-linked cell death (Guigas et al., 2004). Benzodiazepines are commonly prescribed anxiolytics to T2DM patients with history of stress (Okada et al., 1994; Okada et al., 1995). Diazepam is widely prescribed for the treatment of anxiety, insomnia or stress disorders. Diazepam augmented the blood glucose level in hyperglycemic rats; however it did not alter the same in presence of metformin in the animals (al-Ahmed et al., 1989). Thus, the anti-hyperglycemic activity of metformin is not impaired in the presence of diazepam. Despite its promising

neuroprotective properties, the exact mechanism of diazepam in neuroprotection is not fully understood. It has also been reported that diazepam acts on translocator proteins apart from other actions such as gamma-amino butyric acid-A receptor stimulation and hypothermia (Sarnowska et al., 2009). Translocator proteins located at the contact site between outer and inner mitochondrial membrane, and thus regulate the mitochondria-linked apoptosis (Chelli et al., 2004; Marselli et al., 2004). Therefore, both metformin and diazepam have mitochondrial effects apart from other reported mechanisms. As stress can influence diabetaogensis, the management of T2DM by the combination therapy could be a better choice over monotherapy.

Therefore, the study evaluates the anti-hyperglycemic and anti-hypertriglyceridaemic activity of metformin and diazepam in T2DM rats with repeated CRS exposure. The level of corticosterone in the blood and ulcers in the stomach region were estimated as a measure of stress in the T2DM rats with co-occurring stress condition. Further, the potential anxiolytic-like effect of metformin and diazepam was evaluated in the elevated plus maze (EPM) in the above condition. In addition, the anti-diabetic, anti-stress and anxiolytic-like activity of metformin was evaluated in presence of diazepam in the above condition. At the molecular level, the extent of phosphorylation of IRS-1 and Akt were evaluated to elaborate the insulin resistance in the above condition. At the sub-cellular level, mitochondrial function and integrity were investigated in six brain regions such as hippocampus (HIP), hypothalamus (HYP), pre-frontal cortex (PFC), striatum (STR), amygdala (AMY) and nucleus accumbens (NAC) to elaborate the mitochondrial basis of the combination therapy. Oxidative stress markers such as extent of lipid peroxidation (LPO) and antioxidant enzyme activities like superoxide dismutase (SOD) and catalase (CAT) were estimated in the above brain regions to study the mitochondrial-dependent antioxidant mechanism.

Materials and methods

Animals

Male Charles Foster strain albino rats (200–250 g) 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). All experiments were conducted in compliance with the Principles of laboratory animal care (NIH, 2011) guidelines. 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 phospho-IRS-1^{ser307}, total IRS, phosphor-Akt^{ser473}, total Akt 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 co-occurring T2DM and repeated CRS (DMS)

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). Further, two stress sessions 24 hr apart were performed during 08:00 hr to

12:00 hr on 6th and 7th day of streptozotocine injection and were consisting of a 1 hr restraint period (rat restrainers were transparent plastic tubes 15 cm long × 6.5 cm width) in a 4 °C room (Sullivan RM, Szechtman, 1995; Garabadu and Krishnamurthy, 2013).

The Experimental Design

The experimental design consisted of three sets of experiments. The animals were acclimatized for seven days and were randomly divided into five groups viz:- control, diabetes with repeated CRS (DMS), DMS+MET, DMS+DZ and DMS+MET+DZ in each of the experiment. The experimental protocol was followed for 13 days for all experiments. The day animals received the streptozotocine injection was considered as day-1(D-1). The rats were exposed to repeated CRS procedures to all the group animals except control group rats on D-6 and D-7. On D-7, after 1 hr to CRS paradigm, metformin (25 mg/kg, *p.o.*; Yanardag et al., 2005) was administered to DMS+MET and DMS+MET+DZ group animals while diazepam (1 mg/kg, *p.o.*; Beattie, 1977; Kumar et al., 2013) was administered to DMS+DZ and DMS+MET+DZ group rats after 30 min to metformin treatment. This treatment schedule was continued for seven consecutive days i.e., D-13 of the experimental design. The experiment 1 and 2 were performed for the oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) respectively. In experiment 3, all the animals were killed after 1 hr to last dose on D-13 of the experimental schedule by decapitation. The blood and liver were stored immediately at -80 ℃ till further study. The brains were removed and microdissected (Palkovits and Brownstein, 1988) into hippocampus (HIP), hypothalamus (HYP), pre-frontal cortex (PFC), striatum (STR), amygdala (AMY) and nucleus accumbens (NAC) and stored immediately at -80 ℃ 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 13th day of the experimental schedule. Metformin, diazepam and their combination, and vehicle were administered 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 13th day of the experimental schedule. Metformin, diazepam and their combination, and vehicle were administered 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 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).

Estimation of plasma glucose and triglyceride level

On the D-1, 3, 7 and 13 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 (Toleikis and Godin, 1995) to obtain plasma for measuring the glucose, triglyceride and corticosterone levels. The plasma glucose and triglyceride were determined spectrophotometrically (Beckman Coulter DU 7400

UV–VIS Spectrophotometer; Fullerton, CA) in triplicate using the glucose GOD PAP kit (Priman Instrument Pvt. Ltd., India) and triglyceride GPO-PAP kit (Span Diagnostics Ltd., India) respectively.

Estimation of plasma corticosterone level

The plasma corticosterone was quantified in a High Performance Liquid Chromatography (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 CORT was detected at 250 nm using UV detector (Model 2849, Waters, USA). The chromatogram was recorded and analyzed with Empower software (Version 2.0).

Estimation of Ulcer Index

The stomach was cut through greater curvature and a blind observer calculated the ulcer index by following standard protocol (Sairam et al., 2002).

Anxiety-like behavior in EPM test

The plus maze consisted of two opposite open arms, 50 × 10 cm, crossed with two opposite open arms of the same dimensions with walls of 40 cm high. The arms were connected with a central square (10 × 10 cm) to give the apparatus a plus-sign appearance. The maze was kept elevated 50 cm above 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 time spent and the numbers of entries made by the rat, during the next 5 min, on the open arms were recorded as an index of anxiety.

Further, the total arm entries 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).

Western blot analysis

For Western blot analysis, the liver tissues were lysed in buffer containing complete protease inhibitor cocktail. Protein concentrations were determined according to Bradford (1976). A standard plot was generated using bovine serum albumin. An aliquot of each sample were electrophoresed in 10% SDS-PAGE gels for phospho-IRS-1^{ser307}, total IRS, phosphor-Akt^{ser473} and total Akt proteins, transferred to polyvinylidene fluoride membranes and probed with specific antibodies. The membrane was incubated overnight with polyclonal rabbit anti-phospho-IRS-1^{ser307} (1:10,000, Abcam Plc., Cambridge, USA), monoclonal anti-total IRS (1:1000, Abcam Plc., Cambridge, USA), monoclonal anti-phospho-Akt^{ser473} (1:10,000, Abcam Plc., Cambridge, USA) and polyclonal anti-total Akt (1:1000, Abcam Plc., Cambridge, USA) primary antibodies. 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 re-probed overnight with rabbit anti β-actin polyclonal primary antibody (Abcam Plc., Cambridge, USA) 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.

Assessment of Mitochondrial function, integrity and oxidative stress

Mitochondria Isolation Procedure

Mitochondria were isolated by following standard procedure of Pedersen et al. (1978). Briefly, the brain regions were homogenized in (1:10, w/v) ice cold isolation buffer (250 mM sucrose, 1 mM EGTA and 10 mM HEPES–KOH, pH 7.2) followed by centrifugation at 600 × *g*/5 min. The resultant supernatant was centrifuged at 10,000 × *g*/15 min. The resultant pellets were suspended in 1 ml medium (250 mM sucrose, 0.3 mM EGTA and 10 mM HEPES–KOH, pH 7.2) and again centrifuged at 14,000 × *g*/10 min. All centrifugation procedures were performed at 4 ℃. The final mitochondrial pellet was re-suspended in medium (250 mM sucrose and 10 mM HEPES–KOH, pH 7.2) and used within 3 h. The mitochondrial protein content was estimated using the method of Lowry et al. (1951).

Estimation of mitochondrial succinate dehydrogenase (SDH) activity

The mitochondrial SDH was determined by following the method of Sally and Margaret (1989) based on the progressive reduction of NBT to diformazan (dfz) measured at 570 nm. The mean SDH activity of each region was expressed as micromole formazan produced per min per milligram of protein.

Estimation of mitochondrial membrane potential (MMP)

The Rhodamine dye taken up by mitochondria was measured with spectrofluorometer (Hitachi, F-2500, Japan; Huang, 2002). Briefly, the mitochondrial suspension was mixed with TMRM solution and incubated for 5 min at 25 ℃ followed by frequent washings (four times) to remove any unbound TMRM. The florescence emission was read at an excitation λ of 535 ± 10 nm and emission λ of 580 ± 10 nm using slit no. 10. The peak fluorescence intensity recorded was

around 570 ± 5 nm. The intensity of fluorescence was recorded which was considered to be proportional to MMP.

Estimation of mitochondrial lipid peroxidation (LPO)

Mitochondrial MDA content was measured as a marker for LPO described by Uchiyama and Mihara (1978) and modified by Sunderman et al. (1985). Briefly, the chromophore formed in the reaction was measured at 532 nm. The MDA concentrations are expressed as micromoles of MDA/mg of protein.

Estimation of mitochondrial superoxide dismutase (SOD) activity

The activity of SOD was assayed by the method of Kakkar et al. (1984) based on the formation of NADH–phenazine methosulphate–nitro blue tetrazolium formazan measured at 560 nm against butanol as blank. A single unit of the enzyme was expressed as 50% inhibition of NBT reduction/min/mg of protein under the assay conditions.

Estimation of mitochondrial catalase (CAT) activity

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.

Data Analysis

The results were expressed as mean ± S.E.M. The statistical significance for time-course effects on plasma glucose, TG and CORT levels and parameters in the EPM test paradigm were analyzed by two-way analysis of variance (ANOVA) followed by Post-hoc Bonferroni test. All other data sets were analyzed by one-way ANOVA followed by Post-hoc Student Newman–Keuls test. $P<0.05$ was considered to be statistically significant throughout the experimental data analysis.

Results

Metformin and diazepam combination enhances glucose tolerance during OGTT in DMS rats than metformin or diazepam monotherapy

Table-34 illustrates the effect of metformin or diazepam or their combination on plasma glucose levels during OGTT in DMS exposed rats. Repeated measures of two-way ANOVA revealed that there were significant differences in plasma glucose levels among group [$F(4, 100) = 1432$; $P<0.05$], time [$F(3, 100) = 418.1$; $P<0.05$] and an interaction [$F(12, 100) = 52.5$; $P<0.05$] between group and time. Post-hoc analysis showed that metformin but not diazepam monotherapy decreased the glucose level at 30 min after glucose loading compared to DMS rats. Moreover, the combination of metformin and diazepam showed remarkable improvement in glucose response at 30 min after glucose loading compared to metformin monotherapy. This effect persisted upto 120 min after glucose loading during OGTT.

Metformin and diazepam combination increases insulin sensitivity during ITT in DMS rats than metformin or diazepam monotherapy

Table-35 illustrates the effect of metformin or diazepam or their combination on plasma glucose levels during ITT in DMS exposed rats. Repeated measures of two-way ANOVA revealed that there were significant differences in plasma glucose levels among group [$F(4, 100) = 1392$; $P<0.05$], time [$F(3, 100) = 28.5$; $P<0.05$] and an interaction [$F(12, 100) = 1.5$; $P<0.05$] between group and time. Post-hoc analysis showed that metformin but not diazepam monotherapy decreased significantly the plasma glucose level at 30 min after insulin injection compared to DMS rats. Moreover, the combination of metformin and diazepam showed remarkable improvement in insulin sensitivity at 30 min after insulin injection compared to metformin monotherapy. This effect persisted upto 90 min after insulin injection during ITT.

Metformin and diazepam combination reduces DMS-induced increase in the plasma glucose, triglyceride and corticosterone levels in rats than metformin or diazepam monotherapy

Table-36 illustrates the effect of metformin or diazepam or their combination on DMS-induced alterations in plasma glucose, triglyceride and corticosterone levels. Repeated measures of two-way ANOVA revealed that there were significant differences in plasma glucose, triglyceride and corticosterone levels among group ([F (4, 100) = 6220; P<0.05], [F (4, 100) = 746.2; P<0.05] and [F (4, 100) = 350.7; P<0.05] respectively), time ([F (3, 100) = 15570; P<0.05], [F (3, 100) = 2027; P<0.05] and [F (3, 100) = 1525; P<0.05] respectively) and an interaction ([F (12, 100) = 1568; P<0.05], [F (12, 100) = 231.3; P<0.05] and [F (12, 100) = 126.9; P<0.05] respectively) between group and time. Post-hoc analysis showed that there were no significant differences among groups in plasma glucose or triglyceride or corticosterone levels on D-1. Streptozotocine injection caused significant increase in the plasma glucose, triglyceride and corticosterone levels on D-3 of the experimental schedule compared to vehicle treated rats. Further, exposure to repeated CRS augmented the levels of plasma glucose, triglyceride and corticosterone on D-7 compared to control animals. Metformin treatment significantly decreased the DMS-induced increase in all the biochemical parameters in the plasma on D-13 of the experimental schedule. However, diazepam significantly reduced the DMS-induced increase in the level of corticosterone only in the plasma of the rats. Furthermore, metformin and diazepam combination significantly reduced the DMS-induced increase in all the biochemical parameters in the plasma on D-13 compared to metformin and diazepam monotherapy.

Diazepam in combination with metformin reduces gastric ulcer in DMS exposed rats than either metformin or diazepam monotherapy

The effect of metformin or diazepam or their combination on DMS-induced gastric ulcer in rats is depicted in Fig-43. Statistical analysis by one-way ANOVA revealed that there were significant differences in gastric ulcer index [$F(4, 25) = 49.29$; $P<0.05$] among groups on D-13. Post-hoc test showed that DMS induced significant increase in the gastric ulcers in rats compared to vehicle treated animals. Administration of either metformin or diazepam significantly reduced the DMS-induced increase in the gastric ulcers in rats. Moreover, diazepam treated rats showed significant decrease in the DMS-induced gastric ulcers compared to metformin administered animals. Furthermore, administration of both diazepam and metformin significantly decreased the DMS-induced increase in the gastric ulcers compared to metformin and diazepam monotherapy.

Metformin and diazepam combination exhibits better anxiolytic activity in DMS exposed rats than their monotherapy

Fig-44 illustrates the effect of metformin or diazepam or their combination on percentage of open arm entries to total arm entries (A) and percentage of open arm time spent to total arm time spent (B), and total arm entries (C) in EPM test paradigm of DMS exposed rats. Statistical analysis by repeated measures of two-way ANOVA revealed that there were significant differences in percentage open arm entries and time spent among groups ([$F(4, 50) = 40.97$; $P<0.05$] and [$F(4, 50) = 34.65$; $P<0.05$] respectively), time ([$F(1, 50) = 52.99$; $P<0.05$] and [$F(1, 50) = 22.80$; $P<0.05$] respectively) and an interaction between group and time ([$F(4, 50) = 7.66$; $P<0.05$] and [$F(4, 50) = 4.79$; $P<0.05$] respectively). However, there were no significant differences in total arm entries in EPM paradigm among group [$F(4, 50) = 0.72$; $P>0.05$], time

[F (1, 50) = 9.92; P>0.05] and there was no significant interaction between group and time [F (4, 50) = 0.90; P>0.05]. Post-hoc analysis showed that DMS paradigm caused significant increase in the percentage open arm entries and time spent on D-7 compared to control rats and was sustained upto D-13. Metformin and diazepam significantly ameliorated the DMS-induced increase in the percentage open arm entries and time spent in the EPM test. Moreover, treatment of both diazepam and metformin significantly reduced the DMS-induced increase in the percentage open arm entries and time spent compared to metformin and diazepam administration.

Metformin and diazepam combination enhances the serine phosphorylation of IRS-1^{ser307} and Akt^{ser473} in the liver of DMS exposed rats than their monotherapy

Fig-45 and 46 depict the effect of metformin and diazepam or their combination on the DMS-induced alterations in the level of phosphorylation of IRS-1^{ser307} and Akt^{ser473} in the liver tissues respectively. Statistical analysis by one-way ANOVA revealed that there were significant differences in the level of expression of p-IRS-1^{ser307} [F (4, 10) = 62.14; P<0.05] and p-Akt^{ser473} [F (4, 10) = 75.40; P<0.05], and ratio of p-IRS-1^{ser307}/total IRS [F (4, 10) = 34.18; P<0.05] and p-Akt^{ser473}/total Akt [F (4, 10) = 56.26; P<0.05] in the liver tissues among groups. However, there were no significant differences in the level of expression of total IRS-1 [F (4, 10) = 0.47; P>0.05] and Akt [F (4, 10) = 0.21; P>0.05] in the liver tissues among groups. Post-hoc analysis showed that DMS exposure significantly reduced the extent of phosphorylation of IRS-1^{ser307} and Akt^{ser473} in the rat liver tissues. Metformin but not diazepam significantly mitigated the DMS-induced decrease in the extent of phosphorylation of IRS-1^{ser307} and Akt^{ser473} in the liver. Moreover, metformin with diazepam combination further ameliorated the DMS-induced

decrease in the extent of phosphorylation of IRS-1^{ser307} and Akt^{ser473} in the liver compared to metformin monotherapy.

Effect of metformin or diazepam or their combination on DMS-induced alterations in mitochondrial SDH and MMP in discrete brain regions

Fig-47 depicts the effect of metformin, diazepam or their combination on DMS-induced changes in mitochondrial SDH and MMP in different brain regions. Statistical analysis by one-way ANOVA revealed that there were significant differences in percentage in mitochondrial SDH and MMP in HIP ([F (4, 25) = 72.12; P<0.05] and [F (4, 25) = 75.19; P<0.05] respectively), HYP ([F (4, 25) = 50.29; P<0.05] and [F (4, 25) = 70.10; P<0.05] respectively), PFC ([F (4, 25) = 32.54; P<0.05] and [F (4, 25) = 43.08; P<0.05] respectively), STR ([F (4, 25) = 6.91; P<0.05] and [F (4, 25) = 56.40; P<0.05] respectively), AMY ([F (4, 25) = 54.94; P<0.05] and [F (4, 25) = 50.94; P<0.05] respectively) and NAC ([F (4, 25) = 30.04; P<0.05] and [F (4, 25) = 35.41; P<0.05] respectively) among groups. Post-hoc test showed that metformin and diazepam monotherapy significantly reversed the DMS-induced increase and decrease in the mitochondrial SDH and MMP respectively in all the brain regions. Furthermore, administration of combination of metformin and diazepam caused significant reduction in the DMS-induced increase and decrease in the mitochondrial SDH and MMP respectively in all the brain regions compared to their monotherapy.

Effect of metformin or diazepam or their combination on DMS-induced alterations in mitochondrial LPO and activities of SOD and CAT in discrete brain regions

The effect of metformin or diazepam or their combination on DMS-induced changes in mitochondrial LPO and activities of SOD and CAT in different brain regions is illustrated in Fig-48. One-way ANOVA revealed that there were significant differences in percentage in

mitochondrial LPO and activities of SOD and CAT in HIP ([F (4, 25) = 38.73; P<0.05], [F (4, 25) = 22.26; P<0.05] and [F (4, 25) = 35.26; P<0.05] respectively), HYP ([F (4, 25) = 57.62; P<0.05], [F (4, 25) = 25.43; P<0.05] and [F (4, 25) = 43.28; P<0.05] respectively), PFC ([F (4, 25) = 54.30; P<0.05], [F (4, 25) = 41.21; P<0.05] and [F (4, 25) = 32.11; P<0.05] respectively), STR ([F (4, 25) = 31.44; P<0.05], [F (4, 25) = 21.71; P<0.05] and [F (4, 25) = 52.08; P<0.05] respectively), AMY ([F (4, 25) = 24.00; P<0.05], [F (4, 25) = 63.25; P<0.05] and [F (4, 25) = 42.03; P<0.05] respectively) and NAC ([F (4, 25) = 29.49; P<0.05], [F (4, 25) = 45.42; P<0.05] and [F (4, 25) = 46.26; P<0.05] respectively) among groups. Post-hoc test revealed that metformin and diazepam monotherapy significantly decreased the DMS-induced increase in the mitochondrial LPO in all the brain regions. Administration of both metformin and diazepam further decreased the DMS-induced increase in the mitochondrial LPO in all the brain regions compared to metformin and diazepam monotherapy. Moreover, metformin and diazepam monotherapy caused significant increase in the DMS-induced decrease in the activities of mitochondrial SOD and CAT in all the brain regions. Administration of metformin and diazepam combination further increased the DMS-induced decrease in the mitochondrial SOD and CAT in all the brain regions compared to their monotherapy.

Table-34: Effect of metformin, diazepam or their combination on plasma glucose level during OGTT in co-occurring T2DM and RS exposed rats.

	Plasma glucose level (mg/dl)			
Groups	**0 min**	**30 min**	**60 min**	**120 min**
Control	74.3 ± 1.3	143.6 ± 1.8	157.3 ± 3.9	111.5 ± 3.8
DMS	512.4 ± 12.9[a]	969.2 ± 34.3[a]	1202.8 ± 26.0[a]	1192.7 ± 21.8[a]
DMS+MET	420.3 ± 6.6[a,b]	609.6 ± 25.5[a,b]	690.0 ± 23.5[a,b]	555.6 ± 24.2[a,b]
DMS+DZ	499.0 ± 9.7[a,c]	941.9 ± 23.0[a,c]	1156.6 ± 16.2[a,c]	1188.5 ± 29.0[a,c]
DMS+MET+DZ	206.9 ± 4.3[a,b,c,d]	445.5 ± 24.0[a,b,c,d]	448.7 ± 26.0[a,b,c,d]	426.3 ± 9.0[a,b,c,d]

All values are Mean ± SEM (n = 6). [a]P<0.05 compared to Control, [b]P<0.05 compared to DMS, [c]P<0.05 compared to DMS+MET and [d]P<0.05 compared to DMS+DZ [Repeated measures of two-way ANOVA followed by Bonferroni Post-hoc test].

Table-35: Effect of metformin, diazepam or their combination on plasma glucose level during ITT in co-occurring T2DM and RS exposed rats.

	Plasma glucose level (mg/dl)			
Groups	**0 min**	**30 min**	**60 min**	**90 min**
Control	73.2 ± 2.6	58.1 ± 3.4	56.4 ± 3.3	54.3 ± 4.2
DMS	510.5 ± 12.3[a]	448.3 ± 18.9[a]	439.7 ± 19.7[a]	437.0 ± 21.5[a]
DMS+MET	418.4 ± 5.6[a,b]	395.9 ± 4.5[a,b]	393.8 ± 3.7[a,b]	390.0 ± 4.1[a,b]
DMS+DZ	493.3 ± 9.2[a,c]	437.6 ± 9.5[a,c]	431.2 ± 10.6[a,c]	427.0 ± 9.4[a,c]
DMS+MET+DZ	203.1 ± 5.0[a,b,c,d]	146.4 ± 3.9[a,b,c,d]	142.1 ± 3.8[a,b,c,d]	140.0 ± 3.3[a,b,c,d]

All values are Mean ± SEM (n = 6). [a]P<0.05 compared to Control, [b]P<0.05 compared to DMS, [c]P<0.05 compared to DMS+MET and [d]P<0.05 compared to DMS+DZ [Repeated measures of two-way ANOVA followed by Bonferroni Post-hoc test].

Table-36: Effect of metformin, diazepam or their combination on plasma glucose, triglyceride and corticosterone levels in co-occurring T2DM and RS exposed rats.

Groups	D-1	D-3	D-7	D-13
Plasma glucose (mg/dl)				
Control	76.3 ± 1.0	75.8 ± 0.8	75.5 ± 0.6	75.1 ± 0.8
DMS	76.0 ± 2.8	256.3 ± 3.0[a]	568.8 ± 5.8[a]	577.6 ± 2.5[a]
DMS+MET	74.5 ± 2.7	261.1 ± 2.5[a]	572.9 ± 5.1[a]	425.8 ± 4.7[a,b]
DMS+DZ	75.3 ± 1.7	254.3 ± 2.7[a]	566.2 ± 4.8[a]	574.2 ± 1.8[a,c]
DMS+MET+DZ	74.9 ± 2.6	264.6 ± 1.3[a]	574.1 ± 0.7[a]	212.5 ± 5.2[a,b,c,d]
Plasma triglyceride (mg/dl)				
Control	85.9 ± 1.5	87.4 ± 1.6	87.3 ± 1.6	87.1 ± 1.5
DMS	90.5 ± 1.2	195.2 ± 4.1[a]	471.0 ± 9.8[a]	483.3 ± 10.1[a]
DMS+MET	88.8 ± 1.5	194.1 ± 7.0[a]	474.2 ± 11.1[a]	365.6 ± 9.0[a,b]
DMS+DZ	91.3 ± 1.2	196.2 ± 4.8[a]	470.1 ± 9.8[a]	484.1 ± 9.6[a,c]
DMS+MET+DZ	91.7 ± 1.2	200.0 ± 5.7[a]	484.9 ± 12.6[a]	146.0 ± 7.1[a,b,c,d]
Plasma corticosterone (µg/dl)				
Control	15.8 ± 0.4	16.3 ± 0.4	15.9 ± 0.4	16.1 ± 0.5
DMS	15.9 ± 0.3	34.4 ± 1.5[a]	187.7 ± 5.3[a]	191.5 ± 8.8[a]
DMS+MET	15.4 ± 0.6	32.0 ± 1.1[a]	188.4 ± 5.7[a]	120.5 ± 3.9[a,b]
DMS+DZ	15.2 ± 0.7	31.7 ± 1.0[a]	186.7 ± 6.5[a]	107.1 ± 2.6[a,b,c]
DMS+MET+DZ	15.4 ± 0.7	31.3 ± 1.0[a]	191.1 ± 6.0[a]	77.6 ± 5.5[a,b,c,d]

All values are Mean ± SEM (n = 6). [a]P<0.05 compared to Control, [b]P<0.05 compared to DMS, [c]P<0.05 compared to DMS+MET and [d]P<0.05 compared to DMS+DZ [Repeated measures of two-way ANOVA followed by Bonferroni Post-hoc test].

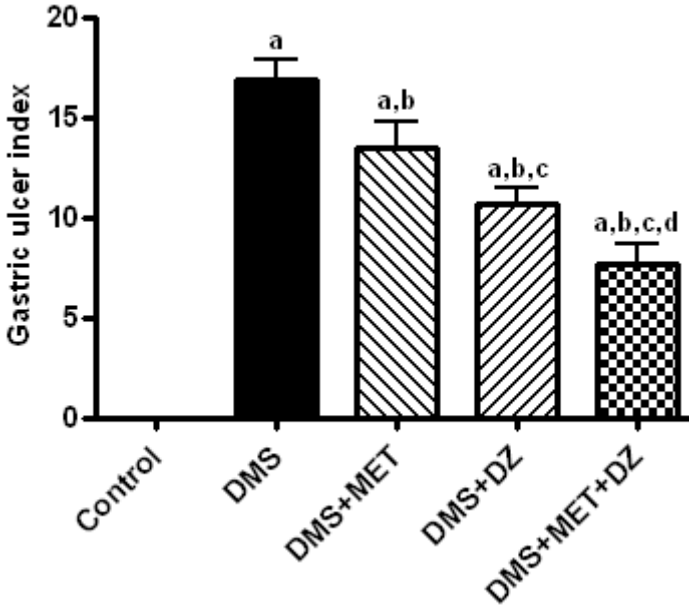

Figure 43: The effect of metformin (MET), diazepam (DZ) and their combination on gastric ulcer in T2DM and repeated CRS paradigm (DMS) exposed rats. All values are Mean ± SEM (n = 6). [a]P<0.05 compared to control, [b]P<0.05 compared to DMS, [c]P<0.05 compared to DMS+MET and [d]P<0.05 compared to DMS+DZ [One-way ANOVA followed by Student Newmann-Keuls test].

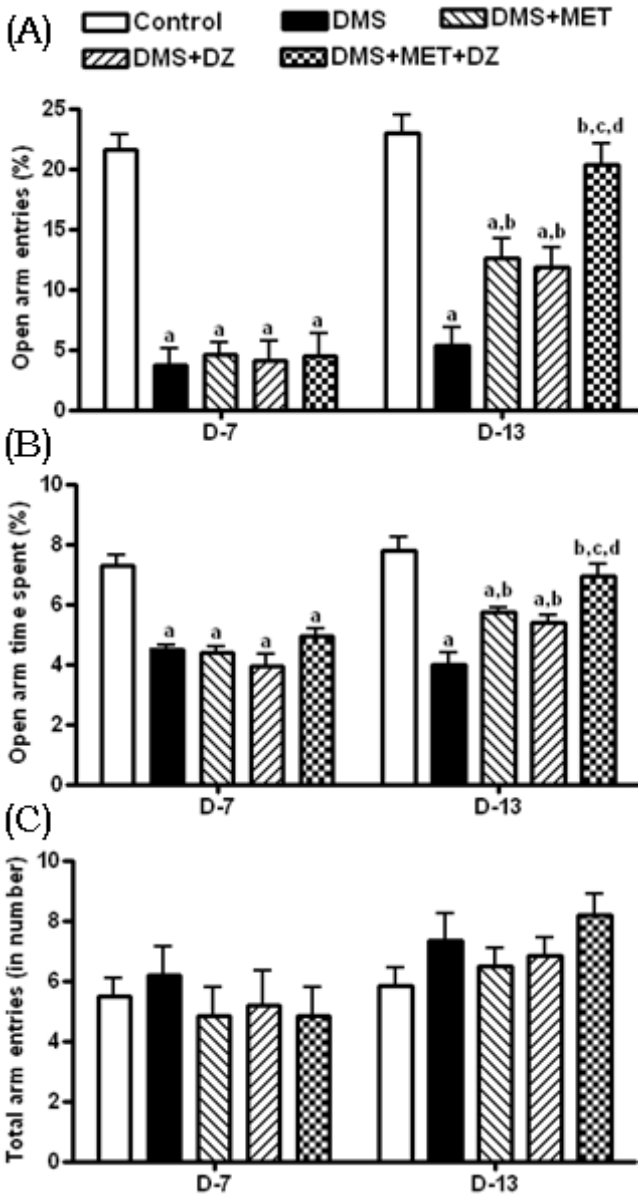

Figure 44: The effect of MET, DZ and their combination on DMS-induced alterations in percentage of open arm entries to total arm entries (A), percentage of open arm time spent to total arm time spent (B), and total arm entries (C) in EPM. All values are Mean ± SEM (n = 6). [a]P<0.05 compared to control, [b]P<0.05 compared to DMS, [c]P<0.05 compared to DMS+MET and [d]P<0.05 compared to DMS+DZ [Repeated measure two-way ANOVA followed by Bonferroni test].

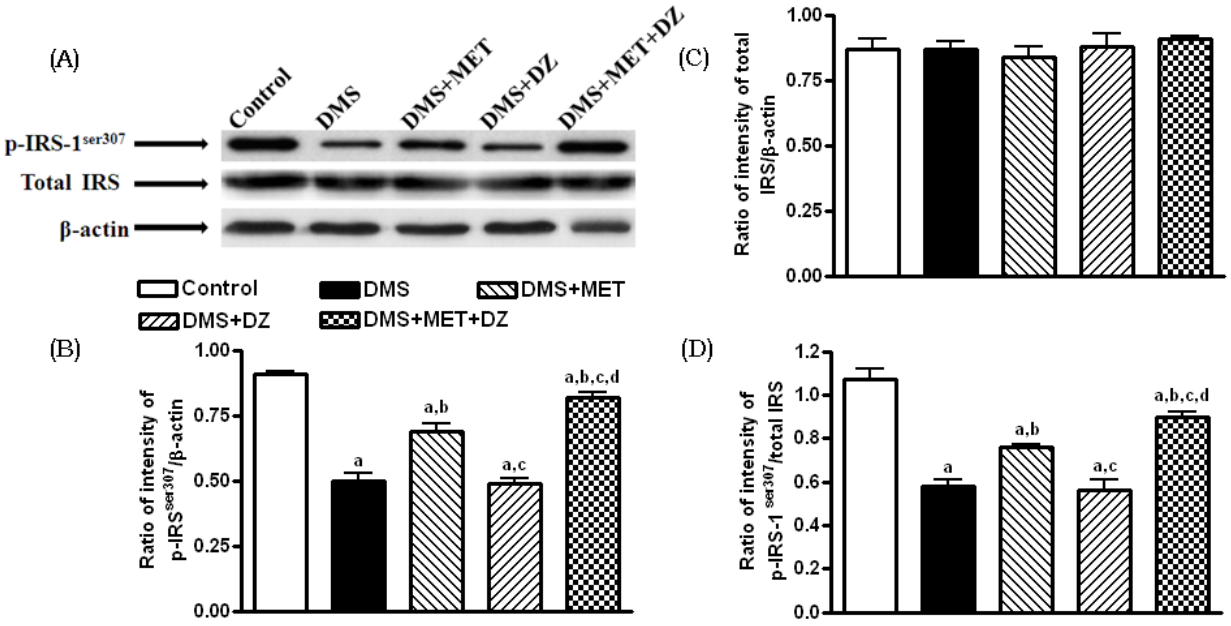

Figure 45: The effect of MET, DZ and their combination on DMS-induced changes in the level of expression of phospho-IRS^{ser307} (p-IRS^{ser307}) and total IRS in the liver tissues. The blots are representative of p-IRS^{ser307} and total IRS (A) in the liver tissues. The results in the histogram are expressed as ratio of relative intensity of levels of protein expression of either p-IRS^{ser307} or total IRS to β-actin, and ratio of relative intensity of level of expression of p-IRS^{ser307} to total IRS. 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 DMS, $^{c}P<0.05$ compared to DMS+MET and $^{d}P<0.05$ compared to DMS+DZ [One-way ANOVA followed by Student Newmann-Keuls test].

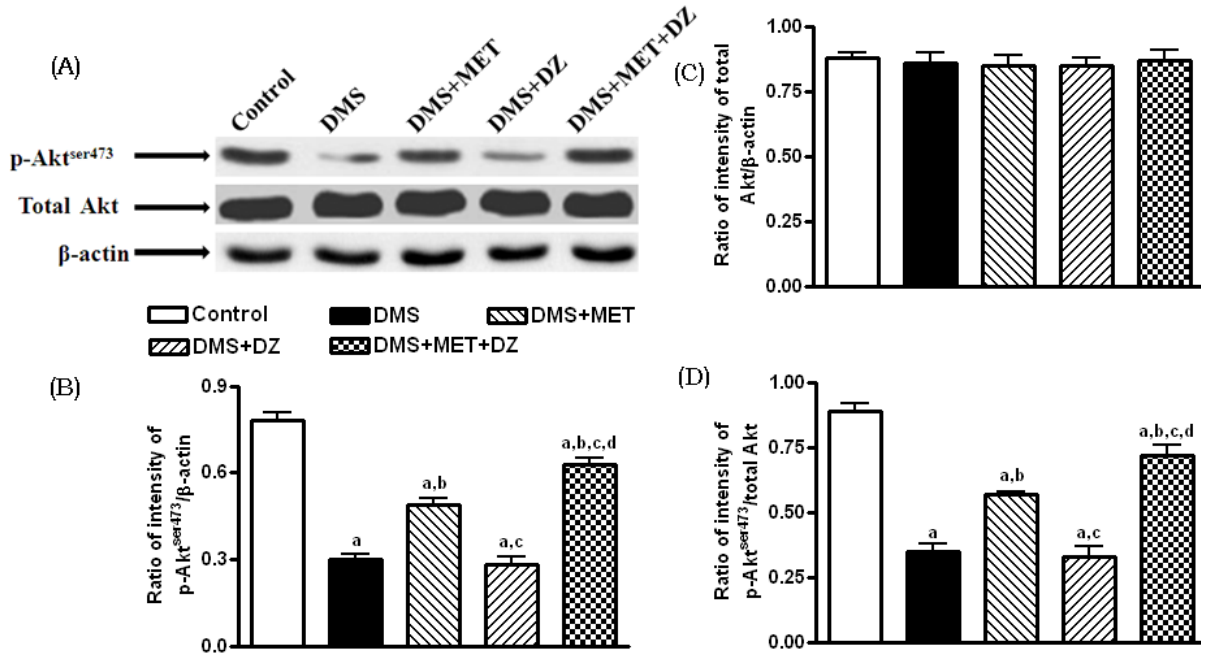

Figure 46: The effect of MET, DZ and their combination on DMS-induced changes in the level of expression of phospho-Akt^{ser473} (p-Akt^{ser473}) and total Akt in the liver tissues. The blots are representative of p-Akt^{ser473} and total Akt (A) in the liver tissues. The results in the histogram are expressed as ratio of relative intensity of levels of protein expression of either p-Akt^{ser473} or total Akt to β-actin, and ratio of relative intensity of level of expression of p-Akt^{ser473} to total Akt. 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 DMS, $^{c}P<0.05$ compared to DMS+MET and $^{d}P<0.05$ compared to DMS+DZ [One-way ANOVA followed by Student Newmann-Keuls test].

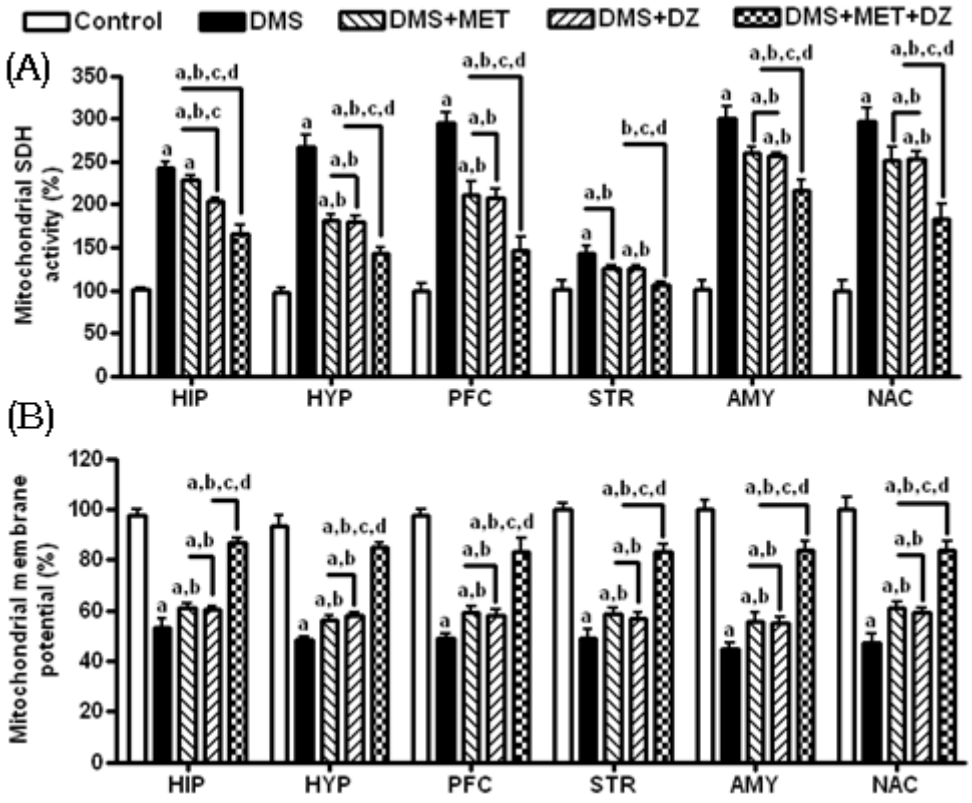

Figure 47: The effect of MET, DZ and their combination on mitochondrial succinate dehydrogenase (SDH) activity and membrane potential in discrete brain regions in DMS exposed rats. All values are Mean ± SEM (n = 6). [a]P<0.05 compared to control, [b]P<0.05 compared to DMS, [c]P<0.05 compared to DMS+MET and [d]P<0.05 compared to DMS+DZ [One-way ANOVA followed by Student Newmann-Keuls test].

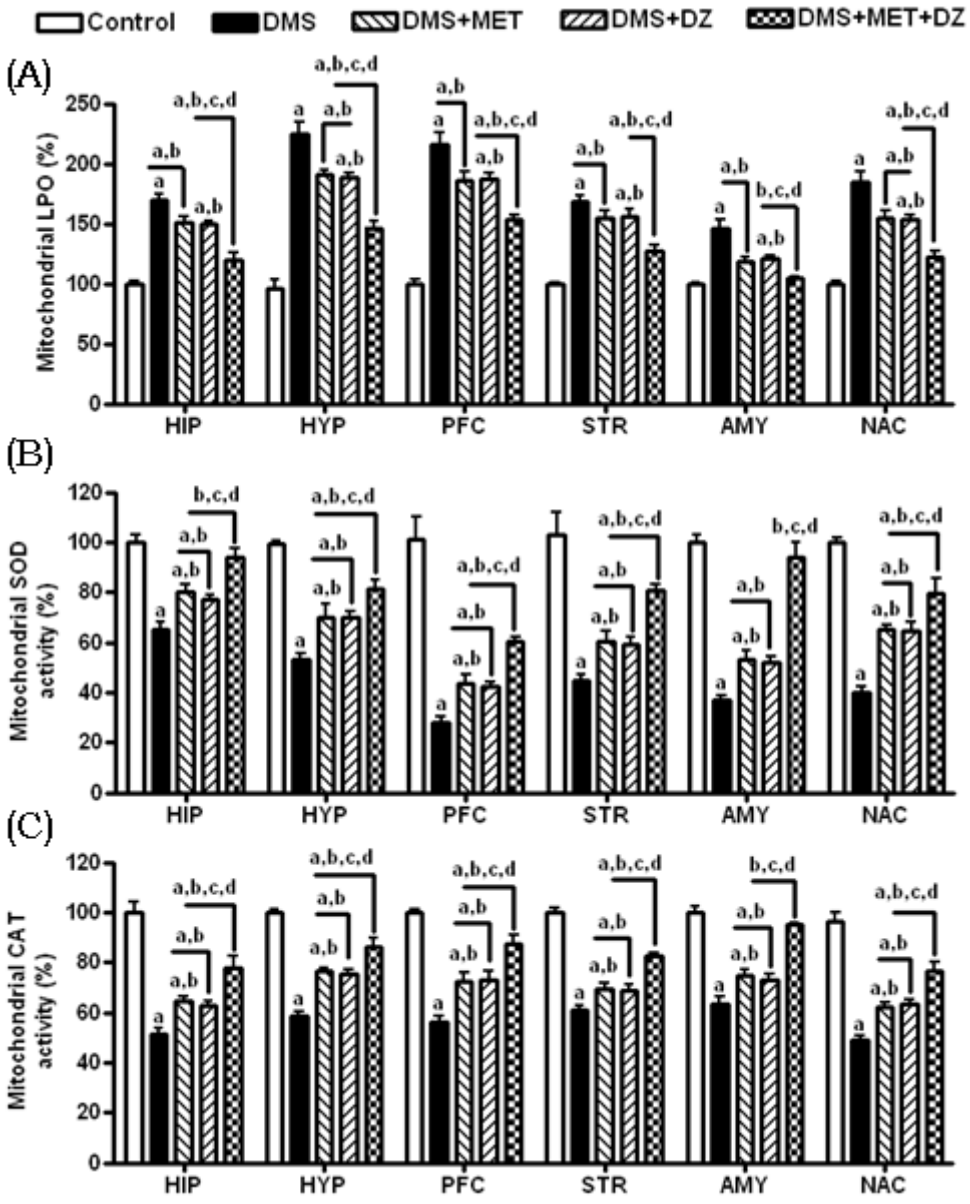

Figure 48: The effect of MET, DZ and their combination on mitochondrial lipid peroxidation (LPO), and superoxide dismutase (SOD) and catalase (CAT) activities in discrete brain regions in DMS exposed rats. All values are Mean ± SEM (n = 6). [a]P<0.05 compared to control, [b]P<0.05 compared to DMS, [c]P<0.05 compared to DMS+MET and [d]P<0.05 compared to DMS+DZ [One-way ANOVA followed by Student Newmann-Keuls test].

Discussion

The primary objective of the present study was to evaluate the anti-diabetic efficacy of metformin and diazepam in co-occurring T2DM and repeated CRS exposed rats. Additionally, the study also examined the effect of co-administration of metformin and diazepam for anti-diabetic, anti-stress and anxiolytic-like activities in the above model. Metformin exhibited anti-diabetic, anti-stress and anxiolytic-like activities in the co-occurring condition of T2DM and stress. However, diazepam showed anti-stress and anxiolytic-like activities but not anti-diabetic activity in the above condition. Metformin and diazepam combination enhanced anti-diabetic, anti-stress and anxiolytic-like activities compared to metformin treatment in co-occurring T2DM and repeated CRS exposed rats. We for the first time report that the combination therapy improved the glucose tolerance and insulin sensitivity in the co-occurring T2DM and stress condition. Further, the combination improved the insulin resistance in the liver of co-occurring T2DM and repeated CRS exposed rats. The combination therapy showed marked improvement in mitochondrial function, integrity and oxidative stress in all the brain regions in the above condition than their monotherapy. Thus, metformin in combination with diazepam would be a better therapeutic option in the management of co-occurring T2DM and stress condition.

To assess the effect of metformin, diazepam and their combination on co-occurring T2DM and stress condition-induced disturbance in the glucose homeostasis, the OGTT and ITT were performed. OGTT is an important index for the evaluation of beta cell function while ITT indicates about the insulin sensitivity (Thomas et al., 2002). The impairment in glucose tolerance and insulin sensitivity during T2DM is reported to be improved by metformin treatment (Wang et al., 2013; Gao et al., 2014). In the present study, the co-occurring condition impaired glucose tolerance and insulin sensitivity. Metformin, but not diazepam monotherapy improved glucose

tolerance in the above co-occurring condition. Interestingly, the improvement of glucose tolerance in the co-occurring condition was better with combination therapy than metformin monotherapy. Moreover, the combination improved insulin sensitivity in the co-occurring condition of T2DM and stress compared to metformin monotherapy. These results suggest the fact that the combination may have potential therapeutic effect on the insulin signaling pathway in the above condition.

Experimental as well as clinical studies report hyperglycemia and aberrant lipid profile in T2DM with stress condition (Okada et al., 2004; Davila et al., 2011; Faulenbach et al., 2012, Garabadu and Krishnamurthy, 2013). Similar to earlier findings, the levels of glucose and triglyceride were elevated with the co-occurring T2DM and stress exposure. Metformin exhibited anti-diabetic activity in terms of reducing glucose as well as triglyceride in the rats exposed to both T2DM and stress paradigm. Metformin decreases T2DM-induced increase in the level of glucose and triglyceride in the plasma of both animals and patients (Karamanos et al., 2011; Chen et al., 2013). However, this is the first report of effect of metformin in the rats exposed to both T2DM and stress paradigm. Further, metformin in presence of diazepam exhibited pronounced anti-hyperglycemic and anti-hypertriglyceridaemic effect than metformin monotherapy in co-occurring T2DM and stress exposed rats. In an earlier report, it has been documented that diazepam treatment augmented the hyperglycemic condition in hyperglycemic rats and this effect was ameliorated with metformin administration (al-Ahmed et al., 1989). However, in the present study diazepam did not alter the hyperglycemic condition in the co-occurring condition of T2DM and stress. This discrepancy could be due to both the mode of induction of diabetes and the presence of stress. These effects indicate that this combination

shows prominent anti-diabetic effect in terms of reducing blood glucose and triglyceride levels in co-occurring T2DM and stress condition.

It is demonstrated that hypercorticosteronemia is observed in both T2DM and stress conditions, and also in their co-occurring situation (Wang et al., 2004; Kumar et al., 2007; de Oliveira et al., 2011; Garabadu and Krishnamurthy, 2013), a result of over-activation of hypothalamic-pituitary-adrenal cortex (HPA)-axis function (Krishnamurthy et al., 2013). Similar to earlier observations, there was significant increase in the level of corticosterone in the plasma in co-occurring T2DM and stress exposed animals in the current study. In addition, this co-occurring condition exhibited significant ulcers in the stomach of the animals providing further evidence as a peripheral marker of HPA-axis dysfunction. Diazepam potentiated the anti-stress activity of metformin in the T2DM rats exposed to repeated stress paradigm in terms of reducing in the plasma corticosterone and gastric ulceration than metformin and diazepam monotherapy. Similar to our findings, metformin regulated plasma corticosterone in T2DM subjects (Cleasby et al., 2003; Carrizo et al., 2009). However, the mechanism of reduction of corticosterone by metformin is still not clear. It has also been postulated that metformin exhibits the anti-diabetic effect by down regulating the glucocorticoid receptors in the brain tissues of rats with hypercorticosteronemia (Cleasby et al., 2003). Diazepam modulates HPA-axis function during stress conditions and thus reduces the level of plasma corticosterone in both animals and patients (Okada et al., 1994; Sarnowska et al., 2009). It has been suggested that activation of corticosteroid receptors play a significant role in the pathogenesis of anxiety-like behaviors in diabetic rats (López-Rubalcava et al., 2013). Further, corticosteroid receptor antagonists synergize the anxiolytic-like activity of diazepam in the diabetic rats (López-Rubalcava et al., 2013). Thus, it can be assumed that the metformin and diazepam in combination exhibited anti-

stress effect in co-occurring diabetic-stress condition probably acting through a common corticosteroid-dependent mechanism. This contention however has to be further investigated.

T2DM patients more vulnerable to stress-related disorders such as anxiety have been associated with poor glycemic control (Lloyd et al., 2005; Fisher et al., 2009). Diabetic patients with anxiety disorders have shown much pronounced hyperglycemia (Anderson et al., 2002). Thus, it is assumed that successful treatment of anxiety may improve glycemic control in T2DM subjects. In the present study, co-occurring T2DM and CRS condition exhibited anxiety-like symptoms on D-7 of the streptozotocine injection in the EPM test paradigm and this effect was sustained upto D-13 of the experimental schedule. The anxiety-like behavior is well documented in diabetes (Hilakivi-Clarke et al., 1990). However, this is the first study where T2DM rats exposed to repeated stress paradigm showed anxiety-like behaviour. Metformin and diazepam treatment for seven consecutive days attenuated the anxiety-like symptoms in experimental rats exposed to both T2DM and repeated CRS paradigm. Metformin in presence of diazepam caused remarkable reduction in the anxiety-like symptoms in experimental animals exposed to both T2DM and repeated CRS paradigm. The results indicate better anxiolytic-like effect of the combination over either metformin or diazepam monotherapy in these conditions in addition to anti-diabetic activity.

The combined results of OGTT and ITT demonstrate that there is impairment in the insulin signaling pathway in the co-occurring T2DM and stress exposed animals. Both metformin and its combination with diazepam therapy showed improvement in the insulin signaling pathway in the above condition. To elaborate the insulin resistance, we evaluated the extent of phosphorylation of IRS-1^{ser307} and Akt^{ser473} in the liver. It has already been documented that there is reduction in the phosphorylation of IRS-1^{ser307} and Akt^{ser473} in the liver of either

T2DM or neurodegeneration (Hotamisligil et al., 1996; Pessin and Saltiel, 2000; Saltiel and Kahn, 2001; Saltiel and Pessin, 2002; Vecina et al., 2014). In the present study, we for the first time report that the phosphorylation of IRS-1^{ser307} and Akt^{ser473} was reduced in the co-occurring condition of T2DM and stress. Further, the combination of metformin and diazepam improved the insulin resistance in part by increasing the phosphorylation of IRS-1^{ser307} and Akt^{ser473} in the liver of the co-occurring T2DM and stress exposed animals.

Mitochondria function and integrity is impaired in both T2DM and stress and also in the co-occurring condition of T2DM and stress in brain tissues (Garabadu and Krishnamurthy, 2013; Cardoso et al., 2013). Similar to earlier observations, the present study revealed that the co-occurring T2DM and stress condition cause hyperactivity of the mitochondrial SDH enzyme and loss in the mitochondrial integrity in all the brain regions (Garabadu and Krishnamurthy, 2013; Cardoso et al., 2013). Further, metformin and diazepam reversed the mitochondrial SDH hyperactivity and loss in integrity in all the brain regions in the T2DM rats also exposed to repeated stress paradigm. It has been reported that metformin restores the brain mitochondrial function in diabetic condition (El-Mir et al., 2008; Pintana et al., 2012). However, this is the first report on effect of metformin in repeated stress exposed diabetic rats. In addition, metformin and diazepam combination further attenuated the mitochondrial SDH hyperactivity and loss in integrity in all the brain regions in co-occurring T2DM and stress condition. Diazepam has been shown to modulate mitochondrial activity in the brain tissues (Sarnowska et al., 2009). Thus, the combination therapy of metformin and diazepam may have potential mitochondrial-dependent activity in addition to glucocorticoid-mediated mechanism in this condition.

The relationship between lipid oxidative damage and mitochondrial function and integrity is well established in T2DM, stress and their co-occurring condition (Fukui et al., 2001; Rasbach

and Schnellmann, 2007; Garabadu and Krishnamurthy, 2013). Similar to earlier findings, the present study reveals that the extent of LPO was higher in the co-occurring condition of T2DM and stress in all the brain regions (Fukui et al., 2001; Rasbach and Schnellmann, 2007; Garabadu and Krishnamurthy, 2013). Metformin and diazepam mitigated the extent of LPO in all the brain regions in T2DM rats also exposed to repeated stress. It has been reported that metformin decreases diabetes-induced increase in LPO in the brain regions (Bhutada et al., 2011). However, this is the first time we report the effect of metformin and diazepam on the extent of LPO in different brain regions in co-occurring T2DM and stress paradigm. The combination of metformin and diazepam attenuated the increase in the extent of LPO in all the brain regions in T2DM rats subjected to repeated CRS paradigm. Moreover, reports also suggest that there is attenuation in the antioxidant defense system in the brain tissues in T2DM, stress and their co-occurring condition (Menabde et al., 2011; Garabadu and Krishnamurthy, 2013). In the present study, the combination of metformin and diazepam mitigated the decrease in the anti-oxidant enzyme activities such as SOD and CAT in all the brain regions in T2DM rats subjected to repeated stress than either monotherapy. Hence, it can be assumed that by improving the antioxidant defense system the combination of metformin and diazepam restores mitochondrial function and integrity, and attenuates mitochondrial oxidative damage in all the brain regions.

In conclusion, metformin exhibited anti-diabetic effect in addition to anti-stress and anxiolytic-like activity in the co-occurring condition of T2DM and stress. Further, metformin along with diazepam showed pronounced anti-diabetic, anti-stress and anxiolytic-like effects compared to metformin monotherapy in the above condition. This indicates synergistic effect of the combination. Further, the combination improved glucose tolerance, and, insulin sensitivity and resistance in the above condition. The combined regimen improved the mitochondrial

function, integrity and oxidative stress in all the brain regions in the co-occurring condition of T2DM and stress. Thus, metformin in combination with diazepam may be a better therapeutic candidate in the pharmacotherapy of co-occurring condition of T2DM and stress.