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Ascorbic acid therapy: A potential strategy against comorbid depression-like behavior in streptozotocin-nicotinamide-induced diabetic rats

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ABSTRACT

This study examined the potency and efficacy of ascorbic acid (AA) in the management of depression-like behavior in diabetic rats. Diabetes mellitus was induced by single intraperitoneal injections of nicotinamide (120 mg/kg) and streptozotocin (65 mg/kg) administered 15 min apart. Diabetic (blood glucose ≥250 mg/dL) rats were subjected to intermittent foot-shocks to induce comorbid depression. Seven groups of diabetes comorbid depressed rats received vehicle (1 mL/kg) or AA (10, 25, 50, 100, 200, or 400 mg/kg) orally for eleven days. Three control groups namely- nondiabetic, diabetic, and depressed rats received the vehicles only. The potency (ED_{50}) and efficacy (E_{max}) of AA against immobility period, hypercorticosteronemia, adrenal hyperplasia, hyperglycemia, hypoinsulinemia, oxidative stress, and inflammatory response were estimated. AA administration caused a dose-dependent decrease ($P < 0.05$) in immobility period with maximum inhibition of 69% (efficacy) at 200 mg/kg and ED_{50} of 14 mg/kg (potency). AA at 200 mg/kg produced the maximal reduction in hypercorticosteronemia (55.1%) and adrenal hyperplasia (52.6%) with ED_{50} of 9.8 and 14.4 mg/kg, respectively. AA at 400 mg/kg produced the maximal reduction in hyperglycemia (35.5%), hypoinsulinemia (32.7%), and lipid peroxidation (82%) with ED_{50} of 18.6, 13.7, and 20.7 mg/kg, respectively. Moreover, AA at 400 mg/kg produced the maximal increase in SOD content (83%), CAT activity (77.9%), and IL-10 level (63%) with ED_{50} of 21.5, 21, and 21 mg/kg, respectively. In conclusion, the present results suggest that AA has therapeutic potential against diabetes comorbid depression but better regulation of hyperglycemia and hypoinsulinemia is required to achieve maximal benefits.

1. Introduction

Ascorbic acid, a natural antioxidant, is primarily consumed through a diet rich in fresh fruits and vegetables [1]. Ascorbic acid is a major free radical scavenger, therefore, prevents cellular damage induced by free radicals [2] and provides protection against diseases (arthritis, atherosclerosis, cancer, diabetes, ischemia) that involves oxidative stress [3,4]. Besides, ascorbic acid acts as a cofactor in the biosynthesis of catecholamines, amino acids, and certain peptide hormones [5]. Low intake of ascorbic acid is recognized as a risk factor in the development of pre-diabetes and metabolic syndrome [6–10]. Ascorbic acid supplementation has been shown to reduce hyperglycemia and prevent the development of diabetic complications [11–15]. Recent studies have shown that depression is one of the major comorbid condition in diabetic patients [16,17]. Substantial evidence indicates that diabetic patients are more vulnerable to depression than non-diabetic patients [18–20]. Earlier findings suggest that daily use of ascorbic acid could reverse depressive-like behavior in rats [21–23]. However, the possible advantage of ascorbic acid therapy in diabetes comorbid depressed rats has not been studied extensively.

There are evidence suggesting that depression and diabetes mellitus share many pathophysiological characteristics [24,25], which includes oxidative stress, inflammation, insulin-resistance, and overactivation of HPA axis [16,26,27]. Chronic hyperglycemia leads to excessive oxidative stress [28,29] and inflammation [30] in diabetic patients resulting in complications such as neuropathy, nephropathy, and retinopathy [31]. Recent literature suggests that excessive oxidative stress, increase in proinflammatory cytokines, and reduction in anti-inflammatory cytokines are associated with depressive symptoms in diabetic patients [29,32–34]. Chronic hyperglycemia is associated with increased formation of free radicals and decreased antioxidant activity [35]. Due to excessive free radical production, the redox balance present in cells get disturbed and contribute to oxidative damage of neuronal components [36]. There is evidence that the brain regions such as prefrontal cortex

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Table 1
Experimental Design.

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	12
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	12
DCD + Ascorbic acid	Diabetic	Ascorbic acid (10 mg/kg)	Intermittent foot-shock	6
		Ascorbic acid (25 mg/kg)		6
		Ascorbic acid (50 mg/kg)		6
		Ascorbic acid (100 mg/kg)		6
		Ascorbic acid (200 mg/kg)		6
		Ascorbic acid (400 mg/kg)		6

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

and hippocampus are more prone to oxidative stress in streptozotocin-induced diabetic rats with depressive-like behavior [37]. A great body of clinical evidence supports the critical role of oxidative stress in the prefrontal cortex of depressive patients [38–42]. Several preclinical studies reported that abnormalities in the prefrontal cortex are responsible for depression-like behavior in normal and diabetic rats [43–45]. It has been shown that oxidative stress in the prefrontal cortex of depressed individuals increases the production of reactive oxygen species leading to lipid peroxidation (LPO) and suppressed anti-oxidative defense (superoxide dismutase (SOD) content and catalase (CAT) activity) [39,40]. Moreover, a latest study has demonstrated that there is an association between inflammatory biomarkers and depression in freshly diagnosed type 1 and type 2 diabetic patients [46]. It is also evident that depressed individuals have significantly lower levels of IL-10 (an anti-inflammatory cytokine) leading to diminished inflammation suppressive activities [47]. Taken together, it can be hypothesized that reduction in oxidative stress might prevent comorbid depression in diabetic condition. Therefore, the present study was undertaken to investigate the potency and efficacy of oral ascorbic acid therapy against depression-like behavior in streptozotocin-nicotinamide-induced experimental diabetic rats. This study evaluated the effects of oral ascorbic acid therapy (10, 25, 50, 100, 200, and 400 mg/kg) on different pathophysiological markers of depression (behavioral despair, hypercorticosteronemia, and adrenal hyperplasia), diabetes (hyperglycemia and hypoinsulinemia), inflammation (brain IL-10 levels), oxidative injury (lipid peroxidation), and free radical scavenging activity (brain catalase activity and superoxide dismutase content).

2. Material and methods

2.1. Animals

Male albino rats weighing 150 ± 30 g were procured from the Central Animal House of Banaras Hindu University, Varanasi, India and acclimatized for seven days in an animal house (Department of Pharmaceutical Engineering and Technology, Indian Institute of Technology, Banaras Hindu University) with standard vivarium conditions (25 ± 2 °C and 12:12 h light/dark cycle) before experimentation. All the experiments were performed in conformity with the guidelines of Central Animal Ethical Committee, Banaras Hindu University and Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment, Forests and Climate Change, Government of India. All efforts were made to minimize animal suffering and pain during experimentation. Rats received commercial food pellets and water ad libitum unless otherwise stated in a specific protocol.

2.2. Materials

Ascorbic acid, streptozotocin, and nicotinamide were purchased from Sigma Aldrich (USA), HiMedia (India), and SD Fine-Chemical Limited (India), respectively. Glucose oxidase peroxidase enzyme kit was procured from Accurex Biomedical Pvt. Ltd. (India). Rat insulin ELISA kit and rat corticosterone ELISA kit were procured from Mercer Expert Assays (USA) and KinesisDx (USA), respectively. Rat IL-10 ELISA kit was procured from Krishgen Biosystems (USA). A microplate-reader (Bio-Rad Laboratories, USA) was used to read the absorbances at a wavelength mentioned in specific procedure. All the remaining chemicals and reagents were purchased from local suppliers.

2.3. Induction of diabetes mellitus in rats

Type 2 diabetes mellitus was induced by intraperitoneal (i.p.) injections each of nicotinamide (120 mg/kg) and streptozotocin (65 mg/kg), as described previously [48,49]. Nondiabetic control rats received single i.p. injections of saline and 0.1 M citrate buffer. All the rats were returned to their respective home cages to receive normal food pellets and 10% sucrose water to minimize hypoglycemic shock. After 72 h of streptozotocin administration, fasting blood samples were collected by tail prick method and glucose levels were measured by using glucometer (Johnson and Johnson Limited, India). Rats with glycemic status ≥250 mg/dL were considered as diabetic and randomly allocated into different groups as per the experimental design in Table 1.

2.4. Induction of comorbid depression in diabetic rats

For the induction of depression, the diabetic rats were exposed to five inescapable foot-shocks (2 mA at 50 Hz, 2 ms duration) with an interval of 10 s, as described previously [48]. Briefly, individual diabetic rats were placed in a chamber with an electrified steel grid floor for 1 min to receive foot-shocks. The rats of the nondiabetic control group were placed in the chamber for 1 min without foot-shocks.

2.5. Experimental design

A dose-response study of ascorbic acid against depression-like behavior in streptozotocin-nicotinamide-induced diabetic rats was designed with six different doses (10, 25, 50, 100, 200, and 400 mg/kg) based on the human dose (1000 mg) shown to reduce blood glucose in diabetic patients [14]. Briefly, the human dose was converted to animal equivalent dose (100 mg/kg) by following the guidance of the United States Food and Drug Administration [50,51]. All other tested doses of ascorbic acid were decided based on the calculated animal equivalent dose (100 mg/kg). Ascorbic acid solutions were prepared in distilled water and administered at 1 mL/kg. The experimental design consisted of four control groups namely- nondiabetic, diabetic, depressed, and diabetic comorbid depressed (DCD) rats that received distilled water and six treatment groups that received different doses of ascorbic acid (Table 1). Diabetic rats were randomly allocated to all groups except the nondiabetic control group, that was allocated nondiabetic rats.

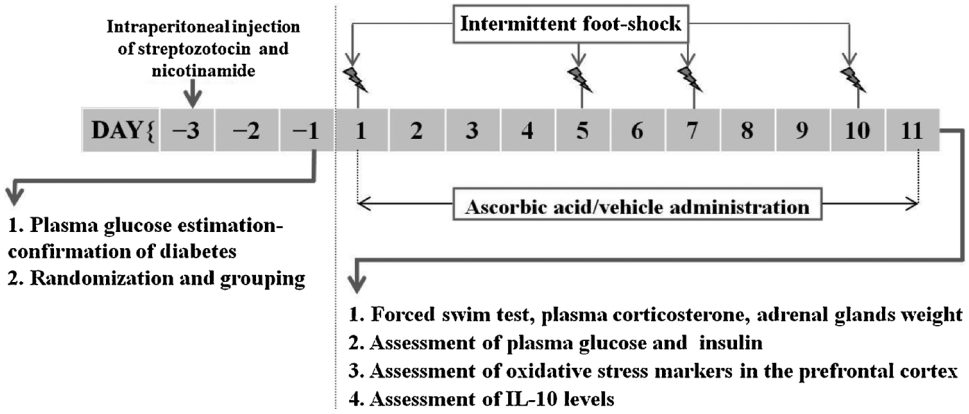

Fig. 1. Schematic representation of timeline of the tasks executed during the experimentation.

Following randomization, rats received different treatments starting from day 1 to day 11. After one hr of distilled water or ascorbic acid administration, the comorbid depression was induced by intermittent foot-shocks (described above) on day 1, 5, 7, and day 10. The timeline of the tasks executed during the experimentation has been represented in Fig. 1.

2.6. Forced swim test

All the rats were allowed to familiarize with the test conditions for 15 min, one hr after the 10^{th} dose of distilled water or ascorbic acid administration. Next day, one hr after the 11^{th} dose of distilled water or ascorbic acid administration, rats were subjected to forced swim test again for five min and immobility periods were recorded as depressive-like behavior or behavioral despair, as described previously [48,52].

2.7. Blood and organ collection

Blood samples, brain, and adrenal glands were collected from each animal and processed, as described previously [48]. The plasma samples were used for estimation of glucose, insulin, and corticosterone. The prefrontal cortex tissue samples were used for estimation of IL-10 levels and the markers of oxidative stress. The isolated adrenal glands were weighed to estimate the wet weight.

2.8. Assessment of plasma glucose, insulin, and corticosterone

The glucose oxidase-peroxidase (GOD-POD) kit was used to estimate the plasma glucose levels in accordance with the manufacturer's instructions. The absorbances were measured at 505 nm using a microplate reader and glucose concentrations were expressed in mg/dL. The plasma insulin and corticosterone levels were estimated using corresponding rat ELISA kits according to the manufacturer's instructions. The absorbances were measured at 450 nm using a microplate reader. The plasma insulin and corticosterone levels were expressed in μIU/mL and ng/mL, respectively.

2.9. Preparation of prefrontal cortex homogenate

Prefrontal cortex homogenate (10% w/v) was prepared in ice-cold 0.02 M sodium phosphate buffer (pH 7.4) using a Teflon-glass homogeniser. The homogenate was centrifuged at 12,000*g* for 45 min at 4 °C, the clear supernatant was collected and used for estimation of oxidative stress markers (lipid peroxidation, superoxide dismutase content, and catalase activity) and IL-10 (an anti-inflammatory cytokine).

2.10. Assessment of oxidative stress markers in the prefrontal cortex

We estimated the markers of oxidative stress such as lipid peroxidation, SOD content, and catalase activity in 10% prefrontal cortex homogenate. Peroxidation of lipids in the prefrontal cortex tissue was estimated by measuring malondialdehyde (the end product of lipid peroxidation), as described previously [53]. Briefly, the prefrontal cortex homogenate was mixed with sodium lauryl sulfate, acetate buffer (pH 3.5), and thiobarbituric acid before heating on a water bath at 95 °C for 60 min. Then, the mixture was allowed to cool at room temperature before adding butanol-pyridine mixture to get a pink colored chromophore. The absorbances were measured using microplate-reader at 532 nm. The levels of lipid peroxidation were estimated from the standard curve of malondialdehyde (MDA) and expressed as nmol MDA/mg protein. SOD activity was determined based on its ability to inhibit the reduction of nitro blue tetrazolium (NBT), as described previously [54]. Briefly, the reaction mixture consisting of NBT (96 mM) and hydroxylamine hydrochloride (20 mM) was mixed with tissue homogenate and the change in absorbances was measured using microplate reader at 560 nm for 2 min at 60 s intervals. NBT reduction occurs in the presence of superoxide radical generated by the auto-oxidation of hydroxylamine. SOD converts the generated superoxide radical into water and hydrogen peroxide, which in turn decreases the reduction of NBT into formazan dye. Percentage inhibition of NBT reduction was calculated by dividing the difference in absorbance of blank (without tissue homogenate) and tissue homogenate samples with the absorbance of blank, multiplied by 100. A 50% inhibition of NBT reduction was considered to be produced by one unit of SOD [54]. Based on the percentage inhibition of NBT reduction in tissue homogenate, SOD content was calculated and expressed as IU/mg of protein. Catalase activity was measured based on its ability to decompose hydrogen peroxide (H_2O_2) as described previously [55]. Briefly, the reaction mixture consists of H_2O_2 and tissue homogenate were incubated for one min. Then the reaction was stopped by adding acetic acid and dichromate reagent and the mixture was boiled in a water bath for 10 min. Dichromate reagent in presence of acetic acid and remaining H_2O_2 was reduced to chromic acetate. Formation of chromic acetate by H_2O_2 was indirectly proportional to catalase activity and was measured using microplate reader at 570 nm. The levels of catalase activity were expressed as μmol H_2O_2 decomposed/min/mg protein. The concentration of protein in the prefrontal cortex homogenate was determined by Bradford assay using bovine serum albumin as a standard [56].

2.11. Assessment of IL-10 levels

The prefrontal cortex IL-10 levels were measured using a rat IL-10 ELISA kit according to the manufacturer's instructions. The absorbances were measured at 450 nm using a microplate reader. The IL-10 levels in

unknown samples were estimated using a standard curve constructed from different concentrations of the IL-10 standard solution and expressed as pg/g of tissue.

2.12. Statistical analysis

Results were reported as mean ± standard error of mean (SEM). Individual data were normalized to the nondiabetic control group, percentage decrease or increase in response as compared to DCD control group was calculated, and median effective doses (ED_{50}) were determined by nonlinear regression analysis using GraphPad Prism (GraphPad Software Inc., USA) version 7.03 for Windows. Where 100% response was not achieved, the percentage increase or decrease in response data were normalized to maximal response through the transform function of GraphPad Prism before calculating ED_{50}. All statistical analyses were carried out using GraphPad Prism. One-way analysis of variance followed by Tukey's multiple comparison test was employed to detect a significant difference between the treatment groups. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. Potency and efficacy of ascorbic acid against comorbid depression

We determined the potency and efficacy of ascorbic acid against immobility period, hypercorticosteronemia, and adrenal hyperplasia by evaluating the effects of different doses (10, 25, 50, 100, 200 and 400 mg/kg) of ascorbic acid in diabetes comorbid depressed (DCD) rats. A significant ($P < 0.05$) increase in immobility period was observed in the DCD control, diabetic control, and depressed control rats as compared to nondiabetic control rats (Fig. 2A). Immobility period was higher in DCD rats compared to diabetic control rats ($P < 0.05$) and depressed control rats ($P > 0.05$). Ascorbic acid administration caused a dose-dependent inhibition ($P < 0.05$) of immobility period with maximum inhibition of 69% (efficacy) at 200 mg/kg and ED_{50} of 14 mg/kg (potency) (upper inset Fig. 2A). In accordance with behavioral results, a significantly ($P < 0.05$) higher plasma corticosterone level was observed in DCD control, diabetic control, and depressed control group as compared to nondiabetic control group (Fig. 2B). The difference in plasma corticosterone levels among DCD control rats, diabetic control rats, and depressed control rats was statistically insignificant ($P > 0.05$). Ascorbic acid administration caused reductions in plasma corticosterone levels starting from 10 mg/kg dose but statistically significant ($P < 0.05$) reductions were observed at doses 25 mg/kg and more (Fig. 2B). Ascorbic acid at 200 mg/kg produced the maximal reduction (55.1%) in plasma corticosterone level with ED_{50} of 9.8 mg/kg (upper inset Fig. 2B). In line with corticosterone data, adrenal gland weight was significantly ($P < 0.05$) increased in DCD control, diabetic control, and depressed control rats compared with nondiabetic control rats (Fig. 2C). Ascorbic acid therapy caused a statistically significant decrease in adrenal gland hyperplasia at doses starting from 25 mg/kg as compared to DCD control rats (Fig. 2C). A maximal reduction (52.6%) in adrenal gland hyperplasia was observed at 200 mg/kg dose of ascorbic acid with ED_{50} of 14.4 mg/kg (upper inset Fig. 2C). The difference in adrenal gland hyperplasia among DCD control rats, diabetic control rats, and depressed control rats was statistically insignificant ($P > 0.05$). An insignificant ($P > 0.05$) change in immobility period, plasma corticosterone, and adrenal gland hyperplasia was observed between diabetic control group and depressed control group.

3.2. Potency and efficacy of ascorbic acid against hyperglycemia and hypoinsulinemia

Plasma glucose and insulin concentrations were estimated to detect whether ascorbic acid has a beneficial role in managing hyperglycemia in diabetes comorbid depression. A significantly ($P < 0.05$) higher plasma glucose levels were observed in DCD control, diabetic control, and depressed control rats compared with nondiabetic control rats (Fig. 3A). Hyperglycemia was higher in DCD control rats compared to depressed control rats ($P < 0.05$) and diabetic control rats ($P > 0.05$). A significantly higher hyperglycemia was observed in diabetic control group compared with the depressed control group ($P < 0.05$). Ascorbic acid administration caused reductions in plasma glucose levels starting from 10 mg/kg dose but statistically significant ($P < 0.05$) reductions were observed at doses 25 mg/kg and more (Fig. 3A). Ascorbic acid at 400 mg/kg produced the maximal reduction (35.5%) in plasma glucose level with ED_{50} of 18.6 mg/kg (upper inset Fig. 3A). Conversely, plasma insulin concentrations were significantly ($P < 0.05$) decreased in DCD control, diabetic control, and depressed control rats as compared to nondiabetic control rats (Fig. 3B). Hypoinsulinemia was higher in DCD control rats compared to depressed control rats ($P < 0.05$) and diabetic control rats ($P > 0.05$). A significantly higher hypoinsulinemia was observed in diabetic control group compared with the depressed control group ($P < 0.05$). Ascorbic acid administration caused a statistically significant reversal of hypoinsulinemia at doses starting from 25 mg/kg as compared to DCD control rats (Fig. 3B). A maximal increase (32.7%) in plasma insulin level was observed at 400 mg/kg dose of ascorbic acid with ED_{50} of 13.7 mg/kg (upper inset Fig. 3B).

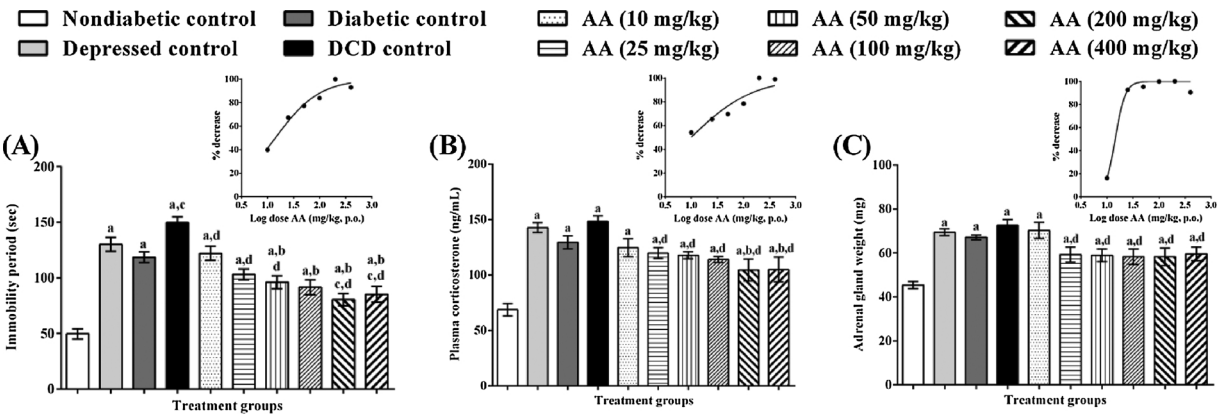

Fig. 2. Effects of different doses of ascorbic acid (AA) on immobility period (A), plasma corticosterone levels (B), and adrenal gland weights (C) in diabetes comorbid depressed (DCD) rats. The upper inset figures indicate the percentage decrease in response compared with DCD control group. [a]$P < 0.05$ versus nondiabetic control group, [b]$P < 0.05$ versus depressed control group, [c]$P < 0.05$ versus diabetic control group, and [d]$P < 0.05$ versus DCD control group.

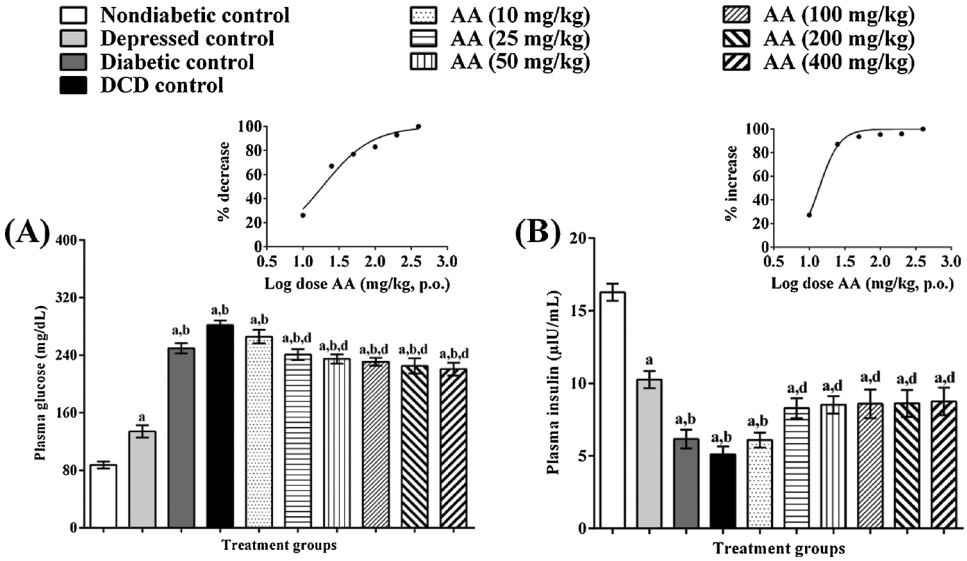

Fig. 3. Effects of different doses of ascorbic acid (AA) on plasma glucose (A) and insulin levels (B) in diabetes comorbid depressed (DCD) rats. The upper inset figures indicate the percentage decrease or increase in response compared with DCD control group. $^{a}P < 0.05$ versus nondiabetic control group, $^{b}P < 0.05$ versus depressed control group, $^{c}P < 0.05$ versus diabetic control group, and $^{d}P < 0.05$ versus DCD control group.

3.3. Potency and efficacy of ascorbic acid against oxidative stress

The alterations in oxidative stress markers due to diabetes comorbid depression and ascorbic acid therapy were studied by estimating malondialdehyde (MDA), the end product of lipid peroxidation, catalase (CAT) activity superoxide, and dismutase (SOD) content in the prefrontal cortex homogenate. Statistically significant higher levels of MDA were observed in the DCD control, diabetic control, and depressed control rats compared with nondiabetic control rats (Fig. 4A). Remarkably higher levels of MDA were observed in DCD control rats compared to depressed control rats ($P < 0.05$) and diabetic control rats ($P < 0.05$). An increased level of MDA was observed in diabetic control group compared with the depressed control group ($P < 0.05$). Interestingly, a dose-dependent decrease ($P < 0.05$) in MDA was observed in ascorbic acid-treated groups as compared to the DCD control group (Fig. 4A). Ascorbic acid at 400 mg/kg produced the maximal reduction (82%) in MDA levels with ED_{50} of 20.7 mg/kg (upper inset Fig. 4A). The rats of DCD control, diabetic control, and depressed control groups showed a significantly ($P < 0.05$) lower SOD content (Fig. 4B) and CAT activity (Fig. 4C) as compared to nondiabetic control rats. SOD content and CAT activity in DCD control rats were significantly lower compared to depressed control rats ($P < 0.05$) and diabetic control rats ($P < 0.05$). A dose-dependent increase ($P < 0.05$) in SOD content (Fig. 4B) and CAT activity (Fig. 4C) was observed in the ascorbic acid-treated groups compared with DCD control group. At 400 mg/kg dose, the ascorbic acid therapy produced the maximal increase (83%) in SOD content with ED_{50} of 21.5 mg/kg (upper inset Fig. 4B) Likewise, the maximal increase (77.9%) in CAT activity was observed at 400 mg/kg dose of ascorbic acid with ED_{50} of 21 mg/kg (upper inset Fig. 4C). An insignificant decrease ($P > 0.05$) in SOD content and CAT activity was observed in diabetic control group compared with the depressed control group.

3.4. Potency and efficacy of ascorbic acid against inflammation in the prefrontal cortex

In order to evaluate whether ascorbic acid was able to attenuate the inflammation associated with diabetes comorbid depression, we analyzed the IL-10 levels in the prefrontal cortex. The brain levels of IL-10 were significantly ($P < 0.05$) decreased in DCD control, diabetic control, and depressed control rats as compared to nondiabetic control rats (Fig. 5). IL-10 levels were significantly ($P < 0.05$) lower in DCD control rats compared to depressed control rats ($P < 0.05$) and diabetic control rats ($P < 0.05$). A decreased level of IL-10 was observed in diabetic control group compared with the depressed control group ($P < 0.05$). In a dose-dependent manner, a statistically significant increase in the levels of IL-10 was observed in ascorbic acid-treated groups as compared to the DCD control group (Fig. 5). At 400 mg/kg

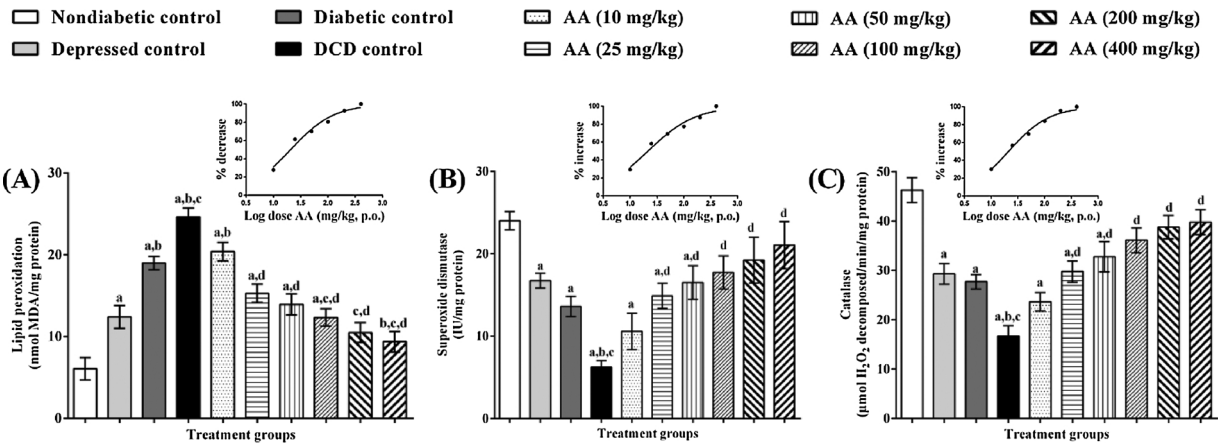

Fig. 4. Effects of different doses of ascorbic acid (AA) on lipid peroxidation (A), superoxide dismutase (B) and catalase activity (C) in diabetes comorbid depressed (DCD) rats. The upper inset figures indicate the percentage decrease or increase in response compared with DCD control group. $^{a}P < 0.05$ versus nondiabetic control group, $^{b}P < 0.05$ versus depressed control group, $^{c}P < 0.05$ versus diabetic control group, and $^{d}P < 0.05$ versus DCD control group.

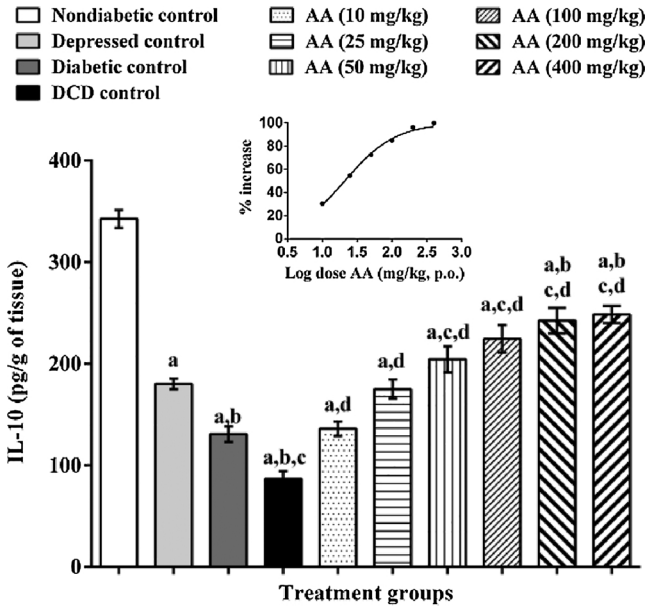

Fig. 5. Effects of different doses of ascorbic acid (AA) on brain IL-10 levels in diabetes comorbid depressed (DCD) rats. The upper inset figure indicates the percentage increase in response compared with DCD control group. $^{a}P < 0.05$ versus nondiabetic control group, $^{b}P < 0.05$ versus depressed control group, $^{c}P < 0.05$ versus diabetic control group, and $^{d}P < 0.05$ versus DCD control group.

dose, the ascorbic acid therapy produced the maximal increase (63%) in IL-10 level with ED_{50} of 21 mg/kg (upper inset Fig. 5).

4. Discussion

The consequences of metabolic changes in diabetic condition are the primary cause of depression that alters the quality of life of diabetic patients [57,58]. The increase in the prevalence of diabetes has increased the incidence of diabetes comorbid depression, which is affecting approximately 25% of the diabetic population [18,20]. There is a large unmet need to develop therapeutic interventions for the management of diabetes comorbid depression [18,59]. Besides its well-known antioxidant activity, ascorbic acid has been shown to produce antidepressant-like activity in animal models of depression [21]. This study explored the effects of ascorbic acid against comorbid depression-like behavior in streptozotocin-nicotinamide-induced diabetic rats and estimated the potency and efficacy against immobility period (forced swim test), hypercorticosteronemia, adrenal hyperplasia, hyperglycemia, hypoinsulinemia, oxidative stress, and inflammatory response.

The pathophysiology of diabetes comorbid depression involves alterations in both behavioral and biochemical parameters [37]. The forced swim test is one of the most widely used behavioral tests for studying the antidepressant-like activity of drug candidates [60]. The duration of immobility during the test period indicates the depressive-like behavior in rats [61]. In the recent past, Moretti et al reported that oral administration of ascorbic acid at 10 mg/kg reduces immobility period when mice were subjected to the tail suspension test [21]. In line with Moretti et al, in this study, ascorbic acid caused a significant reduction in the immobility period when rats with diabetes comorbid depression were subjected to forced swim test but 100 percent efficacy was not achievable even at 400 mg/kg dose. There are evidence suggesting a relationship between depressive-like behavior in rats and hyperactivity of hypothalamic-pituitary-adrenal (HPA) axis [62,63]. Chronic hyperglycemia in diabetes mellitus has been implicated in hyperactivation of HPA axis [64]. One of the explanations for HPA axis hyperactivity in diabetes is hyperglycemia-induced oxidative stress [65]. In hyperglycemic condition, increased absorption of glucose by neurons activates the polyol pathway, an alternate route of glucose metabolism [65]. Activation of the polyol pathway increases the generation of reactive oxygen species and depletes the levels of glutathione reductase [65]. The increase in oxidative stress induces the production of proinflammatory cytokines (TNF-α, IL-1β, IL-6) [66], which are responsible for hyperactivation of HPA-axis and hypersecretion of corticosterone [67,68]. In addition to overproduction of proinflammatory cytokines, a hyporesponsiveness of IL-10 (an anti-inflammatory cytokine) has been implicated in diabetic patients [34,69]. Low levels of IL-10 results in the development of depressive behavior [47,70], primarily through activation of HPA axis [71]. Hyperactivity of HPA axis is also associated with decreased sensitivity of glucocorticoid negative feedback [68,72]. Increased production of corticosterone and decreased sensitivity of glucocorticoid negative feedback in chronic hyperglycemic condition results in long-term exposure of corticosterone to neurons leading to neuronal damage, dendritic atrophy, reduced neurogenesis, decrease synaptic plasticity, and down-regulation of brain-derived neurotrophic factor [73,74]. The outcome of long-term corticosterone exposure leads to reduction in serotonin levels in the brain, which is the major cause of depression [75]. In this study, the combination of two different stressors (hyperglycemia and intermittent foot-shocks) increased the levels of oxidative stress markers and reduced the prefrontal cortex IL-10 levels. The diabetic-only control group showed significantly higher depression-like behavior that was similar to the findings of Husain et al [49]. The depressed-only control group showed changes in depression markers, diabetes marks, oxidative markers, and anti-inflammatory marker possibly due to hyperactivation of HPA axis frequently observed in a depressed condition. These findings from the control groups suggest that both the stressors involve a common pathophysiological mechanism, consisting of oxidative stress and inflammation, leading to depressive-like behavior in rats.

Ascorbic acid therapy produced a profound antioxidant activity in the prefrontal cortex, increased the levels of IL-10, and reduced the levels of corticosterone in diabetes comorbid depressed rats. Chronic activation of the HPA axis and increased production of corticosterone in hyperglycemic condition are associated with trophic stimulation of the adrenal gland by adrenocorticotrophic hormone and result in adrenal hyperplasia [76]. In this study, we observed a severe adrenal hyperplasia in diabetes comorbid depressed rats, which was reduced to some extent by ascorbic acid therapy possibly due to reduction in HPA-axis activation and corticosterone secretion.

It is well known that chronic hyperglycemia-induced oxidative stress is one of the major risk factors for the development of depression in diabetes mellitus [77]. Hyperglycemic condition increases glucose autoxidation, activates polyol pathway, and increases protein glycation leading to the generation of oxidative free radicals in the brain [30]. Increased levels of oxidative stress in neurons have been reported to trigger neuronal damage by peroxidation of polyunsaturated lipids present on the plasma membrane of neurons [78]. Lipid peroxidation is the primary neuronal damage by oxidative free radicals, which alters neuronal structural integrity and viability in the brain of diabetic rats [79]. Ascorbic acid supplementation has been shown to prevent lipid peroxidation in the liver and brain of diabetic rats [80] and mice with ulcerative colitis [81]. Likewise, decreased levels of tissue antioxidant enzymes such as catalase and SOD has been reported in diabetic rats [79] and has been implicated in the development of depression [82]. In the present study, we observed that ascorbic acid therapy reversed the oxidative stress by restoring the brain prefrontal cortex levels of lipid peroxidation, SOD content, and catalase activity in diabetes comorbid depressed rats. Our results suggest that the antidepressant activity of ascorbic acid may be due to the antioxidant activity that helps in restoring neuronal integrity and viability. In the past, several research reports showed similar antioxidant activity against depressive-like behavior in streptozotocin-induced diabetic rats using free radical scavengers such as *Allium sativum* [83], *Rosa canina* [84], anandamide [85], taurine [86], hesperidin [87], and ginger [88].

Streptozotocin-nicotinamide has been extensively used for induction of type 2 diabetes mellitus in rats [82,89–92]. After administration, streptozotocin rapidly enters into the pancreatic beta cells via GLUT 2 transporters [93] and leads to DNA fragmentation and generation of reactive oxygen species resulting in the destruction of pancreatic beta cells and subsequent hypoinsulinemia and hyperglycemia [94]. In this study, we observed a significant reduction in plasma insulin levels and a significant increase in plasma glucose levels in diabetes comorbid depressed rats. Ascorbic acid therapy significantly increased the insulin levels and decreased the plasma glucose levels in diabetes comorbid depressed rats, possibly due to reduction in oxidative stress. This finding supports the hypothesis that exogenously administered ascorbic acid may improve glycemic control as diabetic patients have higher ascorbic acid requirement to compensate ongoing oxidative stress [7,95]. Moreover, it has been shown that the streptozotocin-induced diabetic rats have lower plasma, liver, and kidney levels of ascorbic acid but normal brain levels [96]. Kashiba et al further explained that oxidative loss, decreased biosynthesis, and increased urinary excretion are the possible factors responsible for lower levels of ascorbic acid in streptozotocin-induced diabetic rats [96]. In the present study, the exogenous administration of ascorbic acid may have increased plasma and tissue levels of ascorbic acid that protects the pancreatic cells leading to improved glycemic control. Although ascorbic acid therapy significantly increased the plasma insulin levels and decreased hyperglycemia, the efficacy was minimal. The present findings suggest that abrogating oxidative stress through ascorbic acid therapy alone is not enough to reduce the hyperglycemia, which is a major risk factor for HPA axis hyperactivity, oxidative stress, inflammation, and ultimately depression. To this end, in a recent study, we observed that a combination therapy consisting of metformin and ascorbic acid provides a better control over diabetes comorbid depression [48]. There are two major limitations to this study. First, this study did not evaluate the effects of ascorbic acid in nondiabetic control, depressed control, and diabetic control rats hence, unable to confirm whether the observed effects are specific for the diabetic condition. Second, this study did not estimate the levels of ascorbic acid and oxidative stress in the pancreas to ascertain the mechanism behind the minimal glycemic control achieved through exogenous ascorbic acid administration. Therefore, the above limitations need to be considered while relating the results to another context.

In conclusion, ascorbic acid therapy caused a submaximal attenuation of diabetes comorbid depression in streptozotocin-nicotinamide-induced diabetic rats. Ascorbic acid therapy was found highly efficacious in reducing oxidative stress but submaximal efficacy was observed against hyperglycemia and inflammatory response. Considering hyperglycemia as the major contributor to diabetes comorbid depression, further studies combining low doses of antidiabetic agents with ascorbic are warranted.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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