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An inhibitor of soluble epoxide hydrolase ameliorates diabetes-induced learning and memory impairment in rats

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Abstract

Background: Pharmacological inhibition of soluble epoxide hydrolase (sEH) enhances the synaptic function in the CNS and has a protective role in cognitive decline. We hypothesized that the sEH inhibitor TPPU might prevent the diabetes-induced decline in learning and memory which is associated with alteration in the level of neurotransmitters and oxidative stress.

Methods: Type 1 diabetes was induced in rats and the animals were treated with TPPU for 8 weeks. The learning and memory functions were assessed by the Barnes maze and a step-down test. Indicators of oxidative stress, levels of neurotransmitters, and activity of acetylcholinesterase were measured in the discrete regions of the brain.

Results: Our results revealed that treatment with TPPU significantly improves learning and memory performance in diabetic rats along with decreasing the level of blood sugar. Moreover, treatment with TPPU significantly prevented the diabetes-induced alteration in levels of neurotransmitters, the activity of acetylcholinesterase and preserved anti-oxidant defence system.

Conclusion: Inhibition of the sEH alleviates diabetes-induced decline in learning and memory.

Keywords

Cognitive impairment; Diabetes; Neurotransmitter GABA; NE and DA; sEH Inhibitor TPPU; Oxidative stress markers GSH and MDA

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Conflict of Interest:

The University of California, Davis holds multiple patents on pharmacological use of sEH inhibitors. Dr. Bruce D. Hammock founded Eicosis LLC. for advancing the clinical use of sEH inhibitors.

1. Introduction

Diabetes mellitus (DM) is one of the most common metabolic diseases whose occurrence and prevalence is increasing. It is among the diseases with high rates of complications which significantly lower the quality of life in patients. Type 1 diabetic patients have deficits in executive function, general intelligence, attention and memory [1].

The hippocampus, a crucial part of the brain for learning and memory, is highly sensitive to uncontrolled peripheral hyperglycaemia. A decrease in general cognitive performance, memory impairment, and atrophy are observed in diabetes [2]. The uncontrolled hyperglycaemia endorses the production of advanced glycation end products (AGEs), a critical mediator in the pathogenesis of diabetic complications [3]. AGEs by binding to their membrane receptors provoke the pro-oxidant and pro-inflammatory environment [4], which imparts neuronal degeneration [5]. Further, diabetes-induced neuronal loss and the allied neurotransmitter alterations are crucial steps leading to impaired cognitive functions. In addition to this risk, the high blood pressure associated with diabetes also leads to shrinkage of the brain and cognitive decline [6]. Pharmacological interventions including reduction of oxidative stress and protecting neurons have shown beneficial effects in diabetes-induced cognitive impairment [7, 8, 9].

Epoxyeicosatrienoic acids (EETs) and other epoxy fatty acids are signalling molecules formed by cytochrome P450 epoxidation of arachidonic acid. Many studies suggest that EETs are beneficial in diabetes, hypertension and in inflammatory diseases [10, 11]. These EETs are generally short-lived because these lipid mediators are easily converted into inactive diols by the enzyme soluble epoxide hydrolase (sEH). Inhibition of this enzyme enhances therapeutic actions of EETs. Selective sEH inhibitors possess multiple pharmacological activities including antihypertensive, analgesic, anti-inflammatory and other effects which protect the brain, heart and kidney from pathological insult [12-16]. Effects of the sEH inhibitors in diabetic cognitive impairment are still unknown. So, in this study, we evaluated the protective activity of the sEH inhibitor, TPPU in streptozotocin (STZ)-induced cognitive impairment in rats. Further, we have quantified the levels of key neurotransmitters including γ-Aminobutyric acid (GABA), dopamine (DA) and norepinephrine (NE/NA, also called noradrenaline), and activity of acetylcholine esterase (AChE) which regulates level of neurotransmitter acetylcholine (ACh).

2. Materials and methods

2.1 Chemicals

The soluble epoxide hydrolase inhibitors were synthesized as described previously [17]. STZ (MP Biomed, India) and glucose kit (Span diagnostic, India) were purchased. All other chemicals and reagents used were of analytical grade.

2.2. Animals and vivarium condition

Male Wistar albino rats aged 3 months were procured from the in-house animal facility of Al-Ameen College of Pharmacy, Bangalore, India. The animals were housed under general laboratory conditions of temperature (25±1°C) and relative humidity (55–75%) with a 12: 12

h light–dark cycle. The animals were fed with standard pellet diet and water *ad libitum*. All the experiments were performed with a prior approval from the Institutional Animal Ethics committee and study protocol complied with Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA) guidelines for the care and use of animals. A complete effort was made to alleviate the stress to the animals.

2.3. Induction of Experimental Diabetes and Treatment

Type 1 diabetes was induced by injecting a single dose of STZ (dissolved in cold citrate buffer, pH 4.5, 52 mg/kg, i.p.) to rats fasted overnight [18, 19]. We standardized the dose and effect of food on diabetogenic potential of STZ on rats before start of the study. Administration of the moderate dose of STZ (52 mg/kg) to fasting animals ensured robust induction of diabetes without mortality. The development of diabetes was confirmed by assessing fasting serum glucose post 72 h injection of STZ using a glucose assay kit (Span Diagnostics Ltd., India) [20]. The rats with glucose levels of 250- 350 mg/dl were considered as diabetic and included in the study. This day was considered as day 1. The rats were divided into four groups with eight animals in each group. Group 1 and 2 animals received the vehicle and served as normal control and diabetic control, respectively. The group 3 and 4 animals received TPPU dose of 0.1 and 0.3 mg/kg, respectively for 8 weeks. The doses of TPPU were chosen based on its efficacy to reduce inflammation in our previous studies [15, 16]. Insulin (3 IU/kg, s.c.) was administered twice in a week to diabetic rats to prevent mortality throughout the study. Body weight, blood glucose, and behavioural evaluations were carried out in all animals

2.4 Behaviour test

All behaviour tests were carried out in all animals (n=8, each group) in a sound proof psychopharmacology laboratory between 10 am to 12 pm. The animals were brought to the lab 2 h before the start of experiment to avoid anxiety in animals. The behavioural studies were performed by another experimenter who was blinded about the grouping and treatment.

2.4.1 Barne's maze test—Barne's maze test is used to assess spatial memory, has similarities to Morris Water Maze and to radial arm maze task, but no strong aversive stimuli are applied during the test [21]. Strong aversive stimuli like water or shock are likely to produce stress in the animal, influencing the performance of the task. With the Barnes maze, animals receive reinforcement to escape from the open platform surface to a small dark recessed chamber located under the platform called an “escape box”. The method was performed according to the method of Rosenfeld et al [22].

2.4.2. Step down test—The passive avoidance response was assessed in an apparatus with the dimension of 34 cm × 34 cm × 20 cm. It consisted of grid floor through which a 20 mV electric shock was delivered. In the centre of the chamber, a shock-free zone (SFZ) was placed. After the 8 weeks of treatment, rats were exposed to a 5-min learning course, during which they were permitted to move freely throughout the chamber before being placed on the platform. If the animals stepped down from the platform, they were exposed to an electric foot shock (36 V, AC). After 24 h of trial, test was evaluated to measure step down latency [23].

2.5. Biochemical parameters

After the behaviour test, fasting blood glucose level was measured in all animals. Then animals were further subdivided into 2 subsets (n=4) for measuring (i) activity of AChE and the level of neurotransmitters in the discrete regions of brain and (ii) oxidative stress in whole brain. Memory is dependent on the optimum levels of neurotransmitters in the hippocampus and cerebellum of brain, and the levels of neurotransmitters are altered in memory dysfunction including diabetes-induced memory dysfunction. Whole brain of the diabetic is subjected to oxidative stress. Therefore, we measured the levels of neurotransmitters in the discrete regions of the brain whereas the oxidative parameters were studied in the whole brain.

2.5.1. Plasma glucose levels—Blood was collected from the tail vein in microcentrifuge tubes containing heparin. Plasma was separated and blood glucose was estimated by GOD–POD kit as per the manufacturer's instructions.

2.5.2 Quantification of activity of acetylcholinesterase (AChE) and level of neurotransmitters—After the completion of behavioural test, the animals were sacrificed, brains (n=4) were isolated and different parts of brains were dissected out immediately and stored at −80 °C.

The hippocampus were homogenized separately in 0.1M phosphate buffer and used for quantification of activity of AChE and the level of GABA. The activity of AChE was measured according to a published method [24]. The levels of GABA in homogenates were measured by its reaction with ninhydrin in the presence of glutamate to form a fluorescent product which was measured by a spectrofluorometric method [25].

The cerebellums were homogenized separately in 25 ml of HCl–butanol (0.85mL of concentrated HCl was added to 1L of N-butanol) for about 1 min, then centrifuged for 10 min at 2000 rpm. The aqueous phase was used for noradrenaline (NA) and dopamine (DA) quantification by a spectrofluorometric method [26].

2.5.3 Measurement of oxidative stress—Brains of the remaining animals (n=4) were dissected out, washed with saline, chopped over ice and homogenates (10%, w/v) were prepared with 0.1M (pH 6.8) phosphate buffer. The homogenates were centrifuged at 3,000 g for 10 min at 4°C using Sorvall™ refrigerated centrifuge and the supernatants were used for estimation of oxidative stress. Lipid peroxidation or formation of malondialdehyde (MDA) was quantified by the method of Slater and Sawyer [27]. Glutathione (GSH) was quantified by the method of Moron et al [28].

2.6. Statistical analysis

Results are expressed as mean ± standard error of mean (S.E.M) of 4-8 observations using 4-8 mice. Statistical significance with respect to diabetic control was evaluated using one way ANOVA followed by Dunnett's test using GraphPad Prism 5 (Version 5.0, GraphPad Software Inc., San Diego, CA).

3. Result

3.1. TPPU alleviates diabetes-induced decline in cognitive function

Memory retention was assessed after 5 days of training and the data were analyzed using a repeated-measures ANOVA.

In the Barnes maze test, the diabetic rats had difficulty in locating the escape box and there was a significant decrease in latency to reach escape box by the diabetic rats compared to normal rats. Administration of TPPU had a dramatic effect in minimizing the memory impairment in a dose-dependent manner (Fig. 1A).

In the step down test, the step down latency (SDL) of diabetic rats was significantly shorter than that of normal control animals. The shorter SDL was significantly reversed by treatment of TPPU in a dose-dependent manner (Fig. 1B).

3.2. TPPU decreases fasting blood glucose level of diabetic rats

Compared with normal rats (97±1), a significant (*** $p < 0.001$) higher level of blood glucose (309±6 mg/dl) was observed in diabetic rats throughout the experiment. However, level of fasting blood glucose (FBG) was significantly (*** $p < 0.001$) decreased to 279±4 mg/dl and 219±5 mg/dl with administration of 0.1 mg/kg and 0.3 mg/kg of TPPU, respectively on day 56.

3.3. TPPU minimizes a decrease in the activity of AChE

The activities of AChE in the hippocampus of diabetic rats were significantly reduced in comparison to the normal control rats. Treatment with TPPU significantly ameliorated the reduction in the activities of AChE in the hippocampus samples (Fig. 2A).

3.4. TPPU minimizes an alteration in the level of neurotransmitter

The levels of GABA in the hippocampus samples of diabetic rats were significantly increased in comparison to the normal rats. Treatment with TPPU significantly reduced levels of GABA in hippocampus samples in a dose-dependent manner (Fig. 2B).

The levels of NE were reduced while the levels of DA were increased significantly in the cerebellum samples of the diabetic rats when compared to the normal rats. Treatment with TPPU significantly ameliorated the reduction in the levels of NA in comparison to untreated diabetic rats (Fig. 2C). The levels of DA in the cerebellum samples of the diabetic animal was significantly reduced by TPPU in comparison to untreated diabetic rats in a dose-dependent manner (Fig. 2D).

3.5. TPPU decreases oxidative stress in the brains of diabetic rats

The level of MDA was significantly increased in the whole brain of the diabetic rats in comparison to normal control rats. However, treatment of TPPU significantly prevented the increase in the level of MDA in a dose-dependent manner (Fig. 3A).

Depletion of reduced glutathione was observed in the whole brain of diabetic rats compared to normal control rats. However, treatment with TPPU significantly prevented the depletion of reduced glutathione in a dose-dependent manner (Fig. 3B).

4. Discussion

In this study, we demonstrated that the sEH inhibitor TPPU ameliorates the diabetes-induced cognitive impairment in rats with parallel decrease in the level of blood glucose. Type 1 diabetes was induced by STZ which is an extensively used model for the study of diabetes and diabetic complications. Persistent hyperglycaemia significantly limited the cognitive function as observed in behavioral tests. Cognitive performance was evaluated by the Barne's maze and a step down test. Impairment of spatial memory in the diabetic animal was indicated by prolonged latency period in the Barne's maze. Step down latency was shorter in the diabetic animals which indicates memory impairment in these animals. However, when the rats were treated with sEH inhibitors, a reduction in memory impairment was observed. Interestingly, the sEH inhibitor, TPPU ameliorated the negative impact of diabetes on spatial memory as noticed by the lowest latency to locate the escape box. The positive effect of TPPU on memory could be attributed to its efficacy to lower hyperglycemia, modulate the level of neurotransmitter and ameliorate oxidative stress associated with hyperglycemia.

Hyperglycaemia is the one of the reasons for the development of cognitive impairment in type 1 diabetic patients [29]. A good glycaemic control is known to delay cognitive decline. In our study, the treatment with TPPU decreased fasting blood glucose level of diabetic rats. Thus, antihyperglycemic effect of sEH inhibitor might have alleviated the cognitive decline caused by diabetes. Biessels et al. [30] reported that preventative administration of insulin (2-4 IU/day) to diabetic rats ameliorated dysfunction in spatial memory in water maze test with parallel decrease in hyperglycaemia by 3 fold. In our experiment, we observed marginal decrease in hyperglycemia, therefore, the amelioration of memory dysfunction could be due to effect of TPPU on hyperglycemia in addition to its direct effect of on CNS. Microvascular disease [31], and altered cerebral hemodynamic are few of the potential mechanisms [6] responsible for low cerebral blood perfusion and subsequent memory dysfunction in diabetes [13, 32]. Pre-treatment of TPPU to diabetic rat is known to increase the relaxant response of ACh on aorta *ex vivo* [13] and 5, 6-EET is known to dilate cerebral arterioles [33]. Therefore, TPPU also might have ameliorated memory dysfunction by dilating arterioles and increasing blood perfusion in the brain via stabilization of EETs. Diabetes-induced memory dysfunction is associated with cerebrovascular and neuronal inflammation. Hyperglycaemia-mediated release of inflammatory cytokines including TNF-α cause the serious neuronal damages in the hippocampus [34-36]. TPPU-mediated [15, 16] decrease in the level of TNF-α might be responsible for its protective effect in cognitive decline.

In addition to the changes in behaviour associated with altered glucose metabolism, clinical and experimental studies established a negative correlation with alteration in the level of neurotransmitters and cognitive functions.

Dysfunction of cholinergic transmission is evidently observed in neurological disorders and dementia. In non-diabetic models, inhibition of cholinergic activity, decrease and increase in

level of ACh is reported to alter memory function [37]. Administration of ACh receptor blocker, scopolamine (30 μg), to 16 month old Lewis rats in the hippocampus by Blokland et al, [38] decreased the spatial learning process assessed using Morris water-escape task. Administration of scopolamine (3 nmol) by Elvander et al. [39] to medial septum of Sprague-Dawley rats decreased the level of hippocampal ACh and impaired spatial learning in Morris Water Maze test. Administration of cholinomimetic drug carbachol (0.5-1 μg) by same researchers in medial septum increased the release of ACh in hippocampus with parallel decrease of spatial memory. Bunce et al. [40] also reported an impairment of memory assessed by via radial maze model in Sprague-Dawley rats after injection of carbachol (12.5 - 125 ng) to septum. The level and activity of AChE regulates the level of ACh via its metabolism, so AChE can influence memory. In many studies involving evaluation of memory and activities of AChE in brain of diabetic rats report both increase and decrease in the activity of AChE. Schmatz et al. [41] reported about impairment of memory in Wistar rats with an increase in the activity of AChE in the cerebral cortex, hippocampus, striatum, cerebellum, hypothalamus and blood of the rats after 37 days of induction of diabetes by single intraperitoneal administration of 55 mg/kg of STZ. Maciel et al [42] also reported an instance of memory dysfunction in Wistar rats with an increase in activity of AChE in the supernatant of homogenate of cerebral cortex and hippocampus after 50 days of induction of diabetes by intraperitoneal injection of STZ (70 mg/kg). Similarly, Zhou et al. [43] reported memory impairment in Wistar rats with a decrease in the level of ACh in cerebrospinal fluid from day 15 till day 75 after single intraperitoneal administration of STZ (1%). Contrary to these reports, diabetes-induced decrease in activity of AChE is also reported. Jangra et al. [24] reported a cognitive deficit in Sprague–Dawley rats with decrease in the activity of AChE in the homogenate of hippocampus after 8 week of induction of diabetes by administration of STZ (55 mg/kg, i.p.). Ramkumar et al [44] reported a decrease in the activity of AChE in the in the brain of Wistar rats after 12 week of administration of single dose of STZ (100 mg/kg) to neonatal rats though the researchers did not evaluate memory function. We noticed memory dysfunction in Wistar rats after 8 week of induction of diabetes with a parallel decrease in the activity of AChE in the homogenate of hippocampus. High concentration of free radicals in diabetes decrease the membrane fluidity which can influence the activity of AChE [44]. Reduced activities of AChE in the diabetic rat brain results in an accumulation of ACh resulting in cholinergic hyperactivity and neuronal damage. The differential effect of hyperglycemia on activity of AChE could be due to difference in the level of blood glucose level (450 mg/dl vs 300 mg/dl), timing of treatment (adult vs neonatal), difference in strain of rats (Wistar vs Sprague-Dawley) and whether animals were treated with insulin or not.

GABA also influences memory. In our study, GABA levels were found to be upregulated in the hippocampus of diabetic rats and these findings are in line with the report of Sharma et al [45]. GABAergic inhibition has a role in the control of many behaviors such as anxiety, depression, mood, and cognition. A possible mechanism of how GABA affects memory is through its general inhibitory function [46]. The ability of TPPU to decrease the level of GABA might be responsible for its positive effect on memory in diabetic rats.

Norepinephrine enhances attention, and retrieval of both long-term and working memory, and enhances the ability of the brain to respond to inputs [47]. The decrease in the level of

NE in the cerebellum of diabetic animals in our study is in harmony with the results of Ramakrishnan et al. [48]. It is known that deficiency of NE in the brain results in the development of depression-like symptoms [49]. Recently, Ren et al. demonstrated that the sEH inhibitor TPPU confer prophylactic antidepressant effects in the social defeat stress models of depression indicating a direct effect of this compound in CNS [50]. In our study, the treatment with the sEH inhibitor minimized a reduction in the level of NE in cerebellum of the diabetic rat.

An increase in the level of cerebellar DA in experimental diabetes has already been reported earlier [26] which is in agreement with the study. A possible explanation for the increasing levels of the DA could be related to the activity of monoamine oxidase (MAO). Mayanil et al [51] reported a decrease in the activity of MAO at 3 and 8 days after the induction of diabetes. This supports the concept that elevation in the level of DA in diabetic rats is probably due to a decrease in the metabolism of DA in these animals due to a relative deficiency of MAO.

Apart from the effect of TPPU on the level of neurotransmitter, the sEH inhibitor might be strengthening long-term potentiation (LTP) which is a continuous increase in the strength of synapses that are essential for memory. Studies have shown that STZ-induced diabetes negatively affects LTP [52] and results in synaptic plasticity which is one of the possible mechanisms underlying learning and memory [53]. Recent experimentation in an animal model indicated pharmacological inhibition of sEH by TPPU in mice enhances the LTP which could be also one of the mechanism for its protective effect in diabetic cognitive impairment [54]. Vascular cognitive impairment is associated with increase in the activity of sEH, a decrease in its substrates, EETs and an increase in the resulting diols or dihydroxyeicosatrienoic acids (DHETs) [55]. Thus, inhibition of the sEH activity by TPPU may account for its beneficial effect against diabetes-induced cognitive impairment. Interestingly, the LTP is negatively modulated by oxidative stress [56, 57].

Depletion of antioxidant enzymes with an increase in free radical generation is a key mechanism involved in diabetes-induced cognitive impairment. Previous study indicates that the depletion of antioxidant enzymes make neurons and astrocytes more susceptible to injury in STZ-induced diabetes. In the present study, the level of MDA, a significant marker for lipid peroxidation is increased while the level of GSH, a potent endogenous antioxidant is reduced in the brain of diabetic rats. These results are in line with the previous findings of Kuhad et al [58]. Interestingly, the treatment with the sEH inhibitor decreased the level of MDA and increased the level of GSH in diabetic rats. The study of Krishnamurthy et al showed that administration of a sEH inhibitor decreased formation of lipid peroxides and increased level of antioxidant in the heart [59]. Ultimately, hyperglycemia is known to increase the level of oxidative stress [60, 61].

With our current level of understanding, it is not possible to pinpoint a single step in the process by which hyperglycemia accelerates cognitive impairment nor can we determine which are the key mechanism by which TPPU ameliorated the process and by implication epoxy fatty acids. TPPU does cross the blood brain barrier based on the liquid chromatography-mass spectroscopy and biological studies but it does not accumulate in the

brain above levels in the plasma. Therefore, the efficacy of TPPU in alleviating diabetes-induced memory dysfunction could be, at least in part, due to its central effect unlike the earlier sEHI (UC1153 or AR9281) that fails to cross the blood brain barrier. The peripheral effect of the TPPU cannot be ruled out since sEHI are known both to improve insulin production thus reducing diabetes and also to reduce several co morbidities of diabetes by decreasing the endoplasmic reticulum stress initiated by diabetes. Future works are necessary to evaluate whether or not this protective effect of a sEH inhibitor is mediated via central, peripheral or both central and peripheral effects. Use of a sEH inhibitor which does not penetrate blood brain barrier and use of standard anti-hyperglycemic control may be particularly useful in this context.

In conclusion, our findings identified a possible new therapeutic potential of sEH inhibitor, TPPU in ameliorating diabetes-induced cognitive impairment.

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Highlights

1. Streptozotocin-induced diabetes resulted in cognitive dysfunction of rats which were evaluated with Barnes maze and step down test.
2. Diabetes-induced memory impairment was associated with an increase in the level of oxidative stress.
3. An alteration in the level of neurotransmitters in the brain including GABA, dopamine and norepinephrine, and activity of acetylcholinesterase which regulate memory was observed in diabetes.
4. Treatment of the soluble epoxide hydrolase inhibitor TPPU ameliorated the diabetes-induced memory impairment with a parallel decrease in blood glucose level. In addition, TPPU also minimized the alteration in the level of neurotransmitters and activity of acetylcholinesterase.

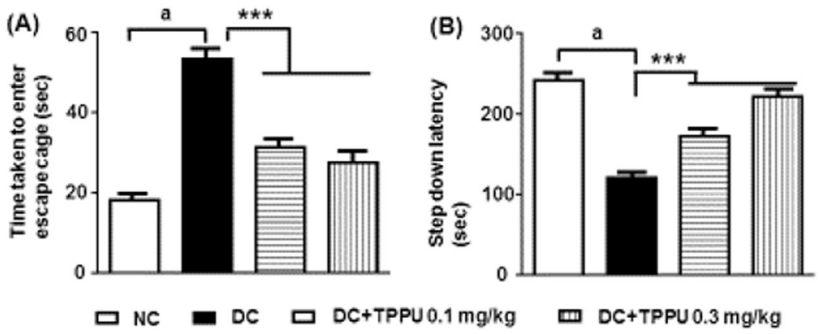

Figure 1:

TPPU decreases diabetes-induced memory dysfunction. Diabetes-induced significant (^{a} $p < 0.001$) memory dysfunction is evident due to increase in time taken to enter escape box (A) and decrease in step-down latency (B) in comparison to normal control animals. Treatment of TPPU significantly increased (*** $p < 0.001$) memory in comparison to diabetic control rats. NC: normal control, DC: diabetic control. Values are represented as mean ± SEM of eight individual observations from 8 animals. One way ANOVA followed by Tukey's multiple comparison test was used for statistical calculation.

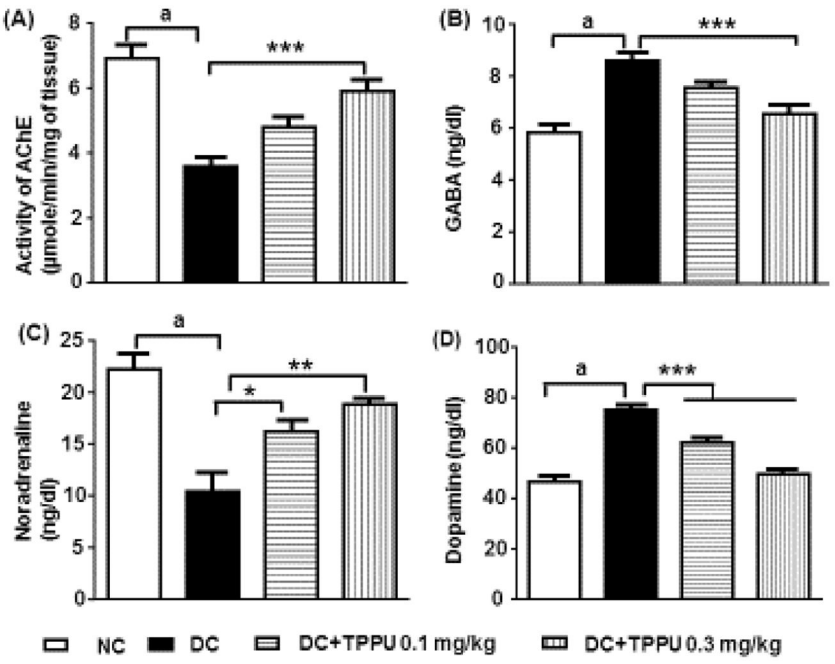

Fig. 2.

TPPU minimizes the alteration in activity of AChE and levels of neurotransmitter in the brain of diabetic rats. (A) The activity of AChE was significantly ($^a p < 0.001$) decreased in the brain of diabetic rats in comparison to normal rats. Administration of TPPU (0.1 and 0.3 mg/kg) significantly prevented the decrease in the activity of AChE in comparison to untreated diabetic rats. (B) The level of GABA in the brain was increased significantly ($^a p < 0.001$) in diabetic rats with respect to normal control rats. (C) In comparison to normal control rats, the level of NA was decreased significantly ($^a p < 0.001$) in the brain of diabetic rats, whereas (D) the level of DA was significantly increased ($^a p < 0.001$) in the brain of diabetic rats when compared to that of normal control rats. Treatment of TPPU to diabetic rats significantly decreased the level of GABA (*** $p < 0.001$) and DA (*** $p < 0.001$) whereas increased (* $p < 0.05$, ** $p < 0.01$) the level of NA in the brain of diabetic rats in comparison to untreated diabetic rats. NC: normal control, DC: diabetic control. Values are represented as mean ± SEM of four individual observations from 4 animals. One way ANOVA followed by Tukey's multiple comparison test was used for statistical calculation.

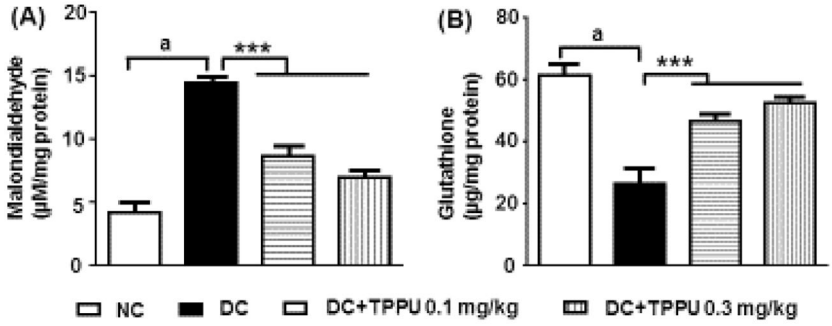

Fig. 3.

TPPU decreases oxidative stress in the brain of diabetic rats. Oxidative stress was increased in the brain of diabetic rats which was evident from significant increase in the level of MDA (^{a} $p < 0.001$) (A) and depletion (^{a} $p < 0.001$) in the level of glutathione (B) in comparison to the normal control rats. Treatment of TPPU decreased the level of MDA (*** $p < 0.001$) and increased the level of glutathione (*** $p < 0.001$) in comparison to diabetic rats. NC: normal control, DC: diabetic control. Values are represented as mean ± SEM of four individual observations from 4 animals. One way ANOVA followed by Turkey's multiple comparison test was used for statistical calculation.