5 Chapter 5: Combination therapy of metformin and ascorbic acid modulates BDNF, caspase, NF-kB, and mitochondrial membrane potential in diabetes comorbid depressed rats

5.1 Introduction

The major source of energy in mammalian brain is glucose (Mergenthaler et al., 2013). It is transported into the brain through GLUT3, which is non-insulin dependent glucose transporter (Mergenthaler et al., 2013). During hyperglycemic condition, increased amount of glucose enters into the brain through GLUT3 transporters (Mergenthaler et al., 2013). In addition to glycolysis pathway, the transported glucose enhances production of sorbitol through polyol pathway and AGE to induce the activation of protein kinase C (PKC), poly ADP ribose polymerase (PARP), and mitogen activated protein kinases/extracellular related kinase (MAPK/ERK) (Yerra et al., 2013). All these pathways contribute to the generation of oxidative free radicals leading to the decreased mitochondrial membrane potential and activation of NF-kB pathway (Yerra et al., 2013). Enhanced NF-kB activation is associated with excessive production of proinflammatory cytokines such as IL-6, TNF-α, and iNOS (Yerra et al., 2013). These proinflammatory cytokines are prerequisite mediators for the initiation and amplification of inflammatory processes in neuronal cells leading to neuroinflammation mediated structural damage to neurons (Yerra et al., 2013). On the other hand, oxidative stress is also responsible for the activation of intrinsic apoptotic pathway. Oxidative stress mediated mitochondrial damage causes the release of cytochrome c which in turn activates caspase 9 (Kudryavtseva et al., 2016). Activated caspase 9 in turn activates caspase 3 which causes apoptosis (Kudryavtseva et al., 2016). Brain derived neurotrophic factor is known as one of the major neurotrophic factors responsible for differentiation, maintenance, and survival of neurons (Dwivedi, 2013). Hyperglycemia mediated neuronal damage leads to decreased

production of BDNF leading to enhanced neuronal death (Bathina and Das, 2015). Degeneration of noradrenergic and seretonergic neurons leads to the development of diabetes induced depression (Markowitz et al., 2011). Ample evidence suggests that low level of BDNF is observed in depressed patients and use of antidepressants reversed these effects (Dwivedi, 2013).

The pathophysiology of diabetes involves increased level of blood glucose and reduced sensitivity of toward insulin. This increase in blood glucose level further increases the sensitivity of HPA axis and increases cortisol level. Hypothalamic-pituitary-adrenal axis dysregulation performs vital roles inside the pathogenesis of diabetic comorbid depression (Menezes Zanoveli et al., 2016). However, the exact relationship between depression and diabetes manifestation is unknown. Therefore, it is necessary to justify the possible relationship between diabetes and depression.

Our previous report implicated that metformin attenuates the diabetic comorbid depression (DCD). However, the pathological mechanisms underlying DCD still unknown. Studies suggested that mitochondrial membrane potential and oxidative stress is a critical underlying mechanism for DCD (Wayhs et al., 2013). Therefore, inclusion of ascorbic acid would improve the mitochondrial dysfunction and may facilitate the action of metformin for the treatment of DCD. In our earlier work, we have investigated 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 pro-inflammatory cytokines TNF-α and IL-6 in the brain). Further, caspase-9, caspase-3 and NF-κB in the prefrontal cortex were estimated to explore the mechanisms involved in the diabetes comorbid depression.

Therefore, this part of the thesis evaluated the effect of metformin and ascorbic acid combination therapy on brain derived neurotropic factor (BDNF), mitochondrial membrane potential, caspase-9, caspase-3, and NF-κB in the prefrontal cortex of diabetes comorbid depressed rats.

5.2 Materials and methods

5.2.1 Experimental design

Rats were randomly allocated into seven experimental groups of six animals each (Table 4). In overnight fasted rats, diabetes was induced by single i.p. injections of nicotinamide and streptozotocin 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).

Table 4: Experimental design of combination therapy of metformin and ascorbic acid in modulation of BDNF, caspase, NF-kB, and mitochondrial membrane potential 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
Depressed control	Nondiabetic	Distilled water (1 mL/kg)	Intermittent foot-shock	6
Diabetic control	Diabetic	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)	Intermittent foot-shock	6
		Ascorbic acid (25 mg/kg)		6

Metformin (25 mg/kg) and ascorbic acid (25 mg/kg)	6

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

Metformin and ascorbic acid (Sigma Aldrich, USA) were dissolved in distilled water for oral administration to DCD rats. The control animals received distilled water only (1 mL/kg, p.o.). In this experiment, the effects of repeated doses of metformin (25 mg/kg/day, p.o.), ascorbic acid (25 mg/kg/day, p.o.), and their combination on depressive-like markers, mitochondrial membrane potential, caspase activation, BDNF expression, and NF-kB expression were examined using diabetes comorbid depressed rats (Figure 5.1). The forced swim test (see section 2.2.6) and organ collection (see section 2.2.7) were performed as described in this thesis previously.

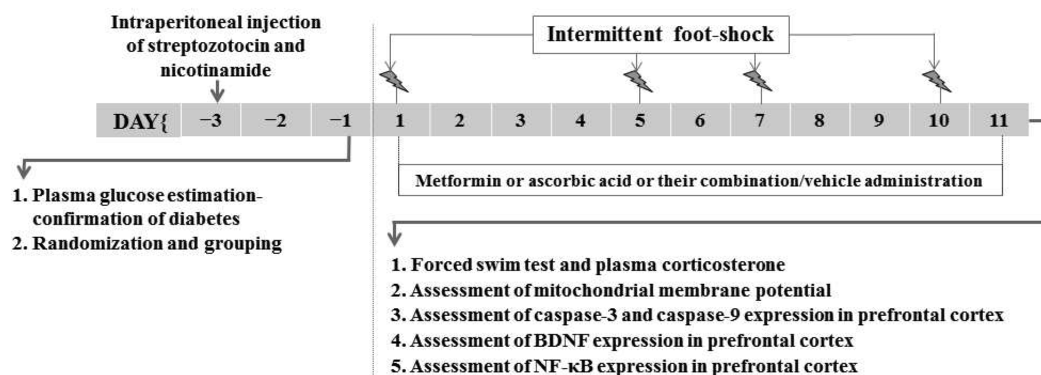

Figure 5.1: Schematic representation of experimental protocol.

5.2.2 Estimation of mitochondrial membrane potential

The mitochondrial membrane potential in prefrontal cortex homogenate was measured using florescent catonic dye teramethyl rhodamine methyl ester at an excitation wavelength of 535 ± 10 nm and emission wavelength at 580 ± 10 nm using emission spectroscopy (Huang, 2002, Prajapati et al., 2017).

5.2.3 Western blotting of BDNF, caspase-9, and caspase-3

Tissue of the prefrontal cortex was isolated and prepared for western blotting as described previously (Ashok et al., 2015). The tissue was washed with PBS and suspended in 100 μl of tissue lysis reagent and kept on ice for 15 min. The tissues were homogenized using a mechanical homogenizer and centrifuged at 15,000×g for 30 min at 4 °C, and the supernatant was collected. Protein content in the samples was quantified using the Bradford reagent (Bradford, 1976). Tissue homogenate protein (50-100 μg) was mixed with appropriate volume of sample loading buffer containing 5% of β-mercaptoethanol and boiled for 15 min at 90 °C. For each sample, proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Tris-Glycine gels containing 10% acrylamide/bis-acrylamide at 100 V. Then, blotted on polyvinylidene difluoride membrane at 16 V for 1 h with transfer buffer. After transfer, blots were stained with Ponceau S to verify equivalent loading of samples and then blocked with 5% skimmed milk for 1 h at room temperature. After two washings with PBS with Tween 20 (PBST), blots were probed overnight with 1:1000 dilutions (in PBS plus 0.2% Tween 20, PBST) of cleaved caspase -9 (37 kDa) and -3 (17 kDa), BDNF (14 kDa), and NF-κB (65 kDa) antibodies at 4 ºC. The blots were washed for 15 min thrice, followed by incubation with horseradish peroxidase-conjugated secondary antibodies in 1:200-dilution for 2 h at room temperature. Blots were developed by Immobilon Western Chemiluminescent HRP Substrate. Relative expression of each protein was determined by densitometric quantification of blots using Versa-Doc Gel Imaging System (BioRad, Hercules, CA). The densiometric analysis was compared with endogenous loading control β-actin.

5.2.4 Real-time PCR (Quantitative Polymerase Chain Reaction, qPCR)

The rat prefrontal cortex was removed and RNA was isolated through TRIzol reagent. Isolated RNA was dissolved in RNase DNase-free water and was quantified before complementary DNA (cDNA) synthesis. A 5 µg of total RNA was used to prepare cDNA using reverse transcription PCR (Superscript III, Life Technologies) according to manufacturer's protocol. qPCR was then performed using SYBR green dye. Optimum primer pairs for BDNF and internal housekeeping control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed using NCBI-Primer BLAST. Primer Sequences used for qPCR techniques were forward- 5'-TGGCTCTCATACCCACTAAGA-3' and reverse- 5'-CGGAAACAGAACGAACAGAAAC-3'. Primers were purchased from GCC Biotech India Pvt Ltd. For qPCR, 40 cycles of the program were set and each cycle comprised a denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s in the Real-Time PCR Instrument (Bio-Rad, USA). Melting curve analysis and mRNA levels were examined using QuantStudio™ Real-Time PCR Software. Changes in mRNA expression were calculated using the Relative Quantity (RQ) equation, $RQ = 2^{-\Delta\Delta CT}$; where delta cycle threshold (ΔCT) denoted a difference in CT values between the target gene and GAPDH, and ΔΔCT signified the differences in the ΔCT value of treated sets compared to control. Results obtained were confirmed by running qPCR products on 2% agarose gel.

5.2.5 Immunohistochemistry

Coronal sections of prefrontal cortex were performed on glass slide. The sections of prefrontal cortex were collected on alum-gelatin and processed by the

avidin-biotin-peroxidase complex immunohistochemical technique. The sections were then washed four-times with phosphate buffer saline (PBS) and then incubated in 3% hydrogen peroxide for 15 min at room temperature to remove endogenous peroxidase. After washing, sections were incubated in a blocking solution (0.01 M PBS with 5% goat serum) for 30 min at room temperature. The sections were then incubated with primary rabbit anti-BDNF polyclonal antibody (1:200, Santa Cruz Biotechnology, CA, USA) overnight at 4 °C, washed with 0.05 M PBS, followed by incubation with secondary goat anti-rabbit antibody (1:1000, Santa Cruz Biotechnology, CA, USA) for 2 h at room temperature. Finally, Nuclear DNA was stained with 4',6-diamidino-2-phenylindole (DAPI; 1.5 μg/ml) at room temperature for 30 min. Images were obtained using an LSM 700 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

5.2.6 Statistical analysis

Results were reported as mean ± standard error of mean (SEM). Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. GraphPad Prism version 7.03 for Windows (GraphPad Software, Inc. CA, USA) was used for statistical analysis. Statistical significance was determined at $P < 0.05$ level of confidence.

5.3 Results

5.3.1 Confirmation of activity against depressive-like markers

In the present study, one way ANOVA revealed that there was a significant ($P < 0.05$) difference among the groups [$F(6, 35) = 50.30$; $P < 0.05$] (Figure 5.2). Post hoc analysis revealed that diabetes control, depressed control, and DCD control rats

showed significant increase in immobility period compared with nondiabetic control rats. Furthermore, in DCD control rats, the increase in immobility period was more significant compared to individual group of diabetes control and depressed control rats. Depressed control rats showed higher immobility period as compared to diabetic control rats. Treatment with monotherapy of metformin and ascorbic acid and their combination significantly reduced the immobility period compared to DCD control rats (Figure 5.2A).

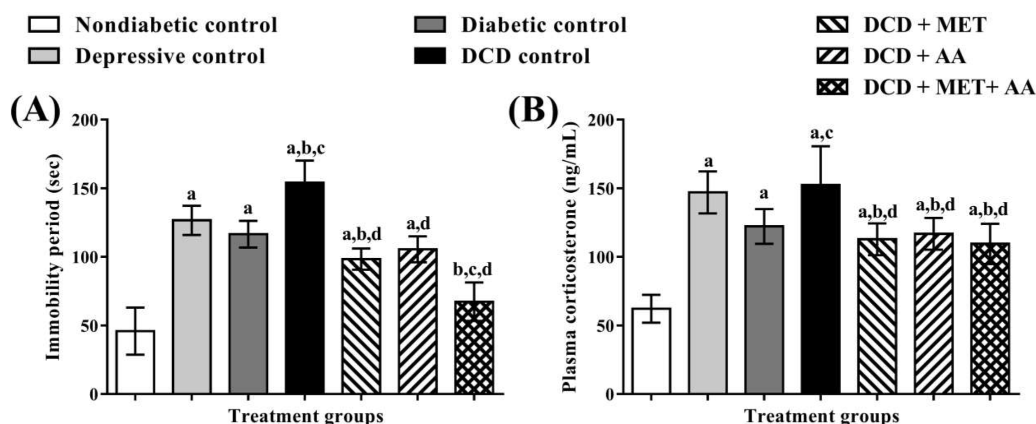

Figure 5.2: Confirmation of activity against depressive-like markers

Effects of metformin (MET), ascorbic acid (AA), or their combination therapy on markers of depression in rats with diabetes comorbid depression (DCD). Immobility period in forced swim test (A) and plasma corticosterone 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 diabetic control, $^{c}P < 0.05$ compared to depressed control, and $^{d}P < 0.05$ compared to DCD control.

Whenever, hypothalamic pituitary adrenal axis gets hyperactivated, it increases the level of plasma corticosterone. In the present study, we observed that the levels of corticosterone were significantly higher [F (6, 35) = 20.73; P < 0.05] in diabetic control, depressed control, and DCD control rats as compared to non-diabetic control rats. Depressed control rats showed increased levels of corticosterone compared to diabetic control rats. On the other hand, metformin, ascorbic acid, and their

combination caused significant decrease ($P < 0.05$) in levels of corticosterone as compared to DCD control rats (Figure 5.2B).

5.3.2 Effect on mitochondrial membrane potential and ROS

In view of diabetes comorbid depression condition, it was observed that mitochondrial damage takes place in neurons (da Silva Dias et al., 2016). In line with that, we observed significantly lower [$F (6, 35) = 39.39$ $P < 0.05$] mitochondrial membrane potential in diabetes control, depressed control, and DCD control rats as compared to nondiabetic control rats. The diabetic control rats showed lower mitochondrial membrane potential compared to depressed control rats. Moreover, treatment with metformin, ascorbic acid, and their combination therapy significantly reversed the mitochondrial membrane potential in DCD rats (Figure 5.3A).

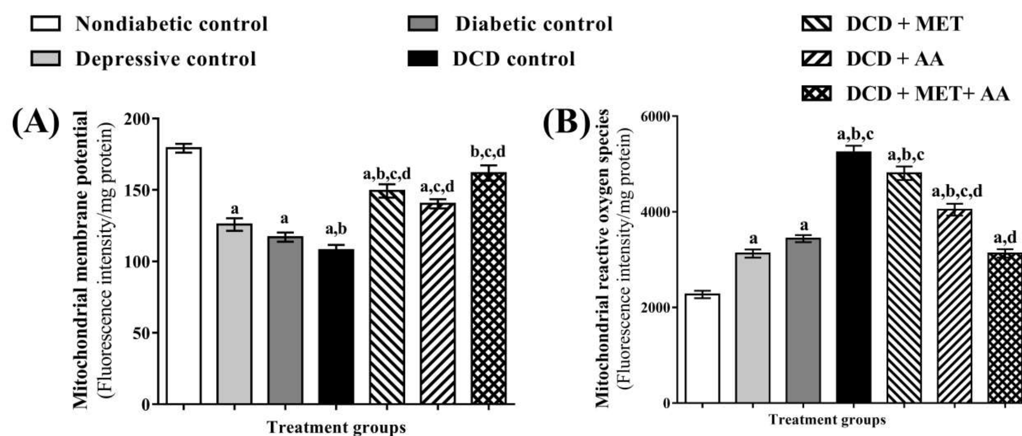

Figure 5.3: Effect on mitochondrial membrane potential and ROS

Effects of metformin (MET), ascorbic acid (AA), or their combination therapy on mitochondrial membrane potential in discrete prefrontal brain regions in rats with diabetes comorbid depression (DCD). Data represent mean ± SEM, n = 6. $^{a}P < 0.05$ compared to nondiabetic control, $^{b}P < 0.05$ compared to diabetic control, $^{c}P < 0.05$ compared to depressed control, and $^{d}P < 0.05$ compared to DCD control.

In accordance to lower mitochondrial membrane potential, a complementary increase [$F (6, 35) = 92.46$ $P < 0.05$] in mitochondrial reactive oxygen species was

observed in diabetic control, depressed control, and DCD control rats as compared to nondiabetic control rats. The diabetic control rats showed lower reactive oxygen species compared with depressed control rats. Moreover, treatment with metformin, ascorbic acid, and their combination significantly reduced the mitochondrial reactive oxygen species in DCD rats (Figure 5.3B).

5.3.3 Effect on caspase -9 and caspase-3 expression in the prefrontal cortex

In view of diabetes comorbid depression condition, we observed significantly higher expression of caspase -9 [$F(6, 14) = 37.98$ $P < 0.05$] and caspase -3 [$F(6, 14) = 29.93$ $P < 0.05$] in diabetic control, depressed control, and DCD control rats as compared to non-diabetic control rats. The diabetic control rats showed higher level of caspase -9 and caspase -3 compared with depressed control rats. Moreover, treatment with metformin, ascorbic acid, and their combination significantly reduced the caspase -9 (Figure 5.4A) and caspase -3 expressions in DCD rats (Figure 5.4B).

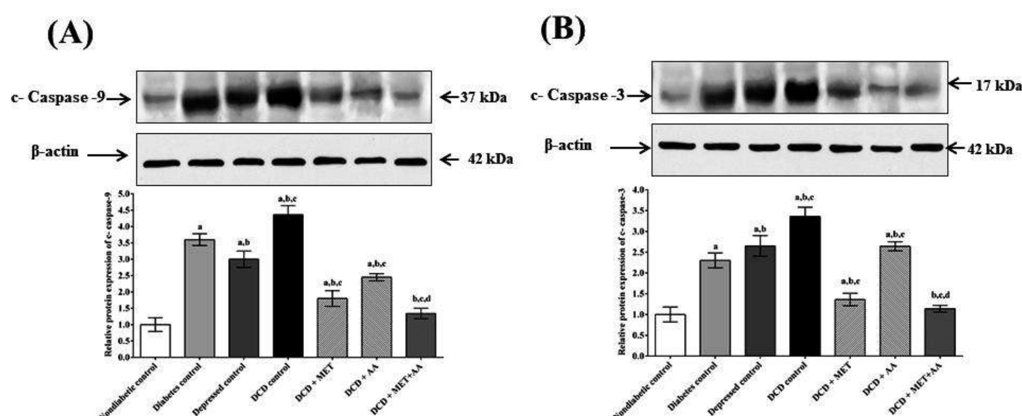

Figure 5.4: Effect on caspase -9 and caspase-3 expression in the prefrontal cortex

Effects of metformin (MET), ascorbic acid (AA), or their combination therapy on c-caspase -9 (A), and -3 (B) protein expression in discrete prefrontal brain regions in rats with diabetes comorbid depression (DCD). Data represent mean ± SEM, n = 3. $^{a}P < 0.05$ compared to nondiabetic control, $^{b}P < 0.05$ compared to diabetic control, $^{c}P < 0.05$ compared to depressed control, and $^{d}P < 0.05$ compared to DCD control.

5.3.4 Effect on BDNF expression in the prefrontal cortex

In view of diabetes comorbid depression condition, a significantly lower BDNF mRNA expression [F (6, 14) = 29.93 P < 0.05] (Figure 5.5A) and BDNF protein expression [F (6, 14) = 29.93 P < 0.05] (Figure 5.5B) was observed in diabetic control, depressed control, and DCD control rats and as compared to non-diabetic control rats. A similar trend was observed in immunohistochemistry of prefrontal cortex sections (Figure 5.5C).

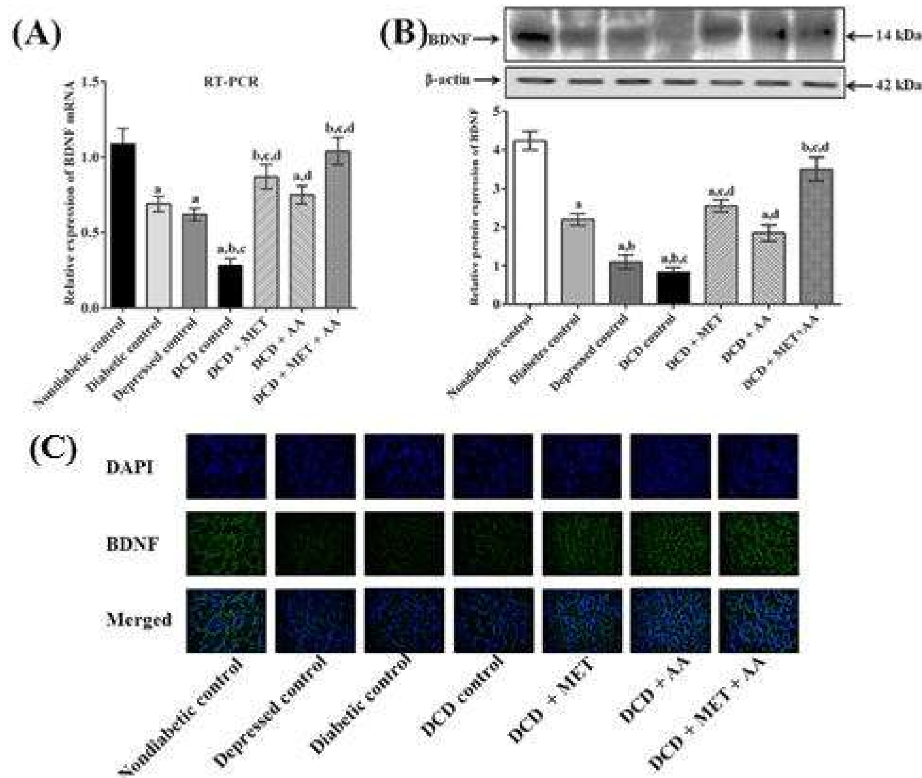

Figure 5.5: Effect on BDNF expression in the prefrontal cortex

Effects of metformin (MET), ascorbic acid (AA), or their combination therapy on BDNF mRNA expression (A), protein expression (B) and representative photomicrograph (40 × magnification) against nuclear- DAPI (C) of the prefrontal cortex in rats with diabetes comorbid depression (DCD). Data represent mean ± SEM, n = 3. ^{a}P < 0.05 compared to nondiabetic control, ^{b}P < 0.05 compared to diabetic control, ^{c}P < 0.05 compared to depressed control, and ^{d}P < 0.05 compared to DCD control.

The depressed control rats showed higher level of BDNF protein expression and BDNF mRNA expression as compared to diabetic control rats. Moreover, treatment with metformin, ascorbic acid, and their combination significantly increased the BDNF expression in DCD rats (Figure 5.5A and Figure 5.5B).

5.3.5 Effect on NF-κB expression in the prefrontal cortex

In this study, we observed a significantly higher [$F (6, 14) = 12.28$ $P < 0.05$] expression of NF-κB in diabetic control, depressed control, and DCD control rats as compared to non-diabetic control rats. Diabetic control rats showed significantly higher NF-κB expression as compared to depressed control rats. Moreover, treatment with metformin, ascorbic acid, and their combination significantly reduced the NF-κB expression in DCD rats (Figure 5.6).

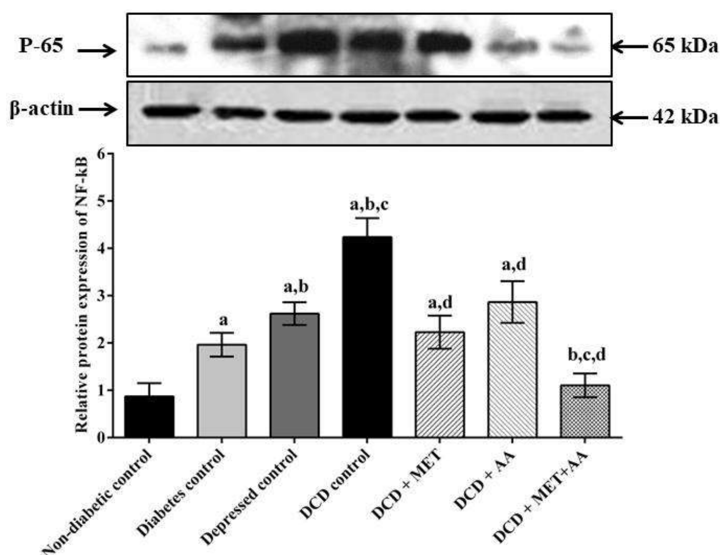

Figure 5.6: Effect on NF-κB expression in the prefrontal cortex

Effects of metformin (MET), ascorbic acid (AA), or their combination therapy on NF-κB protein expression in discrete prefrontal brain regions in rats with diabetes comorbid depression (DCD). Data represent mean ± SEM, n = 3. $^{a}P < 0.05$ compared to nondiabetic control, $^{b}P < 0.05$ compared to diabetic control, $^{c}P < 0.05$ compared to depressed control, and $^{d}P < 0.05$ compared to DCD control.

5.4 Discussion

There is a growing body of evidence suggesting that type 2 diabetes mellitus is associated with increased risk of developing depression (Katon, 2008). Multiple factors contribute the development of depression behavior in diabetic patients in which hyperglycemia induced increased oxidative stress, plasma corticosterone, and decreased BDNF levels in brain, plays a major role (Katon, 2008, Krabbe et al., 2007, Krishnan and Nestler, 2008). In the present study, we observed the development of depressive-like behavior in diabetic comorbid depressed rats through increase in immobility period in forced swim test and hypercorticosteronemia. Treatment with metformin and ascorbic acid combination significantly decreased the immobility time in forced swim test, confirming the antidepressant-like potential of combination therapy. Likewise, plasma corticosterone levels were decreased in metformin and ascorbic acid combination treated rats.

A great body of literature suggests that mitochondrial dysfunction is evident in patients with depression (Shao et al., 2008, Gardner and Boles, 2008, Gardner and Boles, 2011). Mitochondrial dysfunction in the pathophysiology of psychiatric disorders involves a set of impairments that include: (a) disturbances in activity of mitochondrial enzymes, e.g. the oxidative phosphorylation pathway, (b) increased production of damaging ROS and RNS along the mitochondrial respiratory chain, (c) increased mitochondrial DNA (mtDNA) deletions, mutations or polymorphisms, (d) impaired calcium signaling, and (e) impaired energy metabolism. Both increased production and aberrations in detoxification of ROS participate in mitochondrial dysfunction. Mitochondria are major sources of ROS that cause oxidative damage.

Probably, increased ROS and lowered antioxidant levels in depression contribute to mitochondrial dysfunction, apoptosis, and necrosis. The increase in plasma glucose levels during type 2 diabetic mellitus condition favors the absorption of more glucose into the brain through GLUT 1 and GLUT 3 transporters (Vannucci et al., 1997). Increased absorption of glucose into the neurons results in increased metabolism of glucose which in turn causes the overproduction of free radicals from mitochondria (Rovira-Llopis et al., 2017, Pop-Busui et al., 2017). Lipids present in the brain membranes are highly sensitive to oxidation caused by free radicals (Bilici et al., 2001). The relationship between increase in lipid oxidative damage and decrease in mitochondrial membrane potential in diabetes, stress and their comorbid condition is well established (Garabadu and Krishnamurthy, 2014, Cardoso et al., 2013, Rasbach and Schnellmann, 2006). Similar to the earlier findings, we also observed increased lipid peroxidation and subsequent decrease in mitochondrial membrane potential in prefrontal cortex of DCD rats. Metformin and ascorbic acid combination therapy mitigated the extent of lipid peroxidation in prefrontal cortex of DCD rats which ultimately helps in restoring the mitochondrial membrane potential in combination treated rats.

Based on structure and function, caspases are classified into two groups: initiator caspase (caspase-9) and effector caspase (caspase-3) (Li and Sheng, 2012, Elmore, 2007). Mounting evidence in recent years shows that in cells undergoing apoptosis, caspases are activated by two main pathways the extrinsic pathway and the intrinsic pathway. In diabetes comorbid depression, the ongoing neuronal cell damage is characterised by a reduction of mitochondrial transmembrane potential. Thus, revealing the second step of mitochondrial generation of reactive oxygen species.

Finally, mitochondria reduce their volume, and reduce mitochondrial membrane potential. In addition to decrease in mitochondrial membrane potential, oxidative free radicals are also responsible for the release of cytochrome c from mitochondria through its structural damage (Garrido et al., 2006). Released cytochrome c activates caspase 9 which in turn cleaves procaspase 3 into caspase 3 and ultimately causes apoptosis (Boatright and Salvesen, 2003). Programmed cell death is a physiological process commonly defined by an alteration in nuclear morphology and characterised by stepwise degradation of chromosomal DNA (Zamzami et al., 1995).

Hyperglycemia induced apoptosis of prefrontal cortex neurons results in decreased release of BDNF which in turn cause the development of depression (Lee and Kim, 2010a). In the present study, we observed the increased levels of caspase 9 and 3, and decreased levels of BDNF in prefrontal cortex of DCD rats whereas, metformin and ascorbic acid treated rats showed decreased caspase 9 and 3, and increased BDNF levels. Hence, it can be postulated that the antidepressant-like potential of metformin and ascorbic acid combination is may be due the attenuation of oxidative stress induced caspase -9 and caspase -3 activation, and subsequent increase in BDNF levels.

5.5 Conclusion

In this thesis chaptor, our finding showed that combination therapy of metformin and ascorbic acid decreases diabetes comorbid depression by improving mitochondrial membrane potential. Furthermore, combination therapy of metformin and ascorbic acid abrogated oxidative stress induced NF-kB expression, and inhibiting neuroinflammation mediated neuronal damage. In addition, combination

therapy abrogated oxidative stress induced caspase -9 and -3 activity, and hence decreased neuronal apoptosis. On the other hand, combination therapy improved neuronal survival by enhancing the expression of BDNF. Taken together, the current finding suggests that the combination therapy of metformin and ascorbic acid could be a better treatment regimen in the management of patients with diabetes comorbid depression.