Pharmacological Reports 71 (2019) 713–720

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original article

Minocycline reverses diabetes-associated cognitive impairment in rats

Bina K. Mehta, Sugato Banerjee*

Department of Pharmaceutical Sciences and Technology, Birla Institute of Technology, Mesra, Ranchi, Jharkhand, India

ARTICLE INFO

Article history:
Received 12 August 2018
Received in revised form 12 February 2019
Accepted 19 March 2019
Available online 22 March 2019

Keywords:
Cognition
Learning and memory
Neuroinflammation
Type 2 diabetes

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)-1β 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.

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)-1β 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-1β, TNFα and IL-6), nitric oxide, reactive oxygen species, and hydrogen peroxide, all of which have detrimental effect on neurogenesis and neuronal viability [14–21]. Astrogliosis 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

---

* Corresponding author
E-mail address: sbanerjee@bitmesra.ac.in (S. Banerjee).

http://dx.doi.org/10.1016/j.pharep.2019.03.012

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-1β [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 ± 2°C) and humidity (55 ± 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 Electromedical Pvt Ltd, Lucknow, India) was utilized [42]. It comprises of two closed arms with side walls of (500 × 100 x 250 mm) and two open arms (500 x 100 mm). These arms stretched out from a central stage (100 x 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

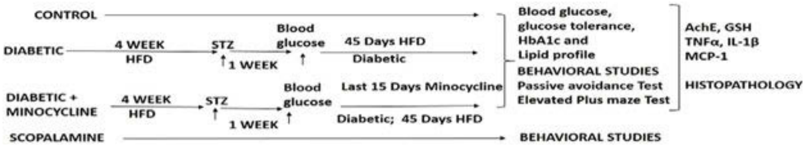

Fig. 1. Experimental work flow.

stress [43]. Using phosphate buffer (pH 8) with 1% Triton X brain tissue (hippocampal; n = 6/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/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-1β, TNF-α and, MCP-1 levels were evaluated for each group (n = 6/group). Levels of TNF-α, IL-1β, 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/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 mm^2. Counts were made in five adjacent fields and the mean number extrapolated to give the total number of neurons per mm^2. 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 5×10^4/0.2 ml cells were plated in 96 welled 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 ± 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 ± 0.1% while HFD-STZ animals showed significantly higher ($p < 0.01$) levels at 10.5±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 ± 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±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.

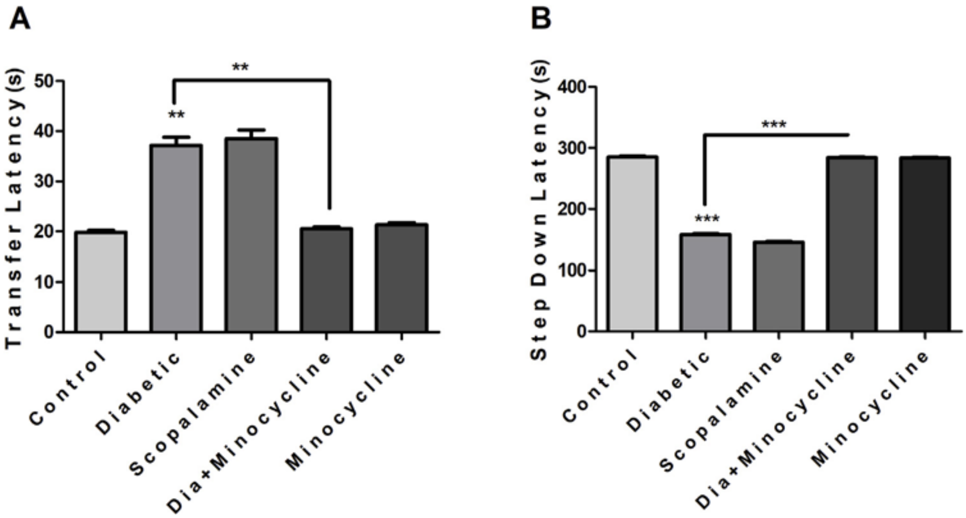

Fig. 2. Effect of type 2 diabetes and minocycline on transfer latency (A) and step down latency (B) in diabetic animals. Scopolamine and HFD-STZ resulted in significant increase ($p < 0.01$) in Transfer Latency (TL) and decrease ($p < 0.001$) in step down latency in diabetic (Dia) group when compared 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 ± 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-1β 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-1β ($p < 0.001$) 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-1β ($p < 0.001$) 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

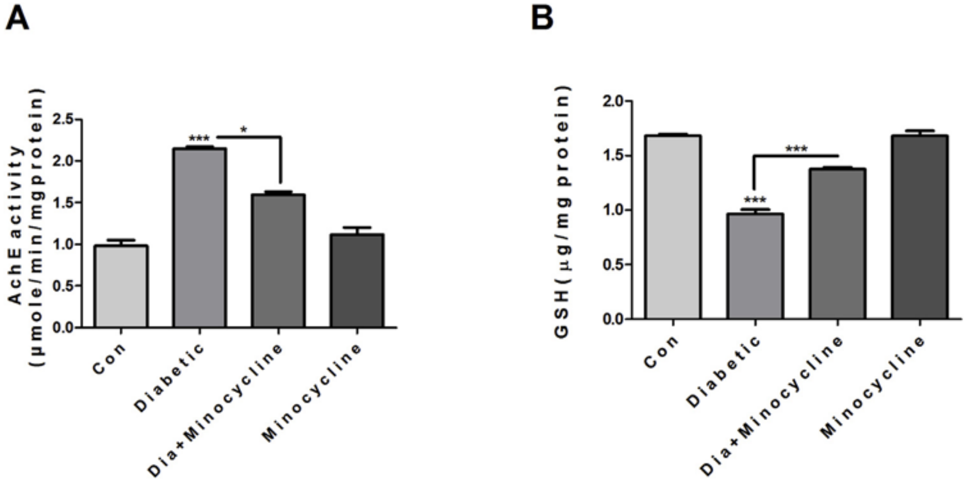

Fig. 3. Effect of type 2 diabetes and minocycline on AChE activity (A) and GSH levels (B) in hippocampal homogenate. HFD-STZ led to considerable decrease ($p < 0.001$) of GSH level. A significant increase ($p < 0.001$) in acetylcholine esterase (AchE) activity was observed in hippocampal homogenate when 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 ± SEM for n= 6. $^{*}p < 0.05$, $^{***}p < 0.001$.

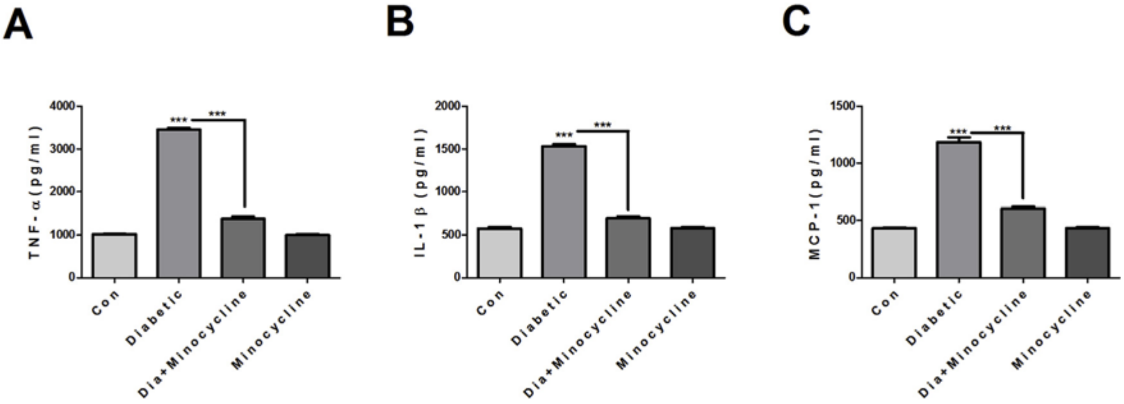

Fig. 4. Effect of type 2 diabetes and minocycline on levels of TNF-α (A), IL-1β (B), and MCP-1(C) in hippocampal homogenate. HFD-STZ led to significant increase ($p < 0.001$) in Tumour necrosis factor (TNF)-α, Interleukin (IL)-1β and Monocyte chemoattractant protein (MCP)-1 levels in hippocampal homogenate 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 ± 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 μM 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

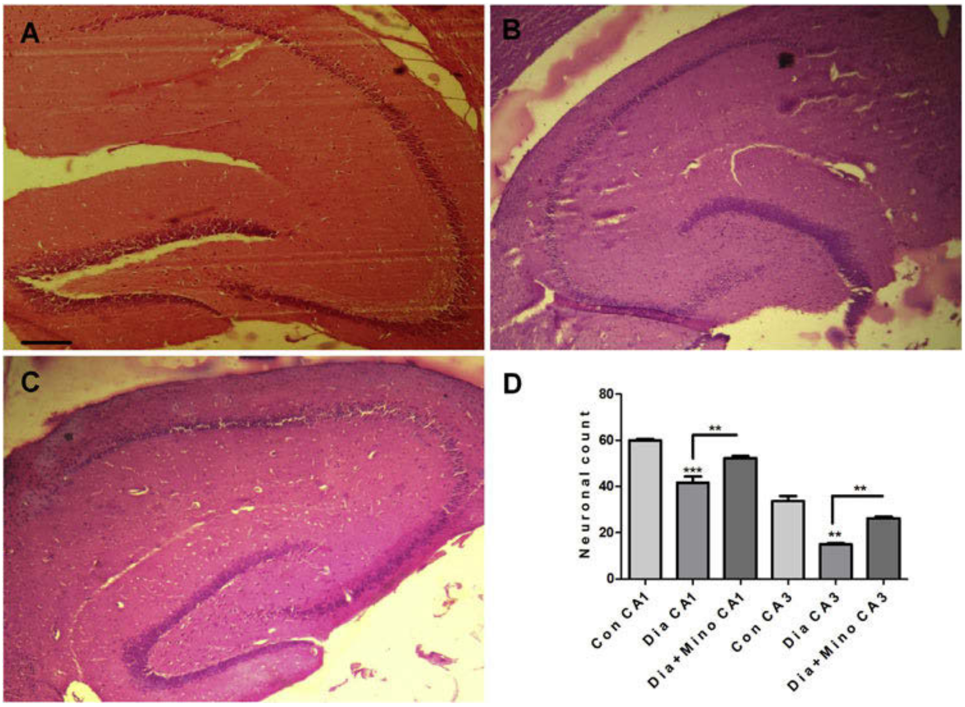

Fig. 5. Photomicrographs of hippocampal sections and neuronal count. A) Control B) Diabetic and C) Minocycline treated diabetic group. D) A significant decrease ($p < 0.001$ and $p < 0.01$) in neuronal count of CA1 and CA3 region respectively of HFD-STZ rat brain was observed when compared with control 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 μm. All values are expressed as mean ± SEM for n=4/group. $^{**}p < 0.01$, $^{***}p < 0.001$.

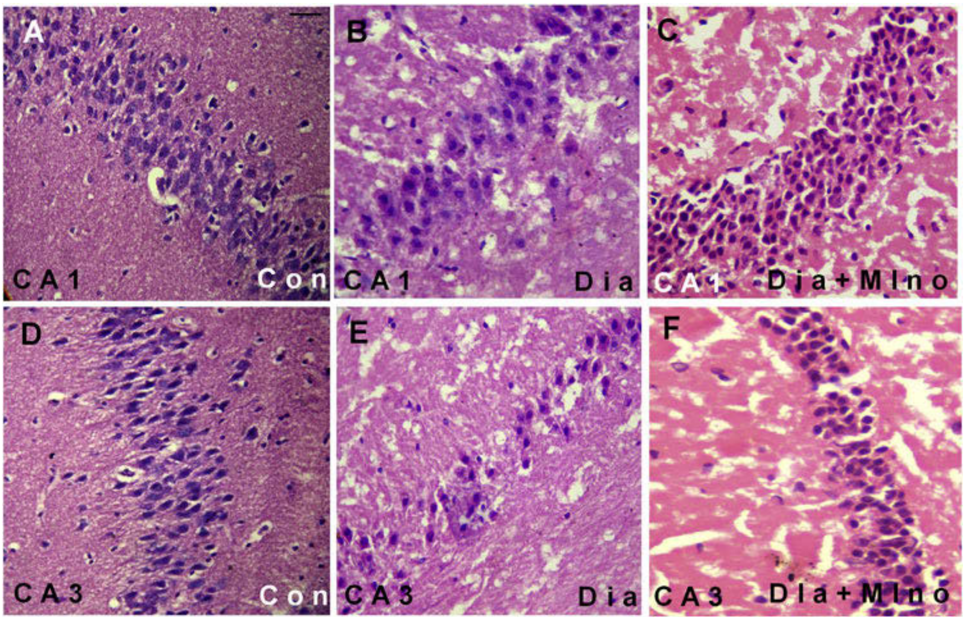

Fig. 6. Effect of T2D and minocycline on hippocampal neurons. Control (Con; A and D): Well-arranged multiple (4–5) layers of neuronal cells with vesicular nuclei was observed. Diabetic (Dia; B and E): Severe loss of neurons making unorganized layers in some areas. Minocycline (Mino) treated Diabetic (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 neurotropic 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 Alzhemer'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-1β [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-1β 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-1β 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

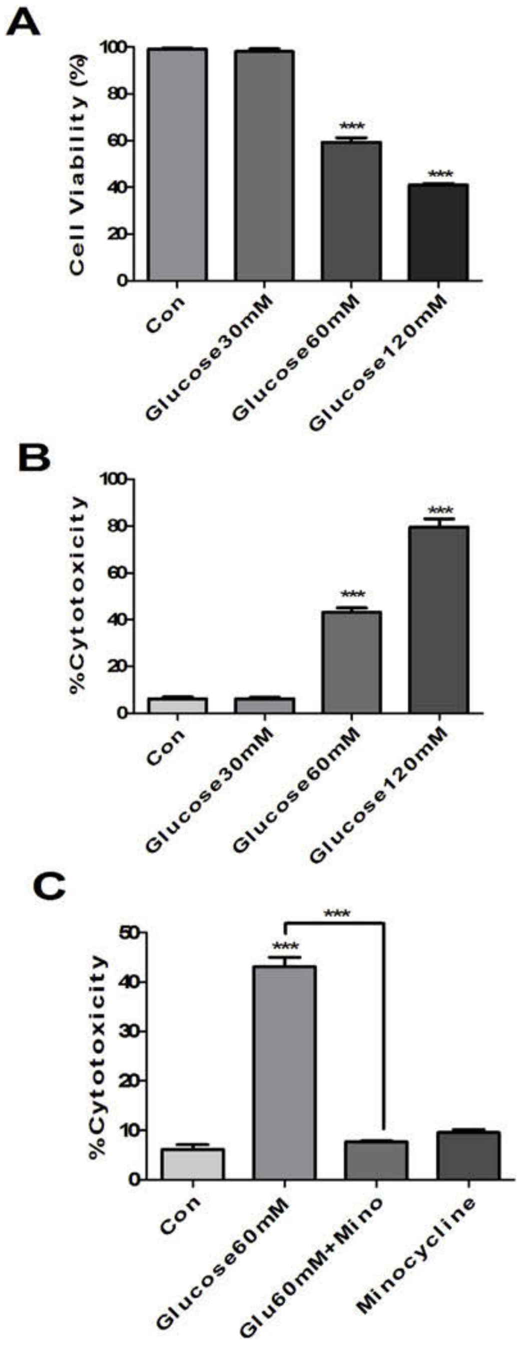

Fig. 7. Effect of glucose on IMR-32 neuroblastoma cells in the presence and absence of minocycline. A) Dose dependent decrease ($p < 0.001$) in cell viability with increased glucose concentration using MTT assay B) Dose dependent decrease ($p < 0.001$) in cell viability with increased glucose concentration 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 ± SEM for n = 10/group. $^{***}p < 0.001$.

protection against hyperglycemia associated neuronal loss. Furthermore, hypercholestemia 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-1β 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 minocycline mediated neuroprotective in HFD-STZ induced diabetic rats.

Funding details

The work has been supported by DST-SERB project no SB/FT/LS-202/2012. The authors sincerely thank Mr Andrew Massey, Department of Pharmaceutical Sciences, University of Tennessee at Memphis, USA for his diligent proof-reading of this manuscript

Conflict of interest

The authors report no conflict of interest

References

[1] Van den Berg E, Kloppenborg RP, Kessels RP, Kappelle LJ, Biessels GJ. Type 2 diabetes mellitus, hypertension, dyslipidemia and obesity: a systematic comparison of their impact on cognition. BBA-Mol Basis Dis 2009;1792:470–81.
[2] Danesh J, Whincup P, Walker M, Lennon L, Thomson A, Appleby P, et al. Low grade inflammation and coronary heart disease: prospective study and updated meta-analyses. Brit Med J 2000;321(7255):199–204.
[3] Alexandraki KI, Piperi C, Ziakas PD, Apostolopoulos NV, Makrilakis K, Syriou V, et al. Cytokine secretion in long-standing diabetes mellitus type 1 and 2: associations with low-grade systemic inflammation. J Clin Immunol 2008;28(4):314–21.
[4] Frances DEA, Ingaramo PI, Ronco MT, Carnovale CE. Diabetes, an inflammatory process: oxidative stress and TNF-alpha involved in hepatic complication. J Biomed Sci 2013;6(6):645–53.
[5] Vikram A, Tripathi DN, Kumar A, Singh S. Oxidative stress and inflammation in diabetic complications. Int J Endocrinol 2014;2014:.
[6] Kampoli A-M, Tousoulis D, Briasoulis A, Latsios G, Papageorgiou N, Stefanadis C. Potential pathogenic inflammatory mechanisms of endothelial dysfunction induced by type 2 diabetes mellitus. Curr Pharm Des 2011;17(37):4147–58.
[7] Helmersson J, Vessby B, Larsson A, Basu S. Association of type 2 diabetes with cyclooxygenase-mediated inflammation and oxidative stress in an elderly population. Circulation 2004;109(14):1729–34.
[8] Basta G, Schmidt AM, De Caterina R. Advanced glycation end products and vascular inflammation: implications for accelerated atherosclerosis in diabetes. Cardiovasc Res 2004;63(4):582–92.
[9] Goldin A, Beckman JA, Schmidt AM, Creager MA. Advanced glycation end products. Circulation 2006;114(6):597–605.

[10] Negrean M, Stirban A, Stratmann B, Gawlowski T, Horstmann T, Götting C, et al. Effects of low-and high-advanced glycation endproduct meals on macro-and microvascular endothelial function and oxidative stress in patients with type 2 diabetes mellitus. Am J Clin Nutr 2007;85(5):1236–43.

[11] Biessels G-J, Kappelle A, Bravenboer B, Erkelens D, Gispen W. Cerebral function in diabetes mellitus. Diabetologia 1994;37(7):643–50.

[12] Hartge MM, Unger T, Kintscher U. The endothelium and vascular inflammation in diabetes. Diab Vasc Dis Res 2007;4(2):84–8.

[13] Nagayach A, Patro N, Patro I. Astrocytic and microglial response in experimentally induced diabetic rat brain. Metab Brain Dis 2014;29(3):747–61.

[14] Creager M, Goldin A, Beckman J, Schmidt A. Advanced glycation end products-Sparking the development of diabetic vascular injury. Circulation 2006;114:597–605.

[15] Giulian D, Li J, Bartel S, Broker J, Li X, Kirkpatrick JB. Cell surface morphology identifies microglia as a distinct class of mononuclear phagocyte. J Neurosci 1995;15(11):7712–26.

[16] Streit WJ, Graeber MB, Kreutzberg GW. Peripheral nerve lesion produces increased levels of major histocompatibility complex antigens in the central nervous system. J Neuroimmunol 1989;21(2):117–23.

[17] Streit WJ, Kreutzberg GW. Response of endogenous glial cells to motor neuron degeneration induced by toxic ricin. J Comp Neurol 1988;268(2):248–63.

[18] Gehrmann J, Matsumoto Y, Kreutzberg GW. Microglia: intrinsic immuneffector cell of the brain. Brain Res Rev 1995;20(3):269–87.

[19] Colton CA, Gilbert DL. Production of superoxide anions by a CNS macrophage. the microglia. FEBS lett. 1987;223(2):284–8.

[20] Banati R, Rothe G, Valet G, Kreutzberg G. Detection of lysosomal cysteine proteinases in microglia: flow cytometric measurement and histochemical localization of cathepsin B and L. Glia 1993;7(2):183–91.

[21] Shen WH, Zhou J-H, Broussard SR, Johnson RW, Dantzer R, Kelley KW. Tumor necrosis factor α inhibits insulin-like growth factor I-induced hematopoietic cell survival and proliferation. Endocrinology 2004;145(7):3101–5.

[22] Banerjee S, Walseth TF, Borgmann K, Wu L, Bidasee KR, Kannan MS, et al. CD38/cyclic ADP-ribose regulates astrocyte calcium signaling: implications for neuroinflammation and HIV-1-associated dementia. J Neuroimmune Pharmacol 2008;3(3):154.

[23] Venters HD, Tang Q, Liu Q, VanHoy RW, Dantzer R, Kelley KW. A new mechanism of neurodegeneration: a proinflammatory cytokine inhibits receptor signaling by a survival peptide. P Natl Acad Sci-Biol. 1999;96(17):9879–84.

[24] Reed M, Meszaros K, Entes L, Claypool M, Pinkett J, Gadbois T, et al. A new rat model of type 2 diabetes: the fat-fed, streptozotocin-treated rat. Metabolis 2000;49(11):1390–4.

[25] Srinivasan K, Viswanad B, Asrat L, Kaul C, Ramarao P. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening. Pharmacol Res 2005;52(4):313–20.

[26] Greenwood CE, Winocur G. Learning and memory impairment in rats fed a high saturated fat diet. Behav Neural Biol 1990;53(1):74–87.

[27] Schmatz R, Mazzanti CM, Spanevello R, Stefanello N, Gutierres J, Corrêa M, et al. Resveratrol prevents memory deficits and the increase in acetylcholinesterase activity in streptozotocin-induced diabetic rats. Eur J Pharmacol 2009;610(1):42–8.

[28] Ghareeb DA, Hussen HM. Vanadium improves brain acetylcholinesterase activity on early stage alloxan-diabetic rats. Neurosci Lett 2008;436(1):44–7.

[29] Pari L, Latha M. Protective role of Scoparia dulcis plant extract on brain antioxidant status and lipid peroxidation in STZ diabetic male Wistar rats. BMC Complement Altern Med 2004;4(1):16.

[30] McNeilly AD, Williamson R, Sutherland C, Balfour DJ, Stewart CA. High fat feeding promotes simultaneous decline in insulin sensitivity and cognitive performance in a delayed matching and non-matching to position task. Behav Brain Res 2011;217(1):134–41.

[31] Leite LM, Carvalho AGG, Ferreira PLT, Pessoa IX, Gonçalves DO, de Araújo Lopes A, et al. Anti-inflammatory properties of doxycycline and minocycline in experimental models: an in vivo and in vitro comparative study. Inflammopharmacology 2011;19(2):99–110.

[32] Tikka T, Fiebich BL, Goldsteins G, Keinänen R, Minocycline Koistinaho J. A tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. J Neurosci 2001;21(8):2580–8.

[33] Adlard PA, Engesser-Cesar C, Cotman CW. Mild stress facilitates learning and exercise improves retention in aged mice. Exp Gerontol 2011;46(1):53–9.

[34] Blum D, Chtarto A, Tenenbaum L, Brotchi J, Levivier M. Clinical potential of minocycline for neurodegenerative disorders. Neurobiol Dis 2004;17(3):359–66.

[35] Swanson RA, Ying W, Kauppinen TM. Astrocyte influences on ischemic neuronal death. Curr Mol Med 2004;4(2):193–205.

[36] Cl Gabriel, Justicia C, Camins A, Planas AM. Activation of nuclear factor-κB in the rat brain after transient focal ischemia. Mol Brain Res. 1999;65(1):61–9.

[37] Krady JK, Basu A, Allen CM, Xu Y, LaNoue KF, Gardner TW, et al. Minocycline reduces proinflammatory cytokine expression, microglial activation, and caspase-3 activation in a rodent model of diabetic retinopathy. Diabetes 2005;54(5):1559–65.

[38] Yrjänheikki J, Tikka T, Keinänen R, Goldsteins G, Chan PH, Koistinaho J. A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. Proc Natl Acad Sci-Biol 1999;96(23):13496–500.

[39] Hunter CL, Bachman D, Granholm AC. Minocycline prevents cholinergic loss in a mouse model of Down's syndrome. Ann Neurol 2004;56(5):675–88.

[40] Xue M, Mikliaeva EI, Casha S, Zygun D, Demchuk A, Yong VW. Improving outcomes of neuroprotection by minocycline: guides from cell culture and intracerebral hemorrhage in mice. Am J Pathol 2010;176(3):1193–202.

[41] Baydas G, Nedzvetskii VS, Nerush PA, Kirichenko SV, Yoldas T. Altered expression of NCAM in hippocampus and cortex may underlie memory and learning deficits in rats with streptozotocin-induced diabetes mellitus. Life Sci 2003;73:1907–16.

[42] Dhingra DM, Parle M, Kulkarni SK. Memory enhancing activity of Glycyrrhiza glabra in mice. J Ethnopharmacol 2004;91(2-3):361–5.

[43] Pocernich CB, Butterfield DA. Elevation of glutathione as a therapeutic strategy in Alzheimer disease. BBA-Mol Basis Dis. 2012;1822(5):625–30.

[44] Sarter M, Bruno JP. Cognitive functions of cortical acetylcholine: toward a unifying hypothesis. Brain Res Rev 1997;23(1):28–46.

[45] Ellman GL, Courtney KD, Andres V. Jr, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7(2):88–95.

[46] Zhang JM, An J. Cytokines, inflammation and pain. Int Anesthesiol Clin 2007;45(2):27–37.

[47] Stranahan AM, Norman ED, Lee K, Cutler RG, Telljohann RS, Egan JM, et al. Diet-induced insulin resistance impairs hippocampal synaptic plasticity and cognition in middle-aged rats. Hippocampus 2008;18(11):1085–8.

[48] Morrison CD, Pistell PJ, Ingram DK, Johnson WD, Liu Y, Fernandez-Kim SO, et al. High fat diet increases hippocampal oxidative stress and cognitive impairment in aged mice: implications for decreased Nrf2 signaling. J Neurochem 2010;114(6):1581–9.

[49] Uranga RM, Bruce-Keller AJ, Morrison CD, Fernandez-Kim SO, Ebenezer PJ, Zhang L, et al. Intersection between metabolic dysfunction, high fat diet consumption, and brain aging. J Neurochem 2010;114(2):344–61.

[50] Kalmijn S. Fatty acid intake and the risk of dementia and cognitive decline: a review of clinical and epidemiological studies. J Nutr Health Aging 2000;4(4):202–7.

[51] Uranga RM, Keller JN. Diet and age interactions with regards to cholesterol regulation and brain pathogenesis. Curr Gerontol Geriatr Res 2010;219683.

[52] Mehta BK, Banerjee S. Characterization of cognitive impairment in type 2 diabetic rats. Indian J Pharm Sci 2017;79(5):785–93.

[53] Beaty HN. Rifampin and minocycline in meningococcal disease. Rev Infect Dis 1983;5(Supplement 3):S451–58.

[54] Fraser A, Gafter-Gvili A, Paul M, Leibovici L. Prophylactic use of antibiotics for prevention of meningococcal infections: systematic review and meta-analysis of randomised trials. Eur J Clin Microbiol 2005;24(3):172–81.

[55] Raghavendra V, Tanga F, DeLeo JA. Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. J Pharmacol Exp Ther 2003;306(2):624–30.

[56] Kielian T, Esen N, Liu S, Phulwani NK, Syed MM, Phillips N, et al. Minocycline modulates neuroinflammation independently of its antimicrobial activity in Staphylococcus aureus-induced brain abscess. Am J Pathol 2007;171(4):1199–214.

[57] Kim H-S, Suh Y-H. Minocycline and neurodegenerative diseases. Behav Brain Res 2009;196(2):168–79.

[58] Zink MC, Uhrlaub J, DeWitt J, Voelker T, Bullock B, Mankowski J, et al. Neuroprotective and anti–human immunodeficiency virus activity of minocycline. JAMA 2005;293(16):2003–11.

[59] Kou W, Banerjee S, Eudy J, Smith LM, Persidsky R, Borgmann K, et al. CD38 regulation in activated astrocytes: implications for neuroinflammation and HIV-1 brain infection. J Neurosci Res 2009;87(10):2326–39.

[60] Tangpong J, Sompol P, Vore M, Clair WS, Butterfield D, Clair DS. Tumor necrosis factor alpha–mediated nitric oxide production enhances manganese superoxide dismutase nitration and mitochondrial dysfunction in primary neurons: an insight into the role of glial cells. Neuroscience 2008;151(2):622–9.

[61] K-j Min, Jou I, Joe E. Plasminogen-induced IL-1β and TNF-α production in microglia is regulated by reactive oxygen species. Biochem Bioph Res Co 2003;312(4):969–74.

[62] Conti G, Scarpini E, Baron P, Livraghi S, Tiriticco M, Bianchi R, et al. Macrophage infiltration and death in the nerve during the early phases of experimental diabetic neuropathy: a process concomitant with endoneurial induction of IL-1β and p75NTR. J Neurol Sci 2002;195(1):35–40.

[63] Ledeboer A, Sloane EM, Milligan ED, Frank MG, Mahony JH, Maier SF, et al. Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. Pain 2005;115(1):71–83.

[64] Mukherjee A, Mehta BK, Sen KK, Banerjee S. Metabolic syndrome-associated cognitive decline in mice: role of minocycline. Indian J Pharmacol 2018;50(2):61–5.

[65] Kraus RL, Pasieczny R, Lariosa-Willingham K, Turner MS, Jiang A, Trauger JW. Antioxidant properties of minocycline: neuroprotection in an oxidative stress assay and direct radical-scavenging activity. J Neurochem 2005;94(3):819–27.

[66] Yenari MA, Xu L, Tang XN, Qiao Y, Giffard RG. Microglia potentiate damage to blood–brain barrier constituents. Stroke 2006;37(4):1087–93.

[67] Bigl K, Schmitt A, Meiners I, Münch G, Arendt T. Comparison of results of the CellTiter Blue, the tetrazolium (3-[4, 5-dimethylthioazol-2-yl]-2, 5-diphenyl tetrazolium bromide), and the lactate dehydrogenase assay applied in brain cells after exposure to advanced glycation endproducts. Toxicol In Vitro 2007;21(5):962–71.

[68] Lobner D. Comparison of the LDH and MTT assays for quantifying cell death: validity for neuronal apoptosis? J Neurosci Meth. 2000;96(2):147–52.

[69] Kupsch K, Hertel S, Kreutzmann P, Wolf G, Wallesch CW, Siemen D, et al. Impairment of mitochondrial function by minocycline. FEBS J 2009;276(6):1729–32.