4 Chapter 4: Metformin and ascorbic acid combination therapy: a potential strategy against diabetes comorbid depression in rats

4.1 Introduction

The pathophysiology of depression in diabetic patient is multifactorial, which includes functional insulin-resistance, inflammation, oxidative stress, decreased activity of norepinephrine (NE) and serotonin (5-HT), and decreased brain-derived neurotrophic factor (Kai et al., 2000, Musselman et al., 2003, Winokur et al., 1988, Lustman and Clouse, 2005, Krabbe et al., 2007). The comorbid depression increases both micro- and macro-vascular complications, the major cause of multi-organ damage and mortality (Semenkovich et al., 2015, Gispen and Biessels, 2000). Previous preclinical studies evidenced an increased risk of development of depression due to decreased functional activity of central neurotransmitters, such as NE and 5-HT in diabetic rats (Trulson and Himmel, 1985, Haider et al., 2013, Arafa et al., 2016). Inflammation and immune activation have been implicated in the pathogenesis of both diabetes and depression (Dantzer et al., 2008, Miller and Raison, 2016). It has been suggested that proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 have the potential to interact with insulin sensitivity and pancreatic β-cell function and induce diabetes (Stuart and Baune, 2012). Depression is associated with overactivation of HPA-axis and overproduction of glucocorticoids leading to aberrant glucose homeostasis (Stuart and Baune, 2012). A recent cross-sectional study has indicated a relationship between inflammation and depression in newly diagnosed diabetic individuals (Herder et al., 2017). Furthermore, vitamin C (ascorbic acid) deficiency (Park et al., 2017) and stressful events (Lloyd et al., 2005) contribute to the development of depressive disorders. Despite vast improvement in our understanding

on diabetes comorbid depression, there is an unmet need to develop therapeutic strategies to treat both diabetes mellitus and comorbid depression.

Earlier studies have reported a lower circulating ascorbic acid levels in patients with diabetes mellitus (Shim et al., 2010, Will and Byers, 1996, Takahashi et al., 2011, Sargeant et al., 2000, Donin et al., 2016). Ascorbic acid supplementation has been shown to produce antidiabetic activity (Afkhami-Ardekani and Shojaoddiny-Ardekani, 2007, Dakhale et al., 2011) and antidepressant activity (Binfaré et al., 2009, Iwata et al., 2014). On the other hand, metformin is a potent oral hypoglycemic agent now recommended as the first-line therapy for diabetes mellitus (type 2) (Viollet et al., 2012). In addition to its hypoglycemic activity metformin has been shown to elicit marked antioxidant activity (Gallo et al., 2005, Nakhjavani et al., 2011, Hou et al., 2010, Esteghamati et al., 2013, Ashabi et al., 2015), neuroprotective activity (Adedeji et al., 2014, Nath et al., 2009), and antiepileptic activity (Zhao et al., 2014). The pleiotropic pharmacological activities of metformin and ascorbic acid makes them suitable for the treatment of diabetes mellitus and comorbid depression, which involves a myriad of pathophysiological characteristics. However, till date, no studies have been conducted to assess the therapeutic potential of metformin and ascorbic acid combination treatment against diabetes mellitus and comorbid depression in rat.

Considering all the pathophysiological factors, it can be hypothesized that a combination strategy, which can abrogate hyperglycemia, inflammation, oxidative stress, and imbalance in neurotransmitter levels, would be a possible option for treating diabetes mellitus and comorbid depression. In the present study, we explored the potential benefits of metformin (25 mg/kg, p.o.) and ascorbic acid (25 mg/kg, p.o.)

combination treatment in a rat model of diabetes mellitus and comorbid depression that primarily focuses on a clinical situation where occurrence of diabetes mellitus leads to depression. Experiments were designed to investigate the effects of combination treatment on the markers of depression (immobility period in forced swim test, plasma corticosterone levels, and adrenal hyperplasia), markers of diabetes mellitus (plasma glucose and insulin levels), brain monoamines (levels of NE and 5-HT in the brain), oxidative stress (lipid peroxidation, superoxide dismutase, and catalase activity in the brain), and inflammatory processes (levels of proinflammatory cytokines TNF-α and IL-6 in the brain).

4.2 Materials and methods

4.2.1 Induction of type 2 diabetes and comorbid depressive-like behavior

We used streptozotocin (dissolved in 0.1 M citrate buffer, pH 4.5) and nicotinamide (dissolved in normal saline) to induce type 2 diabetes mellitus as described previously (see section 2.2.3). The comorbid depressive-like behavior in diabetic rats was induced as described previously in this thesis (see section 2.2.4).

4.2.2 Experimental design

Diabetic rats were randomly allocated into four groups, namely, diabetes comorbid depression (DCD) control, DCD + MET, DCD + AA, and DCD + MET + AA to receive vehicle (1 mL/kg, p.o.), metformin (25 mg/kg, p.o.), ascorbic acid (25 mg/kg, p.o.), or the combination of metformin (25 mg/kg, p.o.) and ascorbic acid (25 mg/kg, p.o.) respectively, for 11 consecutive days. The nondiabetic control group received only distilled water (1 mL/kg, p.o.).

Table 3: Experimental design of combination therapy of metformin and ascorbic acid against diabetes comorbid depression in rats

Group	Glycemic Status	Treatment (p.o.) (Day 1 to Day 11)	Comorbid Depression (Day 1, 5, 7, and 10)	N
Nondiabetic control	Nondiabetic	Distilled water (1 mL/kg)	No intermittent foot-shock	6
DCD control	Diabetic	Distilled water (1 mL/kg)	Intermittent foot-shock	6
DCD	Diabetic	Metformin (25 mg/kg)		6
		Ascorbic acid (25 mg/kg)	Intermittent foot-shock	6
		Metformin (25 mg/kg) and ascorbic acid (25 mg/kg)		6

DCD: Diabetes comorbid depression; N: Number of rats in a group

The doses and duration of metformin and ascorbic acid treatment were based on our dose-response studies that tested the efficacy of metformin and ascorbic acid individually against diabetes and comorbid depression in rats. Metformin (25 mg/mL) and ascorbic acid (25 mg/mL) solutions were freshly prepared in distilled water. The vehicle or drug treatments were initiated along with induction of comorbid depression after the confirmation of diabetes mellitus i.e. 72 h after streptozotocin injection. The timeline of the tasks executed during the experimentation has been represented in Figure 4.1.

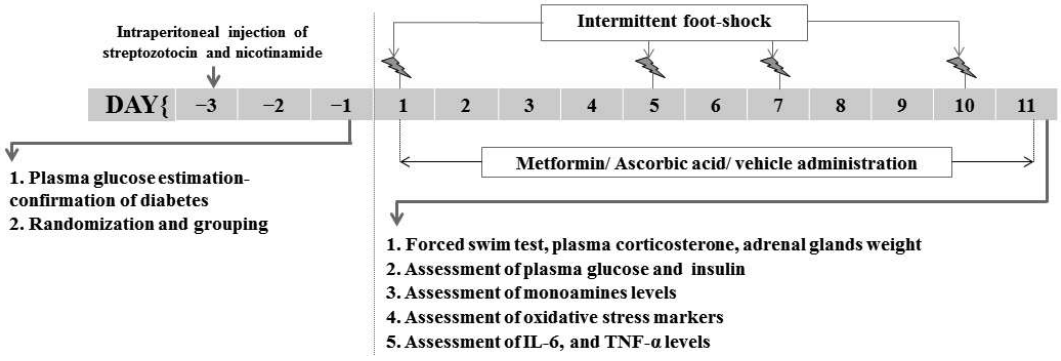

Figure 4.1: Schematic representation of experimental protocol.

4.2.3 Forced swim test

On day 11th, rats were exposed to forced swim test to recorded immobility period as described previously (see section 2.2.6).

4.2.4 Collection of blood, brain, and adrenal glands

After performing forced swim test, blood samples and prefrontal cortex of all animals were collected and processed as described previously (see section 2.2.7).

4.2.5 Estimation of plasma glucose and insulin

Plasma glucose levels were estimated using glucose oxidase peroxidase enzyme kit (Span Diagnostic, India) and an iMark microplate reader (Bio-Rad Laboratories, USA). The absorbance of standard and test samples were measured at 505 nm against the blank and total glucose concentrations (mg/dL) were calculated by dividing the test sample absorbance by standard absorbance multiplied by 100. Plasma insulin levels were estimated using a rat insulin ELISA kit (DRG Instruments GmbH, Germany) and the iMark microplate reader at 450 nm. The plasma insulin concentrations were expressed as μIU/mL.

4.2.6 Estimation of plasma corticosterone

We have measured plasma corticosterone as the surrogate biomarker to assess the comorbid depression (Nade et al., 2009, Bhattacharya et al., 2000, Kenjale et al., 2007, Park et al., 2016). Plasma corticosterone levels were measured using rat corticosterone ELISA kit (DSI S.r.l., Italy) and the iMark microplate reader at 450 nm. The concentrations of corticosterone in test samples were calculated from the standard curve and expressed as ng/mL.

4.2.7 Preparation of brain homogenate

The prefrontal cortex was dissected out using the method described previously (Spijker, 2011) and a 10% (w/v) homogenate was prepared in ice cold phosphate buffer (20 mM, pH 7.4) in a glass homogeniser. The homogenate was centrifuged at 12000×g for 45 min at 4 °C, clear supernatant was collected, and stored at −80 °C until assayed for monoamine levels, oxidative stress, and pro-inflammatory cytokine levels.

4.2.8 Estimation of monoamines in the prefrontal cortex

The monoamines such as NE and 5-HT were estimated using commercially available ELISA kit (Labor Diagnostika Nord GmbH & Co, Germany) and the iMark microplate reader at 450 nm. Assays were carried out in accordance with the manufacturer's instructions.

4.2.9 Estimation of oxidative stress in the prefrontal cortex

Lipid peroxidation (LPO), superoxide dismutase (SOD) content, and catalase activity in prefrontal cortex were quantified as a markers of oxidative stress described previously (see section 2.2.10).

4.2.10 Estimation of proinflammatory cytokines in the prefrontal cortex

The proinflammatory cytokines such as TNF-α and IL-6 in the tissue were measured using specific ELISA kits (Ray Biotech, USA) and the iMark microplate reader at 450 nm. All the assays were conducted according to the manufacturer's protocols. The concentrations of TNF-α and IL-6 in the tissue were expressed in pg/g of tissue.

4.2.11 Statistical analysis

Results were reported as mean ± SEM (n = 6). Statistical analysis was performed using GraphPad Prism version 7.03 for Windows (GraphPad Software Inc., USA). All the data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A value of $P < 0.05$ was set as statistically significant.

4.3 Results

4.3.1 Effects of combination therapy on comorbid depression

The existence of comorbid depression was assessed on day 11 by estimating immobility period (Figure 4.2A) through forced swim test, plasma corticosterone levels (Figure 4.2B), and adrenal hyperplasia (Figure 4.2C) in diabetic rats. The rats with diabetes and comorbid depression showed significantly ($P < 0.05$) higher immobility periods (140.3 ± 4.4 versus 41.2 ± 5.2 sec; Figure 4.2A) compared with nondiabetic controls. Monotherapy of both metformin (92.7 ± 4.5 versus 140.3 ± 4.4 sec; Figure 4.2A) and ascorbic acid (109.2 ± 5.7 versus 140.3 ± 4.4 sec; Figure 4.2A) at 25 mg/kg, p.o. showed significant ($P < 0.05$) decrease in immobility period as compared to the DCD control rats. However, the decrease in immobility period was significantly ($P < 0.05$) higher in metformin monotherapy group compared with ascorbic acid monotherapy group (92.7 ± 4.5 versus 109.2 ± 5.7 sec; Figure 4.2A). The combination (metformin and ascorbic acid at 25 mg/kg, p.o.) therapy showed an additive synergistic effect, with significant decrease ($P < 0.05$) in immobility period (60.8 ± 4.4 versus 140.3 ± 4.4 sec; Figure 4.2A) compared with DCD control rats,

metformin monotherapy (60.8 ± 4.4 versus 92.7 ± 4.5 sec; Figure 4.2A) and ascorbic acid monotherapy (109.2 ± 5.7 versus 140.3 ± 4.4 sec; Figure 4.2A).

Assessment of plasma corticosterone showed significantly ($P < 0.05$) higher levels in rats with diabetes and comorbid depression compared with nondiabetic controls (135.4 ± 3.1 versus 65.4 ± 2.4 ng/mL; Figure 4.2B). Monotherapy with metformin (119.0 ± 2.6 versus 135.4 ± 3.1 ng/mL; Figure 4.2B) or ascorbic acid (122.8 ± 1.9 versus 135.4 ± 3.1 ng/mL; Figure 4.2B) showed a significant ($P < 0.05$) decrease in plasma corticosterone as compared to the DCD control rats. The reduction in plasma corticosterone levels was slightly higher in metformin only treated rats as compared to ascorbic acid only treatment (119.0 ± 2.6 versus 122.8 ± 1.9 ng/mL; Figure 4.2B). In line with results of forced swim test, the combination therapy produced an additive synergistic effect, with significant reductions in plasma corticosterone levels compared with DCD controls (111.1 ± 2.6 versus 135.4 ± 3.1 ng/mL; Figure 4.2B), metformin monotherapy (111.1 ± 2.6 versus 119.0 ± 2.6 ng/mL; Figure 4.2B), and ascorbic acid monotherapy (111.1 ± 2.6 versus 122.8 ± 1.9 ng/mL; Figure 4.2B).

In accordance with increase in corticosterone levels, a significant ($P < 0.05$) adrenal hyperplasia was observed in rats with diabetes and comorbid depression compared with nondiabetic control rats (adrenal gland weight 70.4 ± 3.4 versus 44.3 ± 5.8 mg; Figure 4.2C). A significant ($P < 0.05$) reduction in adrenal hyperplasia was observed with both metformin (adrenal gland weight 58.7 ± 2.6 versus 70.4 ± 3.4 mg; Figure 4.2C) and ascorbic acid (adrenal gland weight 59.6 ± 2.1 versus 70.4 ± 3.4 mg; Figure 4.2C) monotherapy relative to the DCD control rats. Monotherapy of

metformin and ascorbic acid were equally effective in reducing adrenal hyperplasia (adrenal gland weight 58.7 ± 2.6 versus 59.6 ± 2.1 mg; $P > 0.05$; Figure 4.2C). The combination therapy showed significant ($P < 0.05$) reduction in adrenal hyperplasia when compared with DCD control rats (adrenal gland weight 53.9 ± 2.2 versus 70.4 ± 3.4 mg; Figure 4.2C). However, the effect observed with combination therapy was statistically insignificant ($P > 0.05$) when compared with metformin monotherapy (adrenal gland weight 53.9 ± 2.2 versus 58.7 ± 2.6 mg; Figure 4.2C) and ascorbic acid monotherapy (adrenal gland weight 53.9 ± 2.2 versus 59.6 ± 2.1 mg; Figure 4.2C).

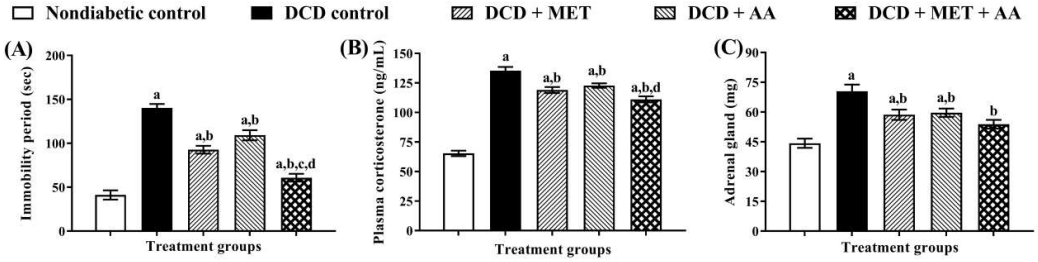

Figure 4.2: Effects of combination therapy on comorbid depression

Effects of metformin, ascorbic acid, or their combination therapy on markers of depression in rats with diabetes and comorbid depression. Immobility period in forced swim test (A), plasma corticosterone levels (B) and adrenal gland weights (C) after 11 days of drug treatment. Data represent mean ± SEM, n = 6. $^{a}P < 0.05$ compared to nondiabetic control, $^{b}P < 0.05$ compared to DCD control, $^{c}P < 0.05$ compared to DCD + MET, and $^{d}P < 0.05$ compared to DCD + AA. DCD- Diabetes with comorbid depression; MET- Metformin (25 mg/kg, p.o.); AA- Ascorbic acid (25 mg/kg, p.o.).

4.3.2 Effects of combination therapy on hyperglycemia and hypoinsulinemia

Plasma glucose and plasma insulin levels were estimated to assess the antidiabetic efficacy of the combination therapy (metformin and ascorbic acid at 25 mg/kg, p.o.) and monotherapy of both metformin (25 mg/kg, p.o.) and ascorbic acid (25 mg/kg, p.o.) after 11 days of administration. The rats with diabetes and comorbid depression exhibited significant ($P < 0.05$) hyperglycemia (279.5 ± 5.9 versus 93.0 ± 3.6 mg/dL; Figure 4.3A) relative to the nondiabetic control rats. Significant ($P < 0.05$)

reductions in plasma glucose (140.6 ± 5.2 versus 279.5 ± 5.9 mg/dL; Figure 4.3A) were observed in both metformin (156.3 ± 6.7 versus 279.5 ± 5.9 mg/dL; Figure 4.3A) and ascorbic acid (236.3 ± 4.9 versus 279.5 ± 5.9 mg/dL; Figure 4.3A) monotherapy group compared with DCD controls. Metformin only treated rats showed significant ($P < 0.05$) decrease in plasma glucose as compared to ascorbic acid only treatment (156.3 ± 6.7 versus 236.3 ± 4.9 mg/dL; Figure 4.3A). Moreover, the combination treatment showed a significant ($P < 0.05$) decrease in the levels of plasma glucose compared with DCD control group (140.6 ± 5.2 versus 279.5 ± 5.9 mg/dL; Figure 4.3A) and ascorbic acid monotherapy (156.3 ± 6.7 versus 236.3 ± 4.9 mg/dL; Figure 4.3A).

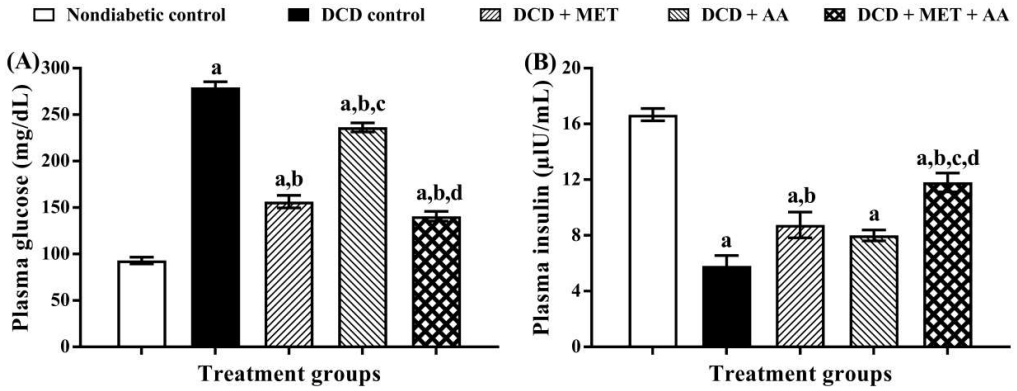

Figure 4.3: Effects of combination therapy on hyperglycemia and hypoinsulinemia

Effects of metformin, ascorbic acid, or their combination therapy on plasma glucose (A) and insulin levels (B) in rats with diabetes and comorbid depression. Data represent mean ± SEM, n = 6. $^{a}P < 0.05$ compared to nondiabetic control, $^{b}P < 0.05$ compared to DCD control, $^{c}P < 0.05$ compared to DCD + MET, and $^{d}P < 0.05$ compared to DCD + AA. DCD- Diabetes with comorbid depression; MET- Metformin (25 mg/kg, p.o.); AA- Ascorbic acid (25 mg/kg, p.o.).

On the other hand, the plasma insulin levels were significantly ($P < 0.05$) reduced in rats with diabetes and comorbid depression relative to the nondiabetic control rats (5.8 ± 0.7 versus 16.7 ± 0.4 µIU/mL; Figure 4.3B). Metformin

monotherapy caused a significant ($P < 0.05$) increase in plasma insulin levels relative to DCD control rats (8.7 ± 0.9 versus 5.8 ± 0.7 μIU/mL; Figure 4.3B) whereas, ascorbic acid monotherapy caused a statistically insignificant ($P > 0.05$) increase in insulin levels compared with DCD control rats (8.0 ± 0.4 versus 5.8 ± 0.7 μIU/mL; Figure 4.3B). The combination therapy caused a significant increase in plasma insulin levels compared with DCD control rats (11.8 ± 0.7 versus 5.8 ± 0.7 μIU/mL; Figure 4.3B), metformin monotherapy (11.8 ± 0.7 versus 8.7 ± 0.9 μIU/mL; Figure 4.3B) and ascorbic acid monotherapy (11.8 ± 0.7 versus 8.0 ± 0.4 μIU/mL; Figure 4.3B).

4.3.3 Effects of combination therapy on brain monoamine levels

The alterations in levels of brain monoamines are the pathophysiological hallmark of depression. We estimated the levels of NE and 5-HT in prefrontal cortex, which is one of the major brain structures implicated in depression. Animals with diabetes and comorbid depression exhibited significant ($P < 0.05$) decrease in NE (264.7 ± 16.4 versus 510.3 ± 10.2 ng/g tissue; Figure 4.4A) and 5-HT (183.6 ± 8.0 versus 355.0 ± 8.4 ng/g tissue; Figure 4.4B) compared with nondiabetic controls. Metformin monotherapy caused significantly ($P < 0.05$) increase in NE levels relative to the DCD control rats (347.0 ± 11.9 versus 264.7 ± 16.4 ng/g tissue; Figure 4.4A) whereas, ascorbic acid monotherapy showed statistically insignificant ($P > 0.05$) increase in NE levels as compared to the DCD control rats (308.8 ± 16.3 versus 264.7 ± 16.4 ng/g tissue; Figure 4.4A). In contrast, rats administered with the combination showed significantly ($P < 0.05$) higher levels of NE compared with DCD control rats (383.6 ± 15.4 versus 264.7 ± 16.4 ng/g tissue; Figure 4.4A) and ascorbic acid monotherapy (383.6 ± 15.4 versus 308.8 ± 16.3 ng/g tissue; Figure 4.4A).

In addition to its effect on NE levels, metformin monotherapy caused significant ($P < 0.05$) increase in 5-HT levels compared with DCD control rats (226.6 ± 10.2 versus 183.6 ± 8.0 ng/g tissue; Figure 4.4B) whereas, ascorbic acid monotherapy showed a statistically insignificant ($P > 0.05$) increase compared to the DCD control rats (220.3± 12.9 versus 183.6 ± 8.0 ng/g tissue; Figure 4.4B). The combination therapy showed significantly ($P < 0.05$) higher levels of 5-HT as compared to DCD control rats (271.2 ± 10.3 versus 183.6 ± 8.0 ng/g tissue; Figure 4.4), metformin monotherapy (271.2 ± 10.3 versus 226.6± 10.2 ng/g tissue;Figure 4.4) and ascorbic acid monotherapy (271.2 ± 10.3 versus 220.3 ± 12.9 ng/g tissue; Figure 4.4B).

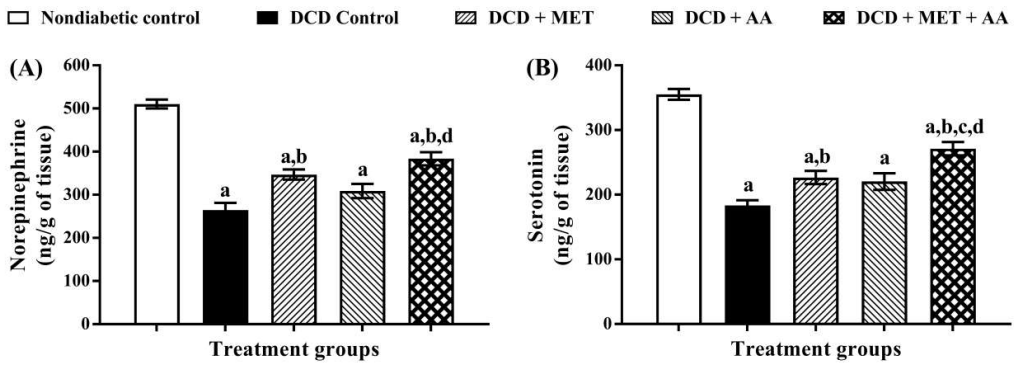

Figure 4.4: Effects of combination therapy on brain monoamine levels

Effects of metformin, ascorbic acid, or their combination therapy on monoamine levels in prefrontal cortex of rats with diabetes and comorbid depression. Norepinephrine levels (A) and serotonin levels (B) after 11 days of drug treatment. Data represent mean ± SEM, n = 6. $^{a}P < 0.05$ compared to nondiabetic control, $^{b}P < 0.05$ compared to DCD control, $^{c}P < 0.05$ compared to DCD + MET, and $^{d}P < 0.05$ compared to DCD + AA. DCD- Diabetes with comorbid depression; MET- Metformin (25 mg/kg, p.o.); AA- Ascorbic acid (25 mg/kg, p.o.).

4.3.4 Effects of combination therapy on oxidative stress

Oxidative stress in the brain, due to diabetes and comorbid depression, was assessed by estimating lipid peroxidation, superoxide dismutase content, and catalase activity in the prefrontal cortex. In comparison to the nondiabetic control rats, DCD control rats showed significantly ($P < 0.05$) higher LPO, which was evidenced by

higher malondialdehyde content (18.1 ± 0.9 versus 7.1 ± 0.4 nmol MDA/mg protein; Figure 4.5A). Ascorbic acid monotherapy showed significantly ($P < 0.05$) decrease in LPO compared with the DCD control rats (11.4 ± 1.0 versus 18.1 ± 0.9 nmol MDA/mg protein; Figure 4.5A) whereas, metformin monotherapy showed a statistically insignificant ($P > 0.05$) decrease relative to the DCD control rats (15.4 ± 1.1 versus 18.1 ± 0.9 nmol MDA/mg protein; Figure 4.5A). In contrast, the combination therapy showed significantly ($P < 0.05$) lower LPO compared with DCD control rats (10.2 ± 0.4 versus 18.1 ± 0.9 nmol MDA/mg protein; Figure 4.5A) and metformin monotherapy (10.2 ± 0.4 versus 15.4 ± 1.1 nmol MDA/mg protein; Figure 4.5A).

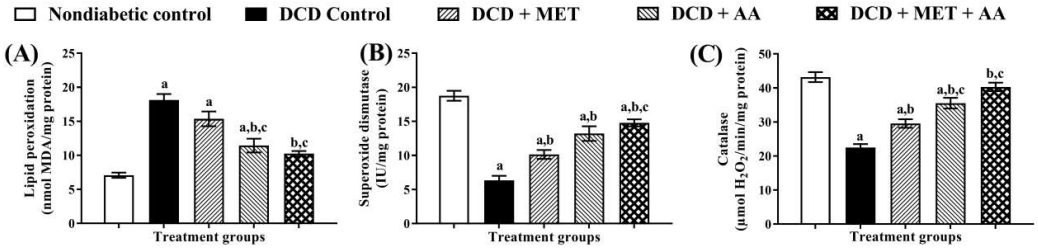

Figure 4.5: Effects of combination therapy on oxidative stress

Effects of metformin, ascorbic acid, or their combination therapy on markers of oxidative stress in prefrontal cortex of rats with diabetes and comorbid depression. Lipid peroxidation (nmol MDA/mg protein) (A), superoxide dismutase (IU/mg protein) (B) and catalase (µmol H_2O_2/min/mg protein) (C) after 11 days of drug treatment. Data represent mean ± SEM, n = 6. $^{a}P < 0.05$ compared to nondiabetic control, $^{b}P < 0.05$ compared to DCD control, $^{c}P < 0.05$ compared to DCD + MET, and $^{d}P < 0.05$ compared to DCD + AA. DCD- Diabetes with comorbid depression; MET- Metformin (25 mg/kg, p.o.); AA- Ascorbic acid (25 mg/kg, p.o.).

On the other hand, SOD content (6.3 ± 0.7 versus 18.8 ± 0.7 IU/mg protein; Figure 4.5B) and CAT activity (22.5 ± 1.1 versus 43.2 ± 1.5 µmol H_2O_2/min/mg protein; Figure 4.5C) was significantly ($P < 0.05$) decreased in DCD control group compared with nondiabetic control group. Treatment with metformin (10.2 ± 0.7 versus 6.3 ± 0.7 IU/mg protein; Figure 4.5B) or ascorbic acid (13.2 ± 1.1 versus 6.3 ±

0.7 IU/mg protein; Figure 4.5B) alone, a significant ($P < 0.05$) increase in SOD content was observed compared with the DCD control rats. SOD content was slightly higher in ascorbic acid monotherapy compared with the metformin monotherapy (13.2 ± 1.1 versus 10.2 ± 0.7 IU/mg protein; $P > 0.05$; Figure 4.5B). The combination therapy showed significantly ($P < 0.05$) higher levels of SOD content compared with the DCD control rats (14.8 ± 0.5 versus 6.3 ± 0.7 IU/mg protein; Figure 4.5B) and metformin monotherapy (14.8 ± 0.5 versus 10.2 ± 0.7 IU/mg protein; Figure 4.5B). In addition, monotherapy of both metformin (29.6 ± 1.2 versus 22.5 ± 1.1 µmol H_2O_2/min/mg protein; Figure 4.5C) and ascorbic acid (35.5 ± 1.5 versus 22.5 ± 1.1 µmol H_2O_2/min/mg protein; Figure 4.5C) showed significant ($P < 0.05$) increase in CAT activity as compared to the DCD control rats. However, monotherapy of ascorbic acid showed significant ($P < 0.05$) increase in CAT activity as compared to the metformin monotherapy (35.5 ± 1.5 versus 29.6 ± 1.2 µmol H_2O_2/min/mg protein; Figure 4.5C). In contrast, the combination therapy showed significantly ($P < 0.05$) higher levels of CAT activity as compared to the DCD control rats (40.3 ± 1.2 versus 22.5 ± 1.1 µmol H_2O_2/min/mg protein; Figure 4.5C) and metformin monotherapy (40.3 ± 1.2 versus 29.6 ± 1.2 µmol H_2O_2/min/mg protein; Figure 4.5C).

4.3.5 Effects of combination therapy on proinflammatory cytokine levels

Diabetes and depression have been implicated in the activation of inflammatory processes with increase in proinflammatory cytokines in the brain. We evaluated the effect of the combination therapy (metformin and ascorbic acid at 25 mg/kg, p.o.) and monotherapy of both metformin (25 mg/kg, p.o.) and ascorbic acid (25 mg/kg, p.o.) on levels of TNF-α and IL-6 in the prefrontal cortex. Significantly ($P < 0.05$) higher levels of TNF-α (295.6 ± 17.9 versus 121.6 ± 10.5 pg/g tissue; Figure 4.6A) and IL-6

(58.2 ± 2.0 versus 10.5 ± 1.6 pg/g tissue; Figure 4.6B) were observed in DCD control rats as compared to nondiabetic control rats. Monotherapy of both metformin (177.9 ± 12.5 versus 295.6 ± 17.9 pg/g tissue; Figure 4.6A) and ascorbic acid (228.9 ± 17.8 versus 295.6 ± 17.9 pg/g tissue; Figure 4.6A) exhibited significantly ($P < 0.05$) lower levels of TNF-α relative to the DCD control rats. However, metformin monotherapy showed lower levels of TNF-α as compared to ascorbic acid monotherapy (177.9 ± 12.5 versus 228.9 ± 17.8 pg/g tissue, Figure 4.6A). Treatment with the combination showed significantly ($P < 0.05$) lower levels of TNF-α compared with DCD control rats (136.8 ± 11.9 versus 295.6 ± 17.9 pg/g tissue; Figure 4.6A) and ascorbic acid monotherapy (136.8 ± 11.9 versus 228.9 ± 17.8 pg/g tissue; Figure 4.6A).

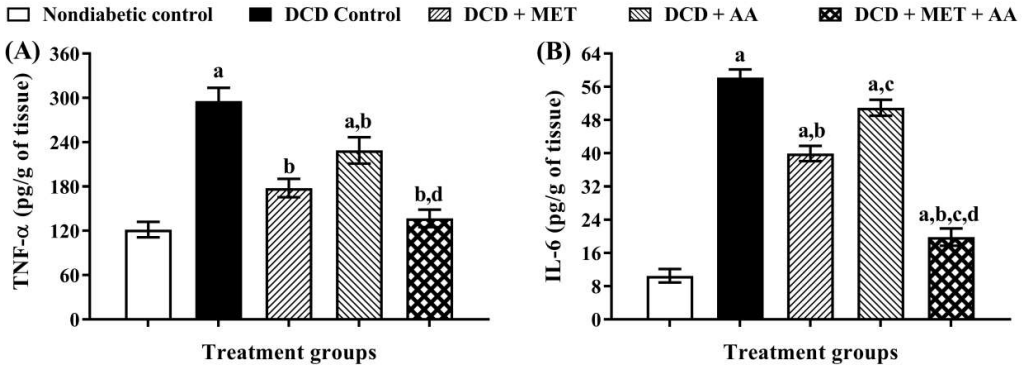

Figure 4.6: Effects of combination therapy on proinflammatory cytokine levels

Effects of metformin, ascorbic acid, or their combination therapy on proinflammatory cytokines in rats with diabetes and comorbid depression. TNF-*α* (A) and IL-6 (B) concentrations in prefrontal cortex after 11 days of drug treatment. Data represent mean ± SEM, n = 6. $^{a}P < 0.05$ compared to nondiabetic control, $^{b}P < 0.05$ compared to DCD control, $^{c}P < 0.05$ compared to DCD + MET, and $^{d}P < 0.05$ compared to DCD + AA. DCD- Diabetes with comorbid depression; MET- Metformin (25 mg/kg, p.o.); AA- Ascorbic acid (25 mg/kg, p.o.).

Metformin monotherapy exhibited significantly ($P < 0.05$) lower levels of IL-6 relative to the DCD control rats (39.9 ± 1.8 versus 58.2 ± 2.0 pg/g tissue, Figure 4.6B) whereas, ascorbic acid monotherapy showed statistically insignificant ($P > 0.05$) decrease as compared to DCD control rats (51.0 ± 1.9 versus 58.2 ± 2.0 pg/g tissue;

Figure 4.6B). In contrast, the combination therapy showed significantly ($P < 0.05$) lower levels of IL-6 compared with DCD control rats (19.8 ± 2.1 versus 58.2 ± 2.0 pg/g tissue; Figure 4.6B), metformin monotherapy (19.8 ± 2.1 versus 39.9 ± 1.8 pg/g tissue; Figure 4.6B), and ascorbic acid monotherapy (19.8 ± 2.1 versus 51.0 ± 1.9 pg/g tissue; Figure 4.6B).

4.4 Discussion

Depression in diabetic patient is a major cause of poor self-care (lower physical activity, unhealthy diet, and lower adherence to medication) that further increases the risk of complications due to diabetes and other metabolic disorders (Lin et al., 2004). So far, tremendous advancement have been made in the diagnosis and treatment strategies of diabetes, but depression in diabetic patients remains underdiagnosed and undertreated (Katon, 2008, Bădescu et al., 2016, Petrak et al., 2015, Petrak and Röhrig, 2018). For the first time, the present study investigated the protective effects of metformin and ascorbic acid combination treatment against diabetes and comorbid depression in rats. Considering the fact that depressive-like behavior was induced in the diabetic rats, the findings of the present study primarily focus on depression in patients who already have diabetes but not vice versa. The results demonstrated that the combination treatment reduces depressive behavior and provides better control over diabetes through modifications in markers of depression, diabetes, oxidative stress, and inflammation. Interestingly, the combination treatment, metformin and ascorbic acid ameliorated diabetes mellitus and comorbid depression in rats at doses approximately $1/4^{th}$ of their human equivalent doses effectively reduced blood glucose in humans (Dakhale et al., 2011), possibly due to differences in species,

treatment duration, and model-specific pharmacological synergism produced by metformin and ascorbic acid.

Depression is manifested by several symptoms such as despair, anhedonia, and cognitive impairment (Guo et al., 2014, Nestler et al., 2002). Forced swim test is one of the important drug screening test in which the immobility period is measured as a marker of depressive-like behavior (Ning et al., 2017). In the present study, a significant increase in the immobility period reflected the severity of depressive-like behavior in diabetic rats that is in agreement with earlier studies (van Donkelaar et al., 2014, Sharma et al., 2010, Yankelevitch-Yahav et al., 2015). Daily oral treatment with ascorbic acid and metformin combination caused a significant reduction in immobility period as compared to monotherapy of both metformin and ascorbic acid, suggesting the protective effect of combination treatment against depressive-like behavior. The present results support the earlier findings that metformin and ascorbic acid, when administered alone, produce antidepressant activity in humans (Guo et al., 2014) and in animal models of depression (Ostadhadi et al., 2015, Binfaré et al., 2009, Iwata et al., 2014, Aswar et al., 2017). A decreased monoamine transmission in the central nervous system has been implicated in the pathogenesis of depression (Krishnan and Nestler, 2008). In the current study, we estimated the monoamine levels in the prefrontal cortex, which is directly involved in clinical depression (George et al., 1994). In agreement with the findings of Miyata and group (Miyata et al., 2007), rats with diabetes and comorbid depression showed significantly low prefrontal cortex monoamine levels due to decreased insulin levels and increased blood glucose. The combination therapy and metformin monotherapy significantly increased the prefrontal cortex monoamine levels similar to the effects observed by

Miyata and group (Miyata et al., 2007) with insulin administration in animals with diabetes and depression. The present findings suggest that reduction in blood glucose and increased plasma insulin levels through antidiabetic effects of metformin and ascorbic acid plays a vital role in reducing depressive-like behavior in forced swim test. Inflammation and oxidative stress in pancreatic islets plays a critical role in the pathogenesis of type 2 diabetes mellitus and beta cell dysfunction (Imai et al., 2016). Administration of both streptozotocin and nicotinamide in rats has been shown to increase oxidative stress and inflammatory cytokines in pancreatic tissue (Palsamy and Subramanian, 2010). Therefore, the increase in plasma insulin levels by metformin or ascorbic acid could be attributed to their protective effects on oxidative stress and inflammation elicited by streptozotocin in the pancreatic beta cells. Interestingly, the combination therapy was highly effective in increasing the plasma insulin levels, possibly due to synergistic effect against oxidative stress and inflammation. As the diabetic patients have a lower circulating level of ascorbic acid, these findings accelerate the hypothesis of co-administration of ascorbic acid with metformin to get better control over hyperglycemia (Adeneye et al., 2007).

Oxidative stress in the brain areas such as prefrontal cortex and hippocampus is a major contributor to the pathophysiology of depression in diabetic subjects (de Morais et al., 2014, Michel et al., 2007). It is reported that oxidative stress in depressive conditions generally lead to reductions in prefrontal cortex size (Michel et al., 2007) and hence may reduce cellular density and neurotransmitter production. Several studies demonstrated that brain membrane lipids are made up of polyunsaturated fatty acids and highly sensitive to oxidation (Bilici et al., 2001, Hall et al., 2016). Lipid peroxidation is regarded as an important biomarker for oxidative

stress, and it has been observed that elevated LPO is associated with increased depressive symptoms (Bilici et al., 2001). In our study, the combination therapy and ascorbic acid monotherapy showed significant reductions in levels of LPO in the prefrontal cortex, whereas rats with diabetes and comorbid depression showed significantly higher levels of LPO. In addition to LPO, a decreased SOD level and CAT activity have been reported in diabetes-associated depression (Maurya et al., 2016). We found that SOD level and CAT activity in prefrontal cortex was lower in rats with diabetes and comorbid depression. In addition, the brain is more susceptible to the effects of reacting oxygen species because of naturally elevated metabolic activities and lower levels of native antioxidant enzyme present (Réus et al., 2016). SOD enzyme converts the superoxide free radicals into H_2O_2 and molecular oxygen, whereas CAT enzyme protects the tissues from highly reactive free hydroxyl radicals via catalyzing the reduction into hydrogen peroxides (Ceretta et al., 2012). The important defense mechanism against disease management is to removal of free radicals O_2^{--} and OH^-. In the present study, the combination therapy and monotherapy of both metformin and ascorbic acid showed an increase in the SOD content and CAT activity in the prefrontal cortex, suggesting enhancement of free radical scavenging is important for cell survival. The overall control over oxidative stress and hyperglycemia by the metformin and ascorbic acid combination might have improved prefrontal cortex health and thereby monoamine transmission.

In accordance with increased immobility/depressive behavior, the plasma corticosterone levels and adrenal gland weights were higher in animals with diabetes and comorbid depression. In the present study, the involvement of two independent stressors, chronic hyperglycemia and intermittent foot-shocks that mimics

environmental stress, contributed in staging a severe depressive condition in rats with diabetes and comorbid depression. It has been reported that chronic stress paradigms in rats generally increase corticosterone secretion, due to increased adrenal corticosterone response to ACTH without affecting sensitivity, along with increased adrenal weight due to hyperplasia in the outer zona fasciculata and hypertrophy of inner zona fasciculata and medulla of adrenal gland (Ulrich-Lai et al., 2006). Similar changes in the morphology and function of the adrenal zona fasciculata of rat were reported in streptozotocin-induced experimental diabetes (Rebuffat et al., 1988). The combination therapy and monotherapy of both metformin and ascorbic acid showed significant reductions in plasma corticosterone levels and adrenal weights, suggesting HPA axis modulatory effects of both metformin and ascorbic acid. In a recent study, it was elucidated that metformin produces rapid antihyperglycemic effect through reductions in ACTH and cortisol secretion via AMP-activated protein kinase/liver X receptor α/pro-opiomelanocortin pathway (Carvalho et al., 2015). On the other hand, ACTH has been shown to increase adrenal vein ascorbic acid concentrations as an integral part of stress response (Padayatty et al., 2007), with a possible intention to reduce the effects of oxidants released during steroidogenesis (Rapoport et al., 1995). ACTH-induced secretion of ascorbic acid in adrenals has also been implicated in the synthesis of norepinephrine (Dhariwal et al., 1989). Taken together, the antidiabetic and antidepressant behavior shown by monotherapy of metformin and ascorbic acid and their combination at low doses could be attributed to their pleiotropic control over HPA axis and monoamine transmission.

In diabetes and comorbid depression, chronic hyperglycemia plays a critical role in immune activation, which is mediated through oxidative stress (Esposito et al.,

2002, Palsamy and Subramanian, 2011, Farooq et al., 2012, Zhou et al., 2017). An increased production of specific proinflammatory cytokines such as TNF-α, IL-6 and IL-10 has been linked to depression and other neurological diseases (Dowlati et al., 2010, Miller et al., 2009). In our study, significantly higher levels of TNF-α and IL-6 were observed in rats with diabetes and comorbid depression, which reconfirmed the role of proinflammatory cytokines in pathogenesis of diabetes and depression. Monotherapy of both metformin and ascorbic acid significantly reduced the levels of inflammatory cytokines in the prefrontal cortex, suggesting that inflammatory pathways in diabetes and comorbid depression are governed by both hyperglycemia and oxidative stress. Further, a synergistic reduction in proinflammatory cytokines by the combination therapy confirmed that optimal control over both hyperglycemia and oxidative stress is required to obtain maximal antiinflammatory effect.

The present study has few limitations that need attention while implicating the results to a specific context. The first limitation is the present study did not examine the bidirectional relationship between diabetes and depression, rather focused on development of depression in already diabetic patients. Another limitation is the absence of a nondiabetic control group that also received the shock makes it difficult to ascertain the individual contribution of streptozotocin-nicotinamide induced diabetes and stress induced by foot-shocks in the severity of depression observed in the forced swim test. Future studies are required to study the combined effects of ineffective doses of metformin and ascorbic acid against diabetes comorbid depression.

4.5 Conclusion

Our finding showed that metformin and ascorbic acid combination therapy produced both antidiabetic and antidepressant activity in rats at doses approximately $1/4^{th}$ of their clinically recommended doses. The combination therapy improved hyperglycemia and depressive-like behavior primarily through synergistic effect on HPA axis and oxidative stress and monoamine transmission. Furthermore, the metformin and ascorbic acid combination therapy abrogated secondary processes such as inflammatory response, frequently observed in patients with diabetes and comorbid depression. Taken together, the current findings suggests that metformin and ascorbic acid combination therapy could be a better treatment regimen in the management of patients with diabetes and comorbid depression. However, further clinical studies are warranted to study the therapeutic potential of metformin and ascorbic acid combination in the designated patient population.