



Original article

Minocycline reverses diabetes-associated cognitive impairment in rats

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ABSTRACT

Background: Minocycline a tetracycline antibiotic is known for anti-inflammatory and neuroprotective actions. Here we determine the therapeutic potential of minocycline against type 2 diabetes associated cognitive decline in rats.

Methods: High fat diet (HFD) and low dose streptozotocin (STZ; 25 mg/kg) were used to induce diabetes in Sprague-Dawley rats. Fasting blood glucose and haemoglobin (Hb) A1c were measured in these animals. Cognitive parameters were measured using passive avoidance and elevated plus maze test. Hippocampal Acetylcholine esterase (AChE), reduced glutathione (GSH), cytokines, chemokine levels were measured and histopathological evaluations were conducted. The diabetic animals were then given minocycline (50 mg/kg; 15 days) and the above parameters were reassessed. MTT and Lactate dehydrogenase (LDH) assays were conducted on neuronal cells in the presence of glucose with or without minocycline treatment.

Results: We induced diabetes using HFD and STZ in these animals. Animals showed high fasting blood glucose levels (>245 mg/dl) and HbA1c compared to control animals. Diabetes significantly lowered step down latency and increased transfer latency. Diabetic animals showed significantly higher AChE, Tumor necrosis factor (TNF)- α , Interleukin (IL)-1 β and Monocyte chemoattractant protein (MCP)-1 and lower GSH levels and reduced both CA1 and CA3 neuronal density compared to controls. Minocycline treatment partially reversed the above neurobehavioral and biochemical changes and improved hippocampal neuronal density in diabetic animals. Cell line studies showed glucosemediated neuronal death, which was considerably reversed upon minocycline treatment.

Conclusions: Minocycline, primarily by its anti-inflammatory and antioxidant actions prevented hippocampal neuronal loss thus partially reversing the diabetes-associated cognitive decline in rats.

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Introduction

Diabetes is a complex disorder with disruptive hyperglycemia leading to secondary complications including cognitive impairment, especially in elderly patients [1]. Chronic hyperglycemia can lead to inflammation [2] as shown by a rise in the plasma cytokines like Tumor necrosis factor (TNF)- α , Interleukin (IL)-1 β and IL-6 in diabetic individuals [3,4]. Insulin resistance during diabetes and associated metabolic disorders like obesity, and dyslipidemia also fuels inflammatory pathways and oxidative stress leading to the development of chronic secondary complications [5]. Chronic inflammation and oxidative stress may lead to cellular damage [6] and potentiate the progression of diabetes associated microvascular or macrovascular complications [7]. Prolonged hyperglycemia also results in the production of

advanced glycation end products (AGEs) which contribute towards neurodegeneration [8–10]. The AGEs also stimulate inflammatory pathways which further enhance oxidative stress, ultimately damaging the neurons [9]. Oxidative stress also progresses due to a decrease in the expression and function of antioxidants, thus making the cells more vulnerable to oxidative insults [11,12]. Diabetes associated central nervous system (CNS) inflammation is associated with microgliosis and astrocyte activation [13]. Activated microglial cells undergo morphological alterations and change from resting ramified to activated type and release various neurotoxic molecules such as proinflammatory cytokines (IL-1 β , TNF α and IL-6), nitric oxide, reactive oxygen species, and hydrogen peroxide, all of which have detrimental effect on neurogenesis and neuronal viability [14–21]. Astroglia primarily results in dysregulation of synaptic glutamate homeostasis, eventually contributing to excitotoxic neuronal damage [22]. The high-fat diet and low dose streptozotocin (STZ) model of type 2 diabetes (T2D) involves feeding the animals with a fatty diet followed by STZ injection. The high fat diet yields insulin resistance and/or

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glucose intolerance while β -cell toxin STZ impairs the secretion of insulin from β -cells. This combination leads to hyperglycemia and insulin resistance resembling the human form of T2D [23,24]. High-fat diet alone has also been reported to impair learning ability in rats [25,26]. Diabetes may also increase AchE and reduced glutathione (GSH) levels in rat brain [27–29]. Minocycline is a second-generation semisynthetic tetracycline-antibiotic, which produces anti-inflammatory and anti-apoptotic actions in the CNS even at low doses because of its high lipid solubility and ability to cross the blood-brain barrier [30,31]. Its antioxidant property has been linked to its neuroprotective and neurorestorative functions as well as hippocampal neurogenesis and synaptogenesis [32]. Minocycline reduces activation of microglia and astrocytes thus inhibiting the release of neurotoxic agents like nitric oxide, TNF- α , and IL-1 β [33–35]. Minocycline can protect neurons and prevent glial dysfunction in diabetic retinopathy [36]. It has also been shown to reduce inflammation and prevent excitotoxicity [32] in cerebral ischemia/reperfusion injury [37,38]. Minocycline may also reverse the loss of cholinergic neurons as shown in a recent study using an animal model of Down's syndrome [39]. In this article, we will show the neuroprotective effect of minocycline against T2D associated cognitive decline in rats.

Materials and methods

Sprague–Dawley (SD) male rats weighing 160–180 g were obtained from National Institute of Nutrition Hyderabad and housed for acclimatization at the Institute's animal house at controlled room temperature ($22 \pm 2^\circ\text{C}$) and humidity ($55 \pm 5\%$) with 12:12 h light and dark cycle. The rats were given commercially accessible regular pellet food with libitum water before dietary modification. The guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India were followed and a prior agreement was considered from the institutional animal ethics committee (Approval No 1972/PH/BIT/2/17/1AEC) for conducting the experiment.

Animal model development

SD rats (Age 8–12 weeks) were divided into two diverse dietary regimens: one group received normal pellet diet (NPD; Amrut Diet, New Delhi, India) and the other group was given a high-fat diet (HFD i.e. NPD + 310 gm/kg Lard; 58% calories from fat); Baroda Earth Private Limited, Baroda). Animals separated into five distinct treatment groups: 1) Controls taking NPD, 2) Non-treated diabetic (HFD +25 mg/kg STZ; Sigma-Aldrich St Lewis, MI, USA), 3) Minocycline (Sun Pharmaceutical Industries Ltd, Mumbai, India) diabetic 4) Minocycline and 5) Scopolamine as amnestic agent (0.4 mg/kg) group. In the minocycline treated group diabetic animals were administered minocycline for the last 15 days while HFD continued for the entire period. The experimental scheme has been depicted in Fig. 1.

Diabetes induction and treatment

One month of dietary modification (HFD) made the animals insulin resistant; next, a single dose of Streptozotocin (STZ) (25 mg/kg) dispersed in citrate buffer (pH 4.4) was given *ip*

following 12 h of fasting. This was followed by 5% glucose solution to avoid hypoglycemic shock. One week after administration of STZ, blood glucose levels were estimated. Animals with fasting glucose of more than 250 mg/dl were considered to be diabetic. Glucose levels were estimated by tail prick each week for a month utilizing One Touch glucose strips (One Touch Ultra; Life Scan, Inc). Hemoglobin (Hb) A1c levels were likewise verified (Gluquant A1c; Meril Life Sciences Pvt Ltd). Minocycline treatment of 50 mg/kg [40] by oral gavage for 15 days was given to diabetic as well as to a group of non-diabetic animals.

Behavioural studies

Passive avoidance

In a passive avoidance test, increases in memory are correlative of prolongation or increase in step-down latency (SDL) [41]. Passive avoidance tests were conducted utilizing an apparatus which comprised of 2 chambers connected to an entryway. One compartment was brightly lit while the other one was kept dark. The two compartments were fitted with a metal grid floor which could be electrified *via* external controls (Medicraft Electromedical Pvt Ltd, Lucknow, India). Animals from each group ($n = 10$) were trained to enter the dark chamber first by placing them in the bright chamber. Once they entered the dark chamber, the entryway was closed. After this underlying conditioning, animals were tasked to move from the bright to the dark chamber, but this time the metal flooring was electrified (1 mA for 5 s). Animals which took more than 60 s to reach the dark chamber were excluded from the study. Following 24 h of training, retention time was measured on days 1, 2 and 7. The time taken by the animals to travel from the bright to the dark chamber with all the four paws in dark compartment i.e., step through latency was measured. During the observation time frame, no current was provided to the metal grid floor. Three successive readings were taken at an interim of 30 min while the cut off time for the experiment was 5 min.

Elevated plus maze

For this study, an elevated plus maze device (Medicraft Electro-medical Pvt Ltd, Lucknow, India) was utilized [42]. It comprises of two closed arms with side walls of ($500 \times 100 \times 250$ mm) and two open arms (500×100 mm). These arms stretched out from a central stage (100×100 mm), and the maze was raised to an elevation of 600 mm from the floor. Before training animals from each group ($n = 10$), they were allowed to acclimate to the maze for a 2-minute period; afterwards, the animals were kept on the end of the open arm facing away from the closed arms. The time taken by the animal to move from the open arm to the closed arms is referred to as transfer latency. Animals which displayed an initial transfer latency greater than 90 s were excluded from the investigation. Transfer latency of the animals of each group was estimated at day 1, 2 and 7.

Estimation of reduced glutathione (GSH)

Glutathione is a potent endogenous antioxidant that deactivates free radicals. Thus, high degrees of total glutathione are commonly seen and this protects the neuronal cells from oxidative

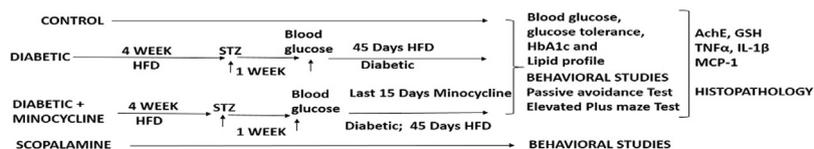


Fig. 1. Experimental work flow.

stress [43]. Using phosphate buffer (pH 8) with 1% Triton X brain tissue (hippocampal; $n = 6/\text{group}$) homogenates were prepared. First, 1 ml of 10% trichloro acetic acid and 1 ml distilled water were mixed with 1 ml tissue homogenate (all of which was kept on ice). The resulting mixture was then centrifuged at 15,000 rpm for 15 min at 4 °C. Next, 4 ml of 5' Dithiobis (2-Nitrobenzoic acid) or DTNB and 1.5 ml phosphate buffer were added to the supernatant and absorbance was estimated at 412 nm. Different concentrations of standard GSH were utilized to prepare the standard curve against which the GSH level was measured. Protein levels in the lysates were estimated using Lowry's protein estimation kit (Bio-Rad Laboratories, Inc).

Estimation of acetylcholinesterase activity (AChE)

AChE hydrolyses and deactivates acetylcholine, the principal neurotransmitter responsible for learning and memory. Therefore, decreased activity or levels of AChE have been associated with impaired cognition [44]. AChE was assessed by utilizing Ellman's technique [42,45] with slight alterations. Brain tissue (hippocampal) homogenate ($n = 6/\text{group}$; 10% w/v) was centrifuged at 15,000 rpm for 15 min at 4 °C. To 0.4 ml of supernatant aliquots, 2.6 ml phosphate buffer (pH 8, 0.1 M) was added; after that 100 μl of DTNB was taken. The absorbance of this solution was estimated at 412 nm. Acetylthiocholine iodide (0.075 M) 20 μl was then added to the above solution and blended thoroughly, again absorbance was estimated at 412 nm at 30 s intervals for 10 min. The change in absorbance per min was observed. One unit of AChE activity was described as the number of micromoles of Ach iodide hydrolysed/min/mg of protein.

Cytokine and chemokine estimation

Elevated pro-inflammatory cytokine concentrations in the CNS have typically been related to neuroinflammation [46]. Alterations in the hippocampal IL-1 β , TNF- α and, MCP-1 levels were evaluated for each group ($n = 6/\text{group}$). Levels of TNF- α , IL-1 β , and monocyte chemoattractant protein (MCP)-1 were estimated in supernatants from hippocampal homogenates utilizing particular cytokine ELISA kit based on manufacturer's guidelines (Sigma-Aldrich, St Louis, MO, USA).

Histopathology

Since hippocampal neuronal density and health are vital for cognition, histopathological analyses of hippocampal sections were conducted. Animals ($n = 4/\text{group}$) were sacrificed by cervical dislocation under ether anesthesia and the whole brain was extracted. Buffered formalin (10% for 2 days; pH 7.6) was used to keep isolated brain for fixation. Tissues were then dehydrated via immersion in increasing concentrations of alcohol (70% to 100%). The tissues were then placed in liquefied wax kept at 60 °C to 70 °C. Once the wax solidified, the tissue was segmented into 5 μm thin segments. Sections were mounted on glass slides and de-paraffinized applying reducing concentrations of alcohol to water (100% ethanol, two changes of 95% ethanol, one of 70% ethanol) so that water-soluble dye can penetrate into the tissue sections followed by rinsing with water. Slides were then treated with Hematoxylin (5 m) and eosin (10 m) stain, dehydrated with graded alcohol (95% and 100%), treated overnight with histoclear, and cover slipped using DPX mountant. For morphological analysis, five serial coronal sections of each mouse from each group were studied quantitatively. Neuronal counts in hippocampus and cortical layer with a microscope (BH-2, Olympus), were performed by two experimenters, blind to the treatments using microscope with final field of 1 mm². Counts were made in five adjacent fields and the

mean number extrapolated to give the total number of neurons per mm². The area size of hippocampus 2 mm from the midline of the brains were measured. CA1 and CA3 neurons were observed under optical microscopes for any basic structural modifications at 5X and 40X magnification. Neurons were counted by using Camera Lucida (The Western Electric and Scientific Works; Haryana India) under an optical microscope under 40X magnification and also using Image J software. Two methods were used in parallel for accurate neuronal count.

Cell viability assays (MTT and LDH)

After 7 days of stabilisation about $5 \times 10^4/0.2 \text{ ml}$ cells were plated in 96 well plates and treated with different concentrations of glucose (30, 60 and 120 mM). They were incubated for 24 h to determine the growth inhibiting activity of glucose in the neuroblastoma cell line. Concentrations of glucose that exhibited 60–70% cell viability were selected for minocycline studies. Different concentrations of minocycline (10, 50 and 100 μM) were added 30 min before glucose treatment (using concentrations which showed 60–70 % cell viability). Glucose induced neurotoxicity (primarily apoptotic) and minocycline mediated protection was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide dye (MTT) assay and Lactate dehydrogenase (LDH) assay.

Statistical analysis

The data were expressed as Mean \pm SEM, and were evaluated by one-way ANOVA followed by Tukey's multiple comparison tests. A value of $p < 0.05$ was considered to be statistically significant. All statistical analyses were carried out using Graph Pad Prism 5.0 software (GraphPad Software Inc. San Diego CA, USA).

Results

Blood glucose level

HFD-STZ animals successfully developed diabetes with high fasting blood glucose (>245 mg/dl). Mean HbA1c levels for controls were $6.5 \pm 0.1\%$ while HFD-STZ animals showed significantly higher ($p < 0.01$) levels at $10.5 \pm 0.2\%$. We have previously reported insulin resistance and hyperlipidemia in the same rat model of T2D [52]. In diabetic animals, fasting blood glucose (252 ± 6.5) and HbA1c levels ($9.6 \pm 0.6\%$) remained largely unaltered after minocycline treatment. Control animals also did not show significant change ($p > 0.05$) in fasting blood glucose (125 ± 8.2) and HbA1c ($6.7 \pm 0.8\%$) upon minocycline treatment.

Behavioral studies

In the passive avoidance test step down latency showed $F(4, 40) = 345.1$, $p < 0.001$. Scopolamine, an amnestic agent (0.4 mg/kg), was found to reduce SDL significantly ($p < 0.001$) compared to control animals. HFD-STZ-induced diabetic animals showed a significant two fold decline in SDL ($p < 0.001$) compared to controls and comparable with scopolamine treated animals suggesting cognitive decline. Diabetes-associated memory loss was partially reversed after 15 days of minocycline treatment as shown by a significant increase in SDL ($p < 0.001$) when compared to diabetic rats (Fig. 2B). Increase in memory reflects as reduced transfer latency (TL) in elevated plus maze test. In the elevated plus maze transfer latency showed

$F(4, 40) = 12.36$, $p < 0.002$. Administration of scopolamine showed a two fold increase in TL ($p < 0.01$) - i.e., rats failed to remember the task hence took longer to enter the closed arms.

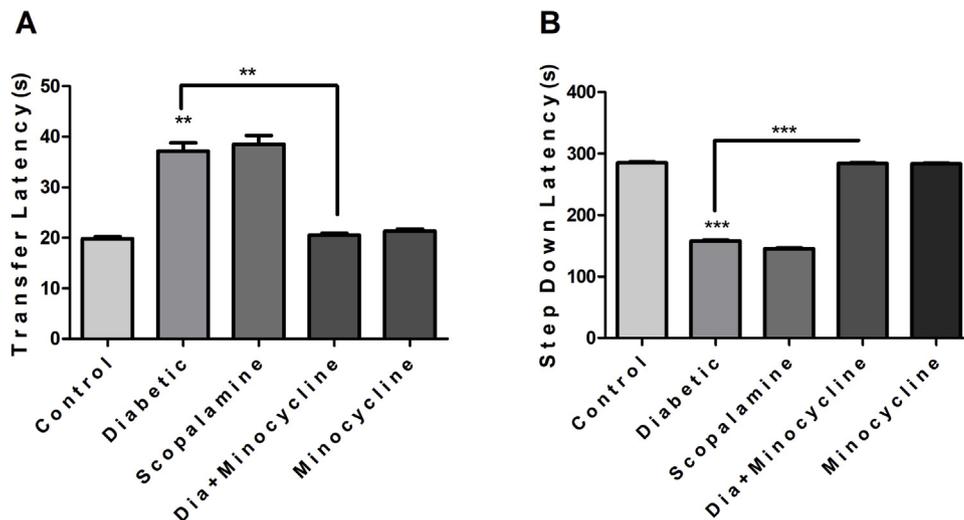


Fig. 2. Effect of type 2 diabetes and minocycline on transfer latency (A) and step down latency (B) in diabetic animals. Scopolamine and HFD-STZ resulted in significant increase ($p < 0.01$) in Transfer Latency (TL) and decrease ($p < 0.001$) in step down latency in diabetic (Dia) group when compared to control group. While minocycline led to reversal of diabetes (HFD+STZ) induced memory impairment as shown by significant decrease in TL ($p < 0.01$) and significant increase in SDL ($p < 0.001$) in minocycline treated group. All values are expressed as mean \pm SEM for $n=10$ /group. ** $p < 0.01$, *** $p < 0.001$.

Similar to the scopolamine group, diabetic animals also showed a significant increase in TL ($p < 0.01$) compared to controls indicating a cognitive decline. This was partially reversed upon minocycline treatment which showed a significant reduction in TL ($p < 0.01$) as compared to diabetic animals (Fig. 2A).

Biochemical estimation in hippocampal lysates

GSH

GSH estimation showed $F(3, 20) = 112.7$, $p < 0.001$. Diabetes led to a 1.5-fold reduction in hippocampal GSH levels ($p < 0.001$) compared to normal animals, suggesting increased hippocampal oxidative stress. However 15 days of minocycline treatment normalized ($p < 0.001$) the GSH levels. (Fig. 3B).

AchE

AchE studies showed $F(3, 20) = 76.1$, $p < 0.001$. Diabetic animals showed a two-fold increase ($p < 0.001$) in hippocampal AchE levels

which was partially reversed ($p < 0.05$) upon minocycline treatment (Fig. 3A).

Effect of minocycline on proinflammatory cytokines and chemokines

In the cytokine estimation, $F(3, 20) = 1254$, $p < 0.001$ and $F(3, 20) = 484.4$, $p < 0.001$ was found for TNF- α and IL-1 β respectively. While MCP-1 estimation showed $F(3, 20) = 243.4$, $p < 0.001$. HFD-STZ induced T2D animals showed a nearly four-fold surge in TNF- α ($p < 0.001$), IL-1 β ($p < 0.001$) and MCP-1 ($p < 0.001$) in the hippocampal lysates compared to controls. Minocycline could successfully reduce the MCP-1 concentrations ($p < 0.001$) to control levels found in healthy rat hippocampus. While minocycline treatment led to a two-fold reduction in the hippocampal TNF- α and IL-1 β ($p < 0.001$) levels in T2D animals. (Fig. 4)

Histopathology

In the histopathological studies neuronal count showed $F(5, 54) = 447.2$, $p < 0.001$. Hippocampal sections from diabetic animals

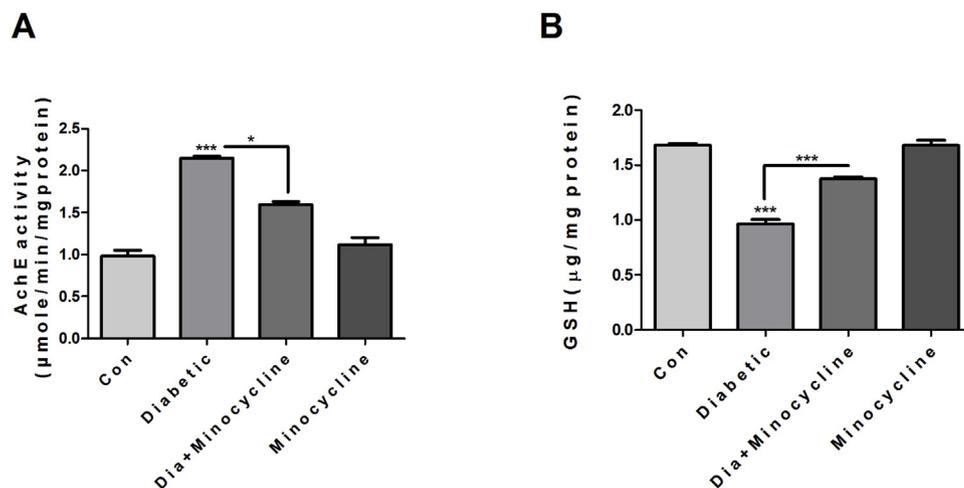


Fig. 3. Effect of type 2 diabetes and minocycline on AChE activity (A) and GSH levels (B) in hippocampal homogenate. HFD-STZ led to considerable decrease ($p < 0.001$) of GSH level. A significant increase ($p < 0.001$) in acetylcholine esterase (AChE) activity was observed in hippocampal homogenate when compared to control group. While post minocycline treatment increased ($p < 0.001$) reduced glutathione (GSH) level and also decreased the AchE activity ($p < 0.05$). All values are expressed as mean \pm SEM for $n=6$. * $p < 0.05$, *** $p < 0.001$.

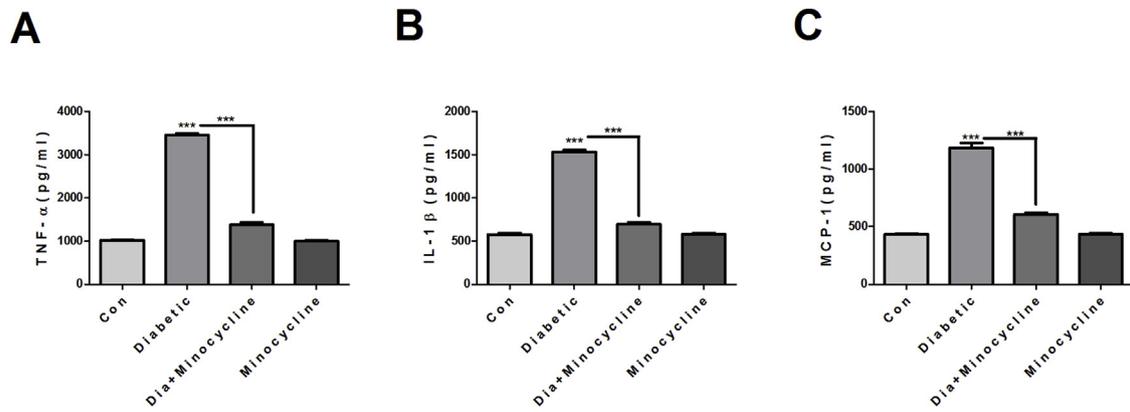


Fig. 4. Effect of type 2 diabetes and minocycline on levels of TNF- α (A), IL-1 β (B), and MCP-1 (C) in hippocampal homogenate. HFD-STZ led to significant increase ($p < 0.001$) in Tumour necrosis factor (TNF)- α , Interleukin (IL)-1 β and Monocyte chemoattractant protein (MCP)-1 levels in hippocampal homogenate when compared to control group. Considerable decrease ($p < 0.001$) in the cytokines and chemokines level was observed after minocycline treatment. All values are expressed as mean \pm SEM for $n = 6$ /group. *** $p < 0.001$.

confirmed the presence of diabetes associated neuronal damage as revealed by a significant decline in the number of CA1 ($p < 0.001$) and CA3 neurons ($p < 0.01$). Interestingly, 15 days of minocycline treatment partially restored neurons in both CA1 ($p < 0.01$) and CA3 ($p < 0.01$) regions, thus confirming the neuroprotective actions of minocycline against diabetes associated damage (Fig. 5). At 40X magnification, control animals showed the presence of several layers of well-toned neurons in conjunction with minimal presence of microglial cells. By contrast, severe microgliosis along with neuronophagia was observed in T2D hippocampal sections, which could explain the reduction in the neuronal count and poor neuronal health in T2D rat brain. However, the minocycline-treated HFD-STZ group showed reduced infiltrations of microglial cells and improved neuronal arrangement, suggesting improved

health and tonicity of the T2D hippocampal neurons after minocycline treatment (Fig. 6).

Cell viability assay

Neuronal treatment with various concentrations of glucose using the IMR-32 cell line displayed cytotoxic effects of glucose. MTT and LDH assays showed a dose dependent decrease in cell viability with an increase in glucose concentration. 60 mM of glucose showed 62% cell survival and 43% cytotoxicity by MTT and LDH assay respectively which was used for further studies. (Fig. 7) However, 5 μ M minocycline pre-treatment significantly reduced ($p < 0.001$) the glucose mediated neurotoxicity as shown by LDH assay. (Fig. 7). LDH assay with minocycline treatment showed $F(3, 36) = 985.4$, $p < 0.001$. Minocycline alone showed little toxicity at

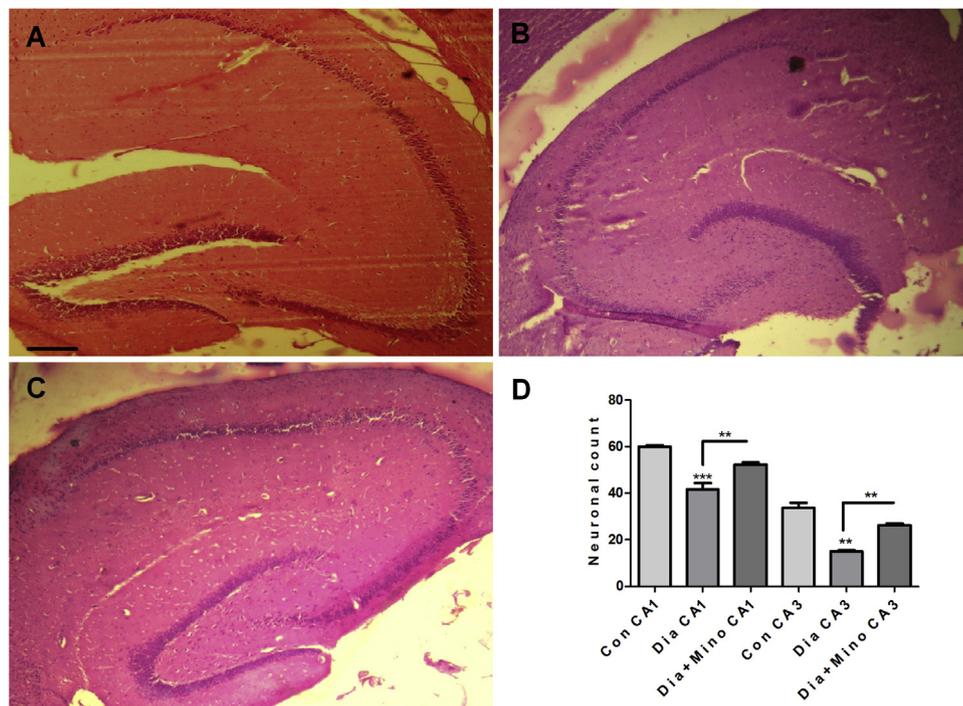


Fig. 5. Photomicrographs of hippocampal sections and neuronal count. A) Control B) Diabetic and C) Minocycline treated diabetic group. D) A significant decrease ($p < 0.001$ and $p < 0.01$) in neuronal count of CA1 and CA3 region respectively of HFD-STZ rat brain was observed when compared with control brain observed under 40X magnification. Minocycline treatment partially reversed this reduction of neuronal count ($p < 0.01$) in diabetic rat hippocampal regions. Representative images showing H and E stained hippocampus regions (CA1 and CA3) of control, diabetes and minocycline treated diabetic groups (5X). Scale bar = 500 μ m. All values are expressed as mean \pm SEM for $n = 4$ /group. ** $p < 0.01$, *** $p < 0.001$.

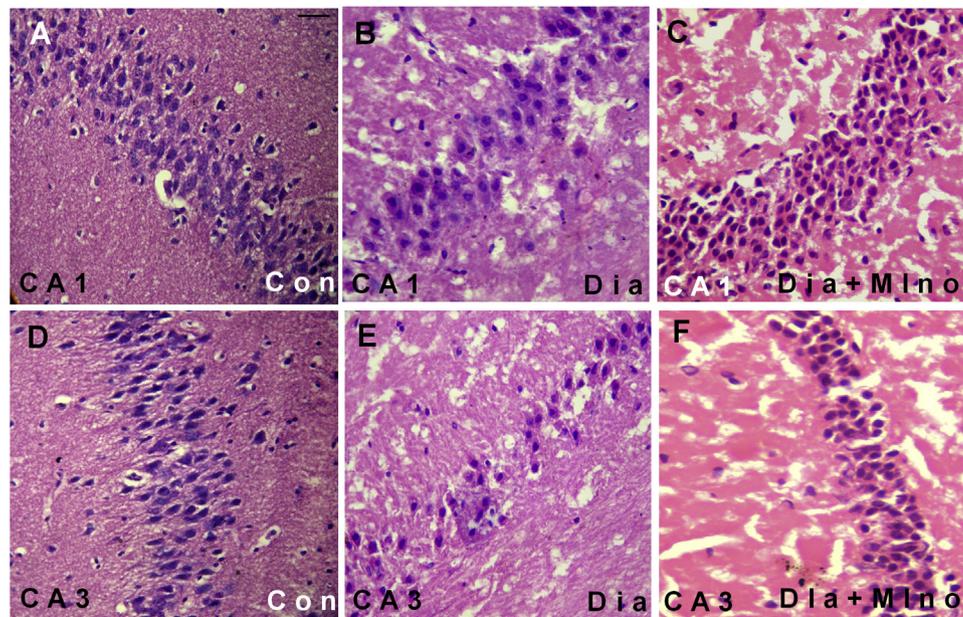


Fig. 6. Effect of T2D and minocycline on hippocampal neurons. Control (Con; A and D): Well-arranged multiple (4–5) layers of neuronal cells with vesicular nuclei were observed. Diabetic (Dia; B and E): Severe loss of neurons making unorganized layers in some areas. Minocycline (Mino) treated Diabetic (C and F): Reduced loss of neurons with greater structural integrity than diabetic brain was observed. Photomicrographs showing CA1 and CA3 region of Hippocampus. Scale bar = 50 μm . Representative images showing Haematoxylin and Eosin (H&E) stained hippocampus regions (40X; n = 4/group).

5 μM concentration but was found to be cytotoxic at higher concentrations (10 μM , 50 μM and 100 μM ; Data not shown) both by MTT and LDH assay.

Discussion

We successfully induced diabetes using HFD and STZ in SD rats. Diabetes led to cognitive decline as revealed by neurobehavioral changes. Diabetic animals showed significantly higher AchE, cytokine and chemokine levels and lower GSH levels with a corresponding reduction in hippocampal neuronal density. Minocycline treatment (15 days) of diabetic animals partially reversed the above neurobehavioral and biochemical changes and improved hippocampal neuronal density in these animals. Cell line studies showed glucose mediated neuronal death, which was also partially reversed upon minocycline treatment. Hence, the present study shows the anti-neuroinflammatory, antioxidant and neuroprotective actions of minocycline in diabetic brain.

Previous reports blame high-fat diets, high blood glucose and insulin resistance for cognitive decline in rats. Primary mechanisms include oxidative stress, reduced brain derived neurotrophic factor (BDNF) levels, microglial activation and neuroinflammation leading to reduced dendritic integrity and the subsequent death of hippocampal neurons [30,47–51]. CA1 and CA3 neurons, involved in various cognitive processes have been shown to be vulnerable to neurotoxic insults inflicted by the activated microglial cells via proinflammatory cytokines and free radicals. We previously reported that HFD along with a low dose of STZ may lead to memory impairment in SD rats. These diabetic animals also showed activation of microglia and astrocytes. Histopathological studies of the diabetic animal brain revealed the presence of a large number of activated microglial cells and severe loss of neurons of CA1 and CA3 neurons [52].

Minocycline has been shown to suppress the stimulation and proliferation of microglial cells. Further, owing to high lipid solubility, minocycline can easily cross the blood-brain barrier a property making it a drug of choice as prophylactic to meningococcal infection and a widely used antibiotic against CNS infections

[53,54]. Minocycline has been shown to be neuroprotective primarily due to its ability to reduce inflammation of the CNS [38,55,56]. It has also been shown to reduce microglial activation and neuroinflammation in various neurodegenerative disorders like an Alzheimer's disease, Parkinson's disease and HIV-1 associated neurocognitive disorder [38,57–59]. Initially, microglial cells are recruited towards sites of CNS injury (MCP-1 mediated) as part of the anti-inflammatory process. However, too much glial activation results in excess release of pro-inflammatory cytokines like TNF- α , IL-1 β [60] and production of reactive oxygen species (ROS) [61], leading to a chronic neuroinflammatory scenario which eventually proves to be neurotoxic. Here we show a minocycline mediated reduction in MCP-1 levels in the diabetic rat brain, which may lead to a reduction in recruitment of activated microglial cells. Hyperglycemia has been independently associated with the cytokine-mediated demyelination process [62] and aggregation of free radicals [31]. Increase in IL-1 β and TNF- α levels in hippocampal brain homogenates with the progression of diabetes and a corresponding cognitive decline has also been reported [55,63]. In the present work, we show prolonged treatment with minocycline may partially reverse diabetes-associated cognitive decline while significantly reducing hippocampal IL-1 β and TNF- α in diabetic animal brain. In a recent study [64], we reported a similar observation in a high fat/high carbohydrate diet-induced mice model of metabolic syndrome. However, these animals showed a higher increase in brain cytokine levels with little change in chemokines compared to HFD-STZ animals. More pronounced oxidative stress and a steep increase in AchE levels with a higher corresponding reduction in hippocampal neuronal density was observed in these metabolic syndrome animals compared to T2D rats. The recovery after minocycline treatment was also less pronounced than in the current work. However, these results may not be comparable since the diet induced metabolic syndrome model is primarily characterised by pronounced hyperlipidemia, central obesity, hypertension and atherogenic changes while HFD-STZ model shows little of the above changes while primarily demonstrated hyperglycaemia and insulin resistance. The cell line studies suggest that minocycline may also provide direct

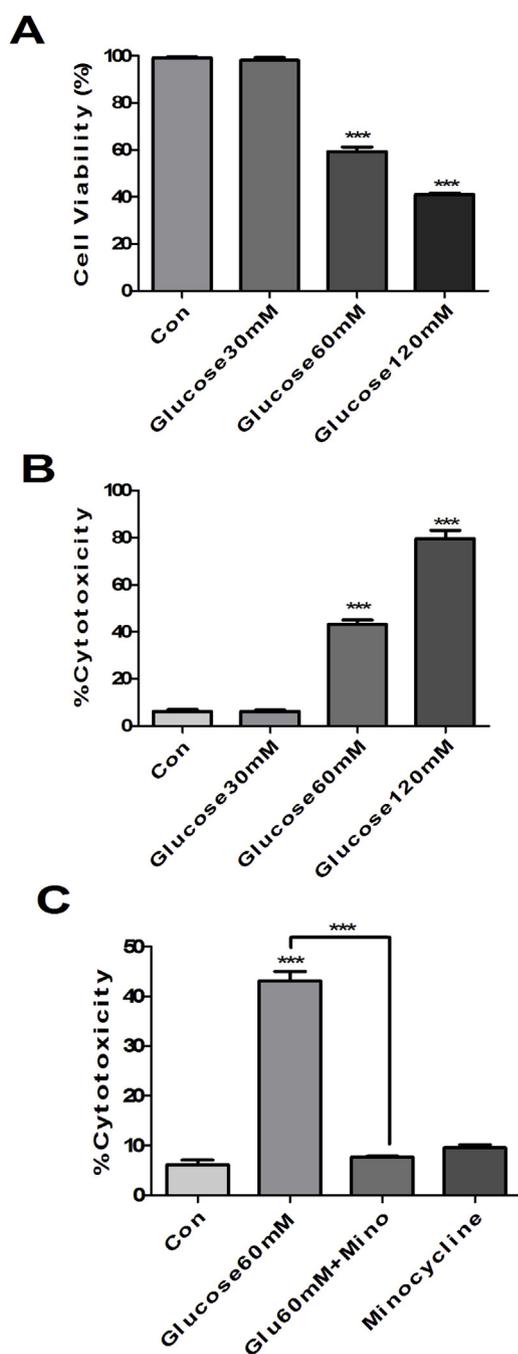


Fig. 7. Effect of glucose on IMR-32 neuroblastoma cells in the presence and absence of minocycline. A) Dose dependent decrease ($p < 0.001$) in cell viability with increased glucose concentration using MTT assay B) Dose dependent decrease ($p < 0.001$) in cell viability with increased glucose concentration using LDH assay C) Improved cell viability ($p < 0.001$) upon minocycline (Mino) pretreatment of glucose treated IMR-32 cells using LDH assay. All values are expressed as mean \pm SEM for $n = 10$ /group. *** $p < 0.001$.

protection against hyperglycemia associated neuronal loss. Furthermore, hypercholesterolemia and hyperglycemia and their associated signalling changes may be involved in metabolic syndrome associated neurotoxicity. Thus, molecular mechanisms involved in cognitive decline associated with metabolic syndrome or HFD-STZ may involve different signalling pathways and the mechanism of neuroprotection by minocycline may vary in these disorders.

Alterations in biochemical parameters occur with the onset of diabetes, which increases the production of free radicals and diminishes antioxidant defense mechanisms. The neuroprotective

actions of minocycline in CNS disorders has also been attributed to its ability to reduce free radical production and increase free radical scavenging, eventually reducing oxidative stress [65,66]. The anti-apoptotic and neuroprotective actions of minocycline may also be due to its suppression of IL-1 β converting enzyme (ICE) which in turn prevents microglial activation. Minocycline has been shown to reduce the oxidation of lipids and proteins, thus enhancing the antioxidant defense system in the spinal cord of the diabetic rats [32,38]. In the present work, we report reduced levels of GSH in the diabetic hippocampus which was found to normalize after minocycline treatment. The above observation indicates a minocycline-mediated reduction of oxidative stress in the diabetic brain. Minocycline has also been shown to be neuroprotective primarily by reducing excitotoxicity. Our neuronal cell line studies have shown a partial reversal of glucose mediated neurotoxicity in LDH assays with low dose of minocycline. However, minocycline was unable to recover the neurons from glucose mediated toxicity in MTT assay (data not shown). Previously it has been shown that both MTT and LDH may correlate with neuronal death but MTT may not correctly quantify neuroprotection, while others have suggested LDH to be more sensitive than MTT to measure neuronal viability [67,68]. Minocycline has also been reported to affect mitochondrial function and induce mitochondria mediated apoptosis [69]. This may explain the above observation and the neurotoxic effects of minocycline at higher doses.

Summary

We conclude that minocycline has antioxidant and anti-inflammatory actions in diabetic brain which may be responsible for minocycline mediated neuroprotection in HFD-STZ induced diabetic rats.

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Conflict of interest

The authors report no conflict of interest

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