Neuroprotective Role of Diosgenin, A NGF Stimulator, Against Aβ (1–42) Induced Neurotoxicity in Animal Model of Alzheimer's Disease

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Abstract

Diosgenin is a neurosteroid derived from the plants and has been previously reported for its numerous health beneficial properties, such as anti-arrhythmic, hypolipidemic, and antiproliferative effects. Although several studies conducted earlier suggested cognition enhancement actions of diosgenin against neurodegenerative disorders, but the molecular mechanisms underlying are not clearly understood. In the present study, we investigated the neuroprotective effect of diosgenin in the wistar rats that received an intracerebroventricular injection of Amyloid-β (1–42) peptides, representing a rodent model of Alzheimer's disease (AD). Animals were treated with 100 and 200 mg/kg/p.o of diosgenin for 28 days, followed by Amyloid-β (1–42) peptides infusion. Animals were assessed for the spatial learning and memory by using radial arm maze and passive avoidance task. Subsequently, animals were euthanized and brains were collected for biochemical estimations and histopathological studies. Our results revealed that, diosgenin administration dose dependently improved the spatial learning and memory and protected the animals from Amyloid-β (1–42) peptides induced disrupted cognitive functions. Further, biochemical analysis showed that diosgenin successfully attenuated Amyloid-β (1–42) mediated plaque load, oxidative stress, neuroinflammation and elevated acetylcholinesterase activity. In addition, histopathological evaluation also supported neuroprotective effects of diosgenin in hippocampus of rat brain when assessed using hematoxylin-eosin and Cresyl Violet staining. Thus, the aforementioned effects suggested protective action of diosgenin against Aβ (1–42) induced neuronal damage and thereby can serve as a potential therapeutic candidate for AD.

Introduction

Alzheimer's disease (AD) is the most prevalent, complex and devastating neurodegenerative disorder in the aging population and clinically characterized by gradual erosion of cognitive functions, particularly decline in perception and language ability, personality disturbance, inability of self-care and death from opportunistic infections (Souza et al. 2016).

Basal forebrain cholinergic neurons (BFCNs) are considered to be prime neurons responsible for learning, memory, and attention, through acetylcholine (ACh) innervation (Baxter et al. 1999). Abnormal formation of intracellular neurofibrillary tangles and extracellular deposition of amyloid beta (Aβ) peptides in the AD brain displayed neuritic atrophy and loss of synapses in BFCNs. Degeneration of BFCNs act as one of the major pathological diagnostic hallmark of AD and is severely affected due to disruption of communication with target neurons in the hippocampus and cortex (Fahnestock et al. 2019). With the age, these affects gets worsen leading to the cascade of pathogenic events causing neuroinflammation, glutamate excitotoxicity, metal dyshomeostasis, oxidative stress and mitochondrial dysfunction in specific parts of the brain (Souza et al. 2016; Lian et al. 2017). BFCNs are essentially dependent on neurotrophic factor, nerve growth factor (NGF), belonging to the neurotrophin family, for their neuronal growth, survival, maintenance, and plasticity by binding with tyrosine kinase A receptor (TrkA) and the p75 pan neurotrophin receptor (p75NTR) (Seiler et al. 1984; Lad et al. 2003). Growing evidence conveys that altered expression of TrkA/ p75 results in basal forebrain cholinergic metabolic dysfunction due to

defective NGF processing and supports accumulation of Aβ (Iulita and Cuello 2014). Interestingly, accumulated Aβ, in AD pathology, in turn causes degeneration of BFCNs and enhances Aβ production by regulating p75NTR over-expression, NGF-induced signalling transduction and internalization of NGF receptors (Zhang et al. 2013).

In recent decades, a number of herbal derived drug compounds, used in traditional medicine, found to improve brain functions by reconstructing neuronal networks and remodelling the atrophic neurites by upregulating NGF expression. For instance, Huperazine A, a novel alkaloid, specific and reversible acetylcholinesterase (AChE) inhibitor, isolated from the Chinese herb Huperzia serrate, elevated NGF levels and thus ameliorated cognitive deficits in AD (Wang et al. 2006). Diosgenin is a one such herbal derived neurosteroid and a promising bioactive biomolecule which has a high industrial value and is the principal precursor compound in the production of therapeutically useful steroidal drugs, including sex hormones and corticosteroids (Masilamoni et al. 2008). It is a natural antioxidant usually derived from Dioscorea rhizome and other herbal drugs, such as Trigonella spp., Polygonatum spp. and Smilax spp with multiple medicinal properties, such as, hypolipidemic (Son et al. 2007), anti-inflammatory (Yang et al. 2017), anti-proliferative (Sethi et al. 2018), anti-arrhythmic (Chen et al. 2015), anti-diabetic (Hua et al. 2016), neuroprotective (Lee et al. 2018), and found to have protective effect against cardiovascular disease (Kalailingam et al. 2014) and skin aging (Kim et al. 2016). Diosgenin, was reported earlier as a potential NGF which efficiently regulates the expression of TrkA and p75 and activates the intracellular signaling cascades involving pathways that are dependent on extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K), which act to prevent nuclear and mitochondrial cell death programs, resulting in neuronal survival and differentiation. In addition, diosgenin boosted memory and exhibited protection and regrowth of axons in Aβ-treated neurons by stimulating $1,25D_3$ -membrane-associated, rapid response steroid-binding protein (1,25D3 -MARRS) in 5XFAD mice (Koh et al. 2016; Tohda et al. 2012). Similarly, diosgenin produced cellular protection against oxidative stress by inhibiting ROS production in neuronal cells in D-galactose induced aging model (Choi et al. 2010; Chiu et al. 2011). All these aforementioned reports provided strong evidences of cognition enhancement property of diosgenin and thus convinced us that diosgenin might be able to attenuate cognitive impairment in the animal model of AD.

Multiple molecular pathogenesis such as tau hyperphosphorylation, Aβ deposition, metal dyshomeostasis, cholinergic hypofunction, glutamate excitotoxicity, neuroinflammation, oxidative stress, and mitochondrial dysfunction act together in complex way in AD progression. Hence there is a need of a broader “one-compound-multi-targets” neuroprotective approach which can collectively target these pathological processes (Pi et al., 2012). In this regard, current study was designed to evaluate the neuroprotective effect of diosgenin on AD related complications in Aβ (1-42) induced wistar rats, representing sporadic AD model. Based on satisfactory results from our studies we can say that diosgenin could be a promising neuroprotectant and therapeutic solution for AD rescue.

Materials And Methods

Drugs and chemicals

Diosgenin and amyloid beta (1–42) peptides were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Aβ (1–42), Tumour necrosis factor- α (TNF- α) and Interleukin-1β (IL-1β) Mouse enzyme-linked immunosorbent assay (ELISA) kits were procured from GenxBio Health Sciences Pvt. Ltd. (Delhi, India). All other chemicals and reagents purchased were of analytical grade only.

Animal procurement

Forty adult male wistar rats, aged 3 months, weighing 250-300 g were provided by central animal facility of JSS College of Pharmacy, Ooty, Tamilnadu, India. The study was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council (US)) and was approved by the Institutional Animal Ethics Committee (IAEC) (JSSCP/IAEC/PhD/Pharmacy Practice/01/2018-19). In order to adapt the environmental conditions of 12- hour light/dark cycle, animals were acclimatized for ten days to the laboratory conditions in polypropylene cages with pellet diet and water ad libitum prior to the commencement of the experiment. All animals were housed five per cage and were maintained under a controlled room temperature of 25 ± 2 °C and relative humidity of 60 ± 5% degree.

Drug preparation

Diosgenin (100 mg/kg and 200 mg/kg) was dissolved in 0.5% carboxymethyl cellulose (CMC) for its oral administration. Aβ (1–42) was firstly dissolved in artificial cerebrospinal fluid (aCSF) at a concentration of 5 μg/μl and incubated at 37 °C for 4 days to allow aggregation before intracerebroventricular (i.c.v.) injection.

Surgical procedure

Rats were anesthetized with ketamine hydrochloride (91 mg/kg, i.p.) and xylazine (9.1 mg/kg, i.p.). After fixing the animals into a stereotaxic apparatus, a burr hole was drilled through the skull above the bilateral hippocampal coordinates (anterior–posterior (AP) = −3.5 mm, medial-lateral (ML) = ± 2.0 mm from the bregma and dorsal-ventral (DV) = 2.7 mm from the skull surface) after midline sagittal incision according to stereotaxic atlas. 2 μl containing 10 μg of Aβ (1–42) was subsequently injected as a single dose over 5 min through a microsyringe into the hole (Rahman et al. 2019). To prevent infection, animals were provided with post-operative care by applying antiseptic over the wound and then transferred to a thermo-regulated chamber to maintain normal body temperature until recovery.

Experimental protocol and drug treatment

Animals were randomly divided into 4 groups, containing 10 rats each. Rats from sham operated (SO) and negative control (NC) groups received 0.5% CMC orally, and were bilaterally infused with 2 μl of aCSF and 2 μl/10 μg of Aβ (1–42) peptides dissolved in aCSF respectively. Test animals received 100 mg/kg/d (Dio 100) and 200 mg/kg/d (Dio 200) p.o. of diosgenin suspended in 0.5% CMC for 28 days followed by

i.c.v. Aβ (1–42) infusion on 28th day. Before initiation of experiments, animals were housed in their respective cages for a week for recovery (Fig. 1). The dose of the diosgenin selected in the present investigation is based on studies conducted earlier (Zhang et al. 2017).

Behavioural cognitive assessments

Radial arm maze (RAM) task

RAM was used as a standard method to assess reference and working memory errors in the animals. RAM apparatus consists of 8 identical arms originating from the centre of the elevated platform, height of about 28 cm. Animals were kept in a food deprived condition overnight. During the experiment, four arms (2, 3, 5, and 7) were randomly baited with food pellets, provided with a number of visual clues and rats were allowed to explore the maze for 5 min. The number of entries into an unbaited arm referred as Reference memory errors and number of re-entries into baited arm referred as working memory errors within 5 min were recorded during the investigation. If the animal visited all the arms before 5 min then the trial was said to be accomplished (Sehgal et al. 2012). After each trial the arms were cleaned with 70% of alcohol.

Passive avoidance test

Passive avoidance test was used to examine the long term memory based on negative reinforcement. The test apparatus consists of a small chamber (illuminated with a 50 W bulb) connected to a dark chamber separated by a guillotine door. All the animals were placed in the bright compartment with opened guillotine door. As the rats entered the dark chamber, door was closed and provided with a low intense foot shock (1 Hz, 5 seconds, 1/5 MA). The total time taken by the animal to transfer from the bright to dark compartment was recorded as step-through latency (STL). After 24 h, retention test was conducted where animals were placed in the illuminated compartment and total time spent in the dark chamber (TDC) was recorded. (Barkur and Bairy 2015).

Brain homogenate preparation

After completion of neurobehavioural assessment, animals were sacrificed under ether anaesthesia. Brains were extracted and cleaned with ice saline. The cerebral hemispheres were separated and the left hemisphere was fixed in neutral buffered formalin (10% v/v) for histological examination. The hippocampus was dissected from the right hemisphere and 10 % (w/v) homogenate of brain sample (0.03 M sodium phosphate buffer, ph-7.4) was prepared by using Teflon glass homogenizer. The homogenate was centrifuged at 1000 rpm at 4°C for 3 min. Supernatant was separated and used for biochemical studies. Hippocampal protein was measured by the method of Lowry et al. (1951) using bovine serum albumin (1 mg/ml) as a standard.

Biochemical assessment

Determination of hippocampal AChE activity

AChE activity was evaluated in rat hippocampus by using Ellman's method (Ellman et al. 1961). The assay mixture consisted of 0.05 ml of supernatant, 3 ml of 0.01M sodium phosphate buffer (pH 8), and 0.1 ml of 0.2 mM 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent). About 0.1 ml of acetylthiocholine iodide was added and the change in the absorbance was measured at the 30 s interval for 5 min at 412 nm. The enzyme activity was expressed in micromoles of acetylthiocholine iodide hydrolysed per min per g of protein. AChE activity was calculated using an extinction coefficient of $13.6mM^{-1}$ cm^{-1} (Khalil and Abass 2017).

Estimation of hippocampal Aβ (1-42), TNF-α and IL-1β levels

Aβ (1-42), IL-1 β and TNF-α levels in the hippocampal homogenate were estimated using commercial ELISA kits as per the manufacturer's instructions.

Estimation of superoxide dismutase (SOD) activity

The activity of SOD in brain hippocampus was analysed by the method suggested by Kakkar et al. (1984). An assay mixture was prepared by adding 1.2 ml of 0.052 M sodium pyrophosphate buffer (pH 8.3), 0.1 ml of 186 µM phenazonium methosulphate, 0.3 ml of 300 µM nitroblue tetrazolium and 0.1 ml brain homogenate. To the mixture, 0.2 ml of 780 µM nicotinamide adenine dinucleotide (NADH) was added to begin the reaction. Reaction mixture was incubated for 90 sec at 30°C followed by addition of 0.1ml of glacial acetic acid to stop the reaction. 4 ml of n-butanol was added to the mixture and stirred vigorously and centrifuged at 4000 rpm for 10 min. The absorbance of organic layer was read at 560 nm. SOD activity was expressed as units/ min/mg protein.

Estimation of catalase (CAT) activity

Catalase activity was measured using spectrophotometric procedure according to the method of Aebi (1984). A mixture of 0.1 ml of brain homogenate and 1.9 ml of 50 mM phosphate buffer was incubated at 25 ºC for 30 min. To this 1.0 ml of freshly prepared 30 mM H_2O_2 was added to initiate the reaction. The change in absorption was taken for 3 min at 240 nm at an interval of 30 sec. CAT activity was expressed as µmol/min/g protein.

Estimation of glutathione reductase (GSR) activity

GSR activity was estimated by the method proposed by Carlberg and Mannervik (1975). The reaction mixture was prepared by adding 0.1 ml of brain homogenate, 1.65 ml of 0.1 M phosphate buffer (pH 7.6), 0.1 ml of 0.1 mM nicotinamide adenine dinucleotide phosphate (NADPH), 0.05 ml of 1 mM oxidized glutathione and 0.1 ml of 0.5 mM ethylenediamine tetraacetic acid (EDTA). The absorbance of each sample was noted at 340 nm. The GSR activity was expressed as nmol/min/mg protein.

Histopathological analysis of brain

The whole brain was stored overnight in neutral buffered formalin (10% v/v) after scarification followed by embedment in paraffin for 4 h. The hippocampus was sectioned at 5 µL thickness using a Leica RM 2135 microtome. Sections were mounted and washed in xylene for deparaffinization followed by rehydration in graded ethanol and finally stained with hematoxylin-eosin (H & E). Another batch of samples were stained with cresyl violet (CV) acetate. Hippocampal neurons morphology were observed and captured at 40 X magnification under light microscopy.

Statistical analysis

The results were analysed using Graph Pad Prism 8.0.2 (263) and values were expressed as mean ± standard deviation (SD). Statistical analysis between groups was measured using one way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparisons. Values were considered to be significant when $p < 0.05$.

Results

Effect of diosgenin on RAM task

In RAM task, animals from NC group showed poor ability in learning the task and made high number of reference ($p < 0.001$, F (3, 36) =45.3) and working ($p < 0.001$, F (3, 36) =61.5) memory errors as compared to SO group. These results showed a significant deficit in spatial cognition after i.c.v. infusion of Aβ (1-42) peptides in NC animals, indicating successful induction of AD like pathology. In contrast, administration of diosgenin significantly reduced reference memory errors at 100 mg/kg (p=0.004) and 200 mg/kg ($p < 0.001$) when compared to NC rats (Fig. 2A). Likewise, working memory errors was also turned down remarkably in Dio 100 (p=0.003) and Dio 200 ($p < 0.001$) animals (Fig. 2 B).

Effect of diosgenin on passive avoidance task

Results from passive avoidance task implicated compromised aversive associative learning in NC rats by significantly reducing STL ($p < 0.001$, F (3, 36) =86) and increasing TDC ($p < 0.001$, F (3, 36) =38) when compared to SO animals. However, rats administered with diosgenin at 100 mg/kg (p =0.007) and 200 mg/kg ($p < 0.001$) significantly enhanced the STL when compared with NC (Fig 3A). On the contrary, TDC was dose dependently reduced in Dio100 (p =0.009) and Dio 200 ($p < 0.001$) animals (Fig. 3B).

Effect of diosgenin on hippocampal AChE activity.

Effect of diosgenin on brain AChE activity is depicted in Table 1. Biochemical analysis of brain hippocampus indicated high AChE activity in NC when compared to SO group ($p < 0.001$, F (3, 36) =19.8). Animals that received diosgenin at 100 mg/kg ($p < 0.01$) and 200 mg/kg ($p < 0.01$) manifested enhanced nootropic effect by exhibiting significant decline in AChE activity as compared to NC rats.

Effect of diosgenin on hippocampal Aβ (1-42) and TNF-α and IL-1β levels.

Significant increase in Aβ (1–42) level in NC rats ($p < 0.001$, F (3, 36)= 85.3) when compared to SO indicated development of animal model of AD. The elevated level of Aβ (1–42) was significantly counteracted by Dio 100 ($p= 0.027$) and Dio 200 ($p =0.007$) animals as compared to NC (Table 1).

Levels of TNF-α ($p< 0.001$, F (3,36)= 42.1) and IL-1β ($p< 0.001$, F (3,36)= 36.3) in the hippocampus were significantly high after Aβ (1–42) infusion in the NC animals as compared to SO group, suggesting AD mediated neuroinflammation. Administration of diosgenin for 28 days improved the inflammatory condition dose dependently by reducing the elevated cytokine levels. At 100 mg/kg, levels of IL-1β ($p= 0.010$) and TNF-α ($p=0.43$) were significantly lowered. Likewise, animals from Dios 200 demonstrated significant reduction in IL-1β ($p=0.005$) and TNF-α ($p=0.011$) levels as compared to NC. The anti-inflammatory effect of diosgenin is represented in Table 1.

Effect of diosgenin on SOD, CAT and GSR activities in hippocampus

As evident from our results, oxidative stress was induced in NC animals after Aβ (1-42) peptide injection, marked by significant reduction in the activities of SOD ($p <0.001$, F (3,36)= 55.1), GSR ($p <0.001$, F (3,36)= 143) and CAT ($p <0.001$, F (3,36)= 31) enzymes when compared to SO. Diosgenin at 100 mg/kg, significantly enhanced the antioxidant activities of SOD ($p <0.001$), GSR ($p= 0.002$) and CAT ($p<0.001$), comparable with the NC. Likewise, at 200 mg/kg, diosgenin improved the SOD ($p <0.001$), GSR ($p <0.001$) and CAT ($p <0.001$) activities more efficiently than 100 mg/kg (Table 2).

Histology of Hippocampus

H&E and CV staining were used to examine the neuroprotective activity of the diosgenin in rat hippocampus. In the current study, H& E staining in SO rats displayed intact neurons with prominent nucleoli in CA1 region of hippocampus. Aβ (1–42) peptides infusion in NC animals resulted in significant ($p < 0.001$, F (3,36)= 53.1) increase in protruding eosinophilic cytoplasm, swelling of neurons, pyknotic nuclei, pyramidal cells shrinkage and dispersed vacuolization identified from H& E staining as compared to SO group (Fig. 4). Diosgenin led to the significant reversal of the condition with mild neuronal toxicity, reduced pyknotic nuclei with lesser number of eosinophilic stained neurons and vacuolization at 100 mg/kg ($p= 0.001$) and 200 mg/kg ($p < 0.001$) when compared to NC. Similarly, on comparison with SO group, extensive neurodegeneration in the hippocampus of NC rats was observed as number of Nissl bodies with blue staining was reduced drastically when assayed by using CV stain, indicating disrupted cell viability ($p < 0.001$, F (3,36)= 22.9). CV positive neurons were dense and easily detected with more blue-stained Nissl bodies in CA1 region of SO hippocampus. Hence, from our experiment, we can convey that neuronal integrity and the number of CV-positive neurons in diosgenin treated groups were significantly improved at 100 mg/kg ($p =0.014$) and 200 mg/kg ($p < 0.001$) as compared to NC (Fig.5).

Discussion

One of the biggest challenge in the world is how to minimize and control the number of patients suffering from AD which is raising day by day (Mahdy et al. 2012). On this context, in the past decades, several

clinical trials were carried out in the hunt of new alternative therapeutics but were failed due to serious adverse effects or no significant therapeutic efficacy (Rahman et al. 2019). As complications associated with AD have complexed the development of the new curative, hence, it is crucial to search for the other strategies. Present study aimed to investigate the neuroprotective effects of diosgenin on Aβ (1–42) peptide induced oxidative stress, plaque accumulation, neuroinflammation and cholinergic dysfunction.

Intracerebroventricular injection of aggregated Aβ (1–42) in rat brain is a conventional AD animal model. Incubation of Aβ (1–42) peptides in aCSF for several days resulted in aggregation of peptides due to conformational transformation from random coil to β-sheet causing enhanced peptide neurotoxic potency (Nakamura et al. 2001). Earlier it was reported that single acute i.c.v injection of aggregated Aβ (1–42) in the rat mimics amyloid load as observed in the brains of AD patients, in both biochemical and histopathological studies. In addition, it also manifested memory dysfunction along with long-lasting disruption of both spatial and contextual fear memories, as well as short-term working memory leading to the neuronal death in rodents (Wang et al. 2014; Fu et al. 2006). Based on these observations, in the present study, we employed aggregated Aβ (1–42) infusion in rat brain as sporadic AD model.

In order to investigate the effect of diosgenin on Aβ (1–42) induced cognition impairment, we performed RAM and passive avoidance task, two behavioural paradigm in the present study. RAM task was used to evaluate learning ability and memory in the rats by measuring the number of spatial memory errors made during specified period. Likewise, passive avoidance task was conducted in order to learn the effect of diosgenin on memory functions by measuring avoidance response of diosgenin treated rats on unpleasant stimulus. Reports from the earlier studies documented that Aβ (1–42) mediated hampered ACh release ensues initiation of cognitive inadequacy in AD pathology, associated with altered memory performances (Xu et al. 2016). Further, Aβ (1–42) peptides infusion in rat brain induces neurobehavioural deficits and underpins the amyloid accumulation by degenerating BFCNs (Turnbull et al. 2018; Baker-Nigh et al. 2015). Similar response was observed in our study. Animals injected with Aβ (1–42) peptides in the brain exhibited deterioration in the short term and long term memory, as significant decline in right choices and latency was observed in RAM and passive avoidance task respectively. These results are in consistent with the former experiments (Yamada et al. 1999; Postu et al. 2018). Diosgenin administration in rats contributed in improvement of spatial learning behaviour task, demonstrating intact learning and memory functions. Few studies conducted earlier represented reversal of impaired learning and memory capacity by diosgenin in D-galactose induced aging rats and in 5XFAD mice model of AD (Chiu et al. 2011; Wang et al. 2018; Tohda et al. 2012). Our results are in congruous with these earlier observations. Previously, scientists have confirmed that increase in NGF biosynthesis initiates signalling cascades accountable for cell survival, growth and release of ACh through the cortico-hippocampal projections (Iulita and Cuello 2014, Mitra et al. 2019). As reported earlier, diosgenin being a potential NGF stimulator, might have produced protective effect on behavioural performances of cognitive impaired rats by improving ACh innervation to the cortex and hippocampus (Koh et al. 2016). In addition, decline in the Aβ (1–42) levels, AChE activity, oxidative stress and neuroinflammatory cytokines by diosgenin treated animals as seen in the current study might be collectively responsible for improved neurobehavioural

deficits. To our knowledge, this is the first study to demonstrate the neuroprotective property of diosgenin in Aβ (1-42) infused AD model.

Elevated brain levels of Aβ, majorly neurotoxic Aβ (1–42) is the primary reason of cognitive decline in AD (Andreeva et al., 2017). Previous studies have demonstrated that direct administration of aggregated Aβ (1–42) into the brains of rodents had caused memory deficits similar to that of AD patients by inducing plaque load (Yamada et al. 1999; Rahman et al. 2019). Our results are in agreement with these reports. Previously, after a series of experimentation, it was reported that Caprospinol, analog of diosgenin, dramatically restored cognitive decline by reducing amyloid deposits in FAB (Fe^{2+}, $A\beta_{42}$ and Buthionic sulfoxime) infused rat model (Lecanu et al. 2004; Lecanu et al. 2009; Lecanu et al. 2010; Papadopoulos et al. 2012). Results from these studies support our findings as we noticed that diosgenin treated rats showed reduced level of Aβ (1–42) when compared to negative control, proving neuroprotective role of diosgenin against plaque load. In recent years, it has been clear that amyloid deposits in the AD brain contributes to neurodegeneration by directly accelerating the membrane-associated oxidative stress and inflammatory responses, leading to the abnormal energy metabolism and the loss of synaptic functions (Tönnies and Trushina 2017). Hence, anti-amyloidogenic effect of diosgenin might be owed to the reduced oxidative stress and neuroinflammation as observed in the current study. However, further studies are needed to be conducted which can clarify the role of diosgenin on other major contributing factors behind Aβ accumulation in brain such as β-secretase, γ-secretase, Aβ-accumulation inhibitors and Aβ-degrading enzymes (Burg et al. 2013).

A large volume of evidence has described about the relationship between Aβ infusion and cholinergic dysfunction in selected AD brain regions. Aβ (1–42) accumulation is associated with loss of cortical synapse and atrophy of BFCNs which is responsible for hampered cholinergic transmission and enhanced AChE activity due to altered NGF metabolism (Counts and Mufson 2005; Mufson et al. 1995; Niewiadomska et al. 2011). Also, reports have confirmed that presence of aggregated Aβ (1-42) in the brain enhances the AChE expression and forms a stable complex with Aβ (Aβ-AChE). These complexes accelerate Aβ toxicity, Aβ deposition, neuronal cell loss, mitochondrial dysfunction and influences astrocyte hypertrophy (Hu et al. 2003). Our study confirmed escalated AChE activity after i.c.v. infusion of Aβ (1–42) in rats which is corresponding to a previous report (Rahman et al. 2019). However, from our research we noticed that administration of diosgenin before AD induction in rats attenuated the elevation of AChE activity dose dependently. Previous study by Koh et al. (2016) demonstrated that diosgenin stimulated NGF biosynthesis via regulating TrkA mediated NGF signalling cascades, resulting in neuronal survival and differentiation of cholinergic neurons. Thus, the probable reason behind diosgenin mediated AChE activity inhibition could be due to enhanced NGF stimulation. Also, reduction in the levels of oxidative stress and neuroinflammatory markers by diosgenin treated animals as observed in the current study could be other contributing factors associated with decreased AChE activity. This is the first study to demonstrate the direct effect of Diosgenin on AChE in an AD model.

Increased plaque load after Aβ (1–42) infusion in the AD brain activates microglial cells which generates pro-inflammatory cytokines (including IL-1β and TNF-α), as well as reactive oxygen species (ROS) and

produces neuroinflammation, causing detrimental effect on neurons (Calsolaro and Edison 2016; Shal et al. 2018). Further, chronic neuroinflammation in turn exacerbate AD complication by providing neurotoxic environment (Essawy et al. 2019). Our study indicated increase in pro-inflammatory cytokines levels (IL-1β and TNF-α) after Aβ (1–42) infusion in the rat brain. Diosgenin treatment before induction of AD like condition, provided a protective effect against Aβ (1–42) induced neuroinflammation by arresting the cytokines levels (IL-1β and TNF-α), strengthening the evidence of anti-inflammatory property of diosgenin. These results are comparable with the previous studies which displayed the ability of diosgenin and its analog in suppressing microglia mediated neuroinflammation in different models by inhibiting the Toll-like receptor (TLR)/nuclear factor kappa B (NF-κB) pathway (Hirai et al. 2010; Zhao et al. 2017; Li et al. 2018, Binesh et al. 2018) and phosphorylated-p38 mitogen activated protein kinase (MAPK) signalling pathways (Wang et al. 2017; Cai et al. 2019; Zhao et al. 2017). Hence, from these reports we can assume that by acting on the similar pathways, diosgenin was able to attenuate elevated cytokines levels in AD like pathogenesis. Moreover, modulation of ACh mediated cholinergic-anti-inflammatory pathway by stimulating NGF biosynthesis by diosgenin as reported from previous studies might support the desired activity (Mitra et al. 2019; Koh et al. 2016). Also, ROS scavenging property of diosgenin as illustrated in this study could be the other possible molecular explanation. To the best of our knowledge, this is the first study to demonstrate anti-inflammatory effect of diosgenin in Aβ (1–42) infused AD rats.

Amyloid accumulation along with the degenerated BFCNs after aggregated Aβ (1-42) infusion in rodents promotes free radical release causing oxidative stress (Boyd-kimball et al. 2004). It is exhibited by impairment in the functions of various endogenous antioxidant enzymes involved in maintenance of ion homeostasis such as superoxide dismutase, glutathione reductase, thioredoxins, and catalase, leading to neuropathological alterations (Feng and Wang 2012; Boyd-kimball et al. 2004). Although, the precise mechanism by which such an oxidative effect occurs is still unclear. It was proposed that senile plaques has high levels of copper which promotes reduction of peptide-bound Cu^{2+} to Cu^{+} and form hydrogen peroxide (H_2O_2) and further reacts with H_2O_2 to form highly reactive hydroxyl free radicals (Butterfield et al. 2013). Generated oxidative stress triggers apoptosis of neuronal cells and hinders the brain functions, resulting in impaired learning and memory deficits (Lennon et al. 1991; Alzoubi et al. 2013; Serrano and Klann 2004; Tuzcu and Baydas 2006). As the brain cells are vulnerable to oxidative stress because of its high oxygen consumption and impaired antioxidant enzymes activities, therefore, regulation of antioxidant enzymatic activities is crucial for developing new therapeutics for the management of neurodegenerative diseases (Bayrakdar et al. 2014). In the present study, reduced activities of antioxidant enzymes such as SOD, GSR and CAT was noticed in negative control rats. Our results are in consistent with the earlier reports (Ledezma et al. 2021; Bayrakdar et al. 2014) and indicates successful induction of oxidative stress after Aβ (1-42) infusion. Known for potent anti-oxidant effect (Son et al. 2007; Jagadeesan et al. 2012), diosgenin ameliorated toxic effect of Aβ on hippocampal SOD, CAT, and GSR activities in diseased rats. Several reports from previous studies stated that NGF attenuates oxidative stress by regulating PI3K, Akt, MAPK-JNK, p53, and NF-κB signalling pathways (Salinas et al. 2003; Chiu et al. 2011; Kaplan and Miller 2000). As diosgenin was earlier reported as a potential NGF regulator (Koh et al. 2016), hence, regulation of these signalling pathways by diosgenin could be the possible

explanation behind its antioxidant potential. Besides, brain inflammatory response to the deposition of extracellular amyloid plaques mediated activated microglia is another source of ROS production (Tönnies and Trushina 2017). As evident from the present study, diosgenin has produced protective effect against Aβ (1-42) induced neuroinflammation, which could be another reason behind the radical scavenging property of diosgenin. This is the first study to demonstrate the antioxidant property of diosgenin against Aβ (1-42) peptides mediated oxidative stress.

Furthermore, neuroprotective role of diosgenin was further supported by histopathological analysis. H & E staining revealed Aβ mediated neuronal damage in CA1 region of the hippocampus of Aβ (1-42) induced AD rats and demonstrated pyknotic nuclei, disorganization of neurons, nuclei swelling and neuronal shrinkage which is in accordant with the previous report (Rahman et al. 2019). Additionally, cresyl violet staining displayed significant morphological changes in CA1 region of the hippocampus of negative control rats with neuron swelling, vacuolization and apoptotic cells upon Aβ (1-42) induction. Diosgenin dose dependently protected the neuronal cells from the deteriorating effect of Aβ (1-42) and intensified the number of healthy neurons with prominent nuclei. This is the first study to demonstrate the histopathological effects of diosgenin in Aβ (1–42) induced AD rats. These improved histopathology outcomes by diosgenin are in consistent with our behavioural and biochemical assessments.

Conclusion

The present work strongly suggests neuroprotective property of diosgenin which was in accordance with the available evidences against AD. The i.c.v. infusion of Aβ (1-42) peptides in wistar rats, demonstrated behavioural disturbance together with plaque load, neuroinflammation, oxidative stress and cholinergic dysfunction. After behavioural, biochemical and histopathological evaluation, our study indicated that oral administration of diosgenin has improved the neurobehavioural performances of Aβ (1-42) peptides infused AD rats. Likewise, diosgenin also attenuated the Aβ (1-42) peptides induced elevated AChE activity and produced protective effect against plaque load, oxidative stress and neuroinflammation mediated neuronal damage. NGF stimulation along with cumulative defensive effects against different AD pathologies by diosgenin could possibly be responsible for its effectiveness against memory impairment in AD rats. However, possibility of introducing diosgenin for the management of AD and its related complications demands further investigation to elucidate the impact of diosgenin on the other neurochemicals involved in AD and identification of molecular mechanism behind its neuroprotective effects.

Abbreviations

1,25D3–MARRS 1,25D_3 -membrane-associated, rapid response steroid-binding protein

ACh Acetylcholine

AChE Acetylcholinesterase

aCSF Artificial cerebrospinal fluid

AD Alzheimer’s disease

Akt Protein kinase B

ANOVA Analysis of variance

AP Anterior–posterior

Aβ Amyloid beta

BFCN Basal forebrain cholinergic neurons

CAT Catalase

CMC Carboxymethyl cellulose

CV Cresyl violet

DTNB 5,5-dithio-bis-(2-nitrobenzoic acid)

DV Dorsal-ventral

EDTA Ethylenediamine tetraacetic acid

ELISA Enzyme-linked Immune Sorbent Assay

ERK Extracellular signal-regulated kinase

GSR Glutathione reductase

H&E Hematoxylin and eosin

H_2O_2 Hydrogen peroxide

i.c.v Intracerebroventricular

IAEC Institutional Animal Ethics Committee

IL-6 Interleukin 6

JNK c-Jun N-terminal kinase

MAPK Phosphorylated-p38 mitogen activated protein kinase

ML Medial-lateral

NADH Nicotinamide adenine dinucleotide

NADPH Nicotinamide adenine dinucleotide phosphate

NF-κB Nuclear factor kappa B

NGF Nerve growth factor

p75NTR p75 pan neurotrophin receptor

PI3K Phosphatidylinositol 3-kinase

RAM Radial arm maze

ROS Reactive oxygen species

SD Standard deviation

SOD Superoxide dismutase

STL Step-through latency

TDC Total time spent in the dark chamber

TLR Toll-like receptor

TNF-α Tumour necrosis factor

TrkA Tyrosine kinase A receptor

Declarations

Declaration of Competing Interest

All authors declare that they have no conflict of interest.

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Author contributions

SS performed the experiments and was a major contributor in writing the manuscript. JA analyzed and interpreted the raw data regarding the Alzheimer's disease. SP conceptualized, reviewed and edited the manuscript. SPD reviewed and edited the manuscript. All authors read and approved the final manuscript.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Ethical approval: All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council (US)) and was approved by the Institutional Animal Ethics Committee (IAEC) (JSSCP/IAEC/PhD/Pharmacy Practice/01/2018-19).

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Tables

Table 1. Effect of diosgenin on hippocampal AChE activity and TNF-α, IL-1β and Aβ (1-42) levels in Aβ (1–42) peptides infused rats.

Groups	AChE activity (μM/min/g protein)	TNF-α (pg/ml)	IL-1β (pg/ml)	Aβ (1-42) (pg/ml)
SO	4.36±1.93	7.70±0.94	13.8±1.69	5.70±1.89
NC	$9.1\pm2.24^{\#\#\#}$	$18.5\pm1.84^{\#\#\#}$	$26.9\pm3.54^{\#\#\#}$	$24.8\pm3.43^{\#\#\#}$
Dio 100	6.19±1.71**	15.2±2.97*	23.3±2.91*	21.0±2.31*
Dio 200	3.37±1.05***	14.9±2.56**	22.6±3.20*	20.3±3.53**

The values were expressed as mean ± SD (n=10) and were tested by one-way ANOVA (df1 = 3, df2 = 36) followed by Tukey post hoc test. Significant change was reported at ###p < 0.001 vs. SO, *p < 0.05, **p < 0.01 and ***p < 0.001 vs. NC group.

Table 2. Effect of diosgenin on hippocampal SOD, CAT and GSR activities in Aβ (1–42) peptides infused rats.

Groups	GSR (nmol /min/mg protein)	CAT (µmol/min/g protein)	SOD (units/ min/mg protein)
SO	33.9±1.86	244±43.3	3.14±0.508
NC	20.2±1.33$^{###}$	194.7±19.6$^{###}$	0.781±0.33$^{###}$
Dio 100	22.9±1.61**	171±40.0***	1.79±0.44***
Dio 200	26.6±1.53***	177±31.2***	2.09±0.352***

The values were expressed as mean ± SD (n=10) and were tested by one-way ANOVA (df1 = 3, df2 = 36) followed by Tukey post hoc test. Significant change was reported at ###p < 0.001 vs. SO, **p < 0.01 and ***p < 0.001 vs. NC group.

Figures

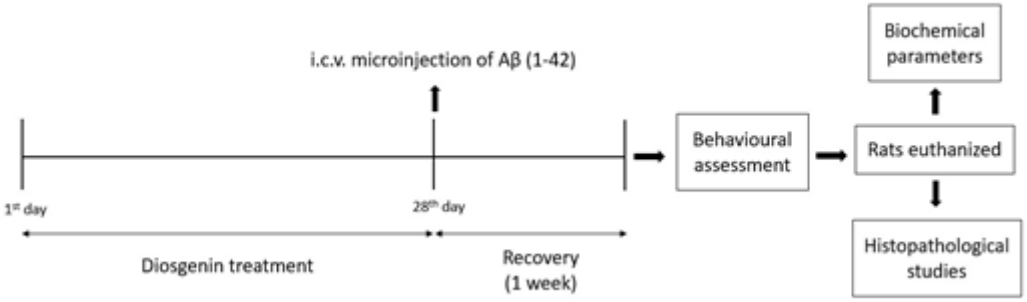

Figure 1

Diagrammatic scheme of experimental procedure

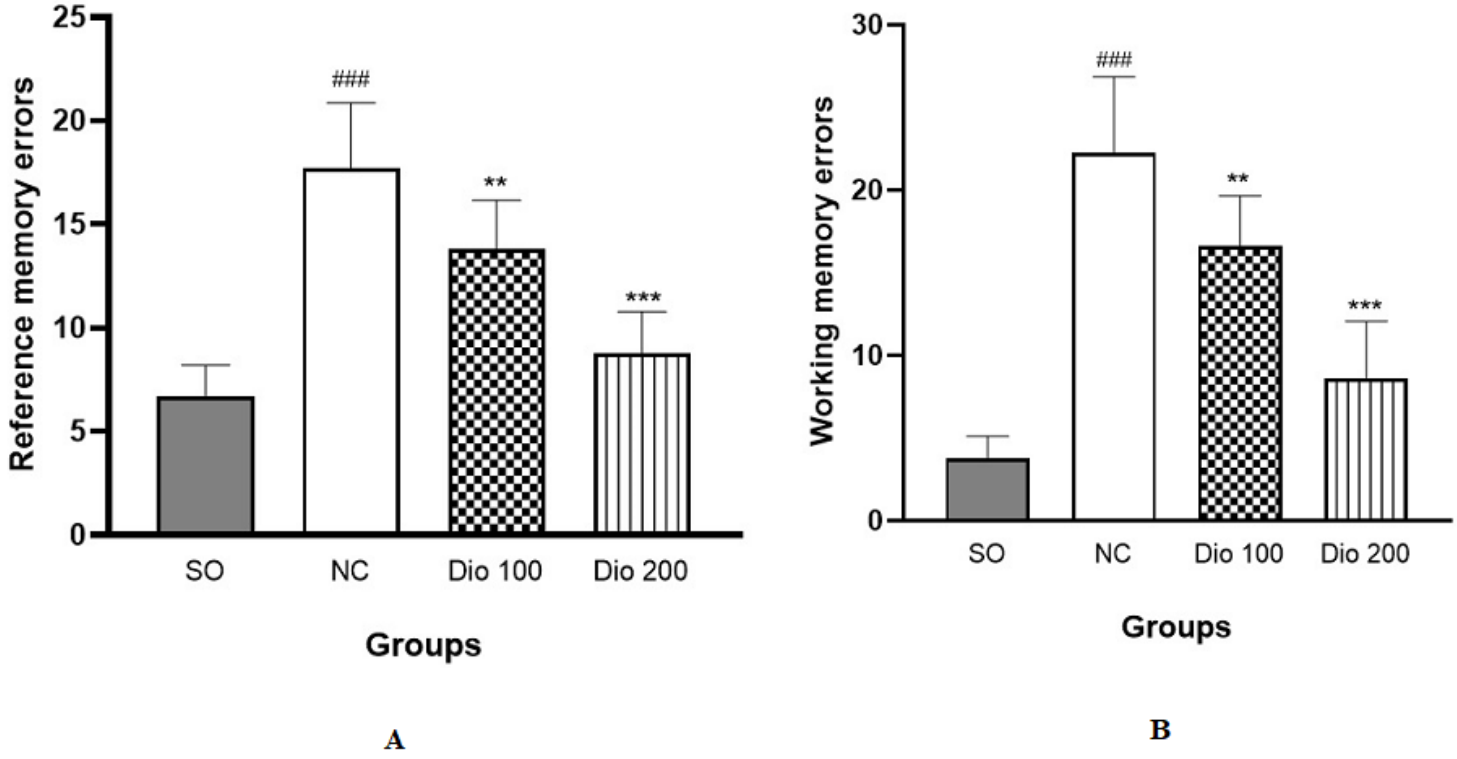

Figure 2

Effect of diosgenin on passive avoidance task in Aβ (1-42) infused rats. (A) step-through latency, (B) total time spent in the dark compartment. The values were expressed as mean ± SD (n=10) and were tested by one-way ANOVA (df1 = 3, df2 = 36) followed by Tukey post hoc test. Significant change was reported at ###p < 0.001 vs. SO, **p < 0.01 and ***p < 0.001 vs. NC group.

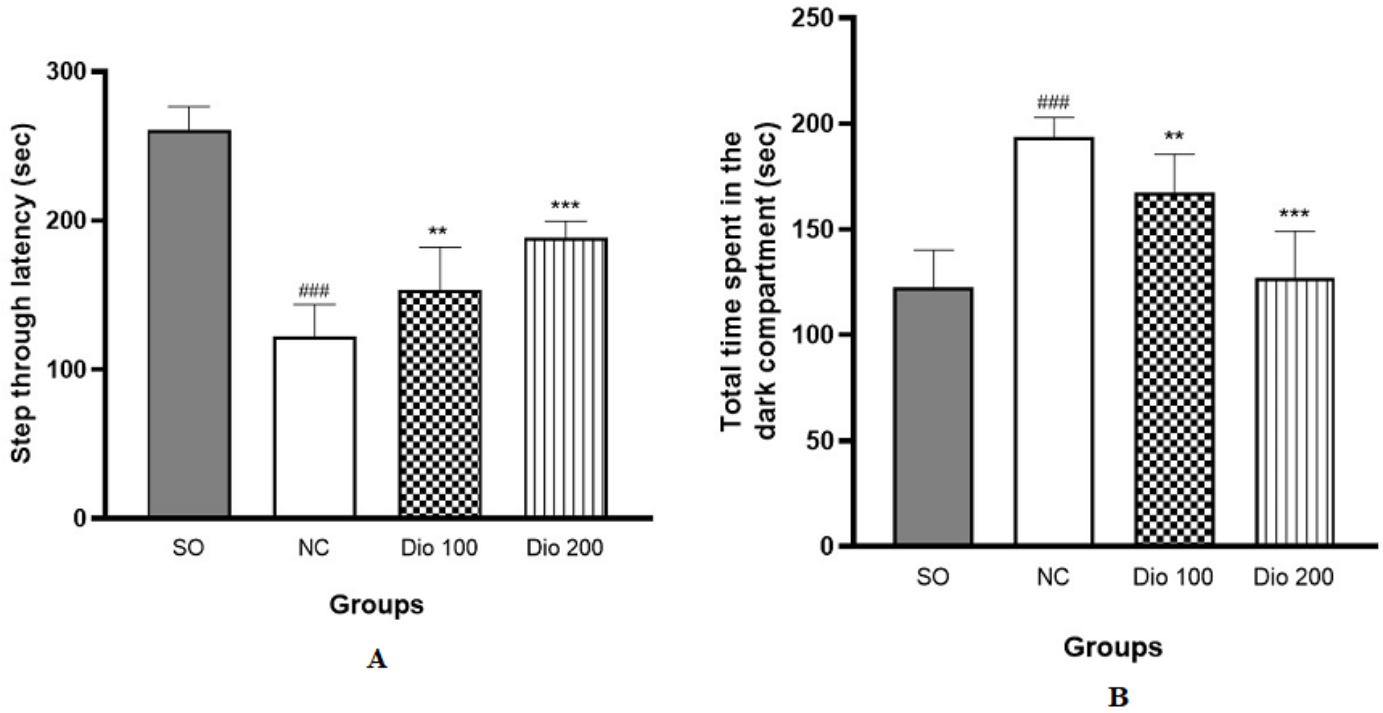

Figure 3

Effect of diosgenin on passive avoidance task in Aβ (1-42) infused rats. (A) step-through latency, (B) total time spent in the dark compartment. The values were expressed as mean ± SD (n=10) and were tested by one-way ANOVA (df1 = 3, df2 = 36) followed by Tukey post hoc test. Significant change was reported at ###p < 0.001 vs. SO, **p < 0.01 and ***p < 0.001 vs. NC group.

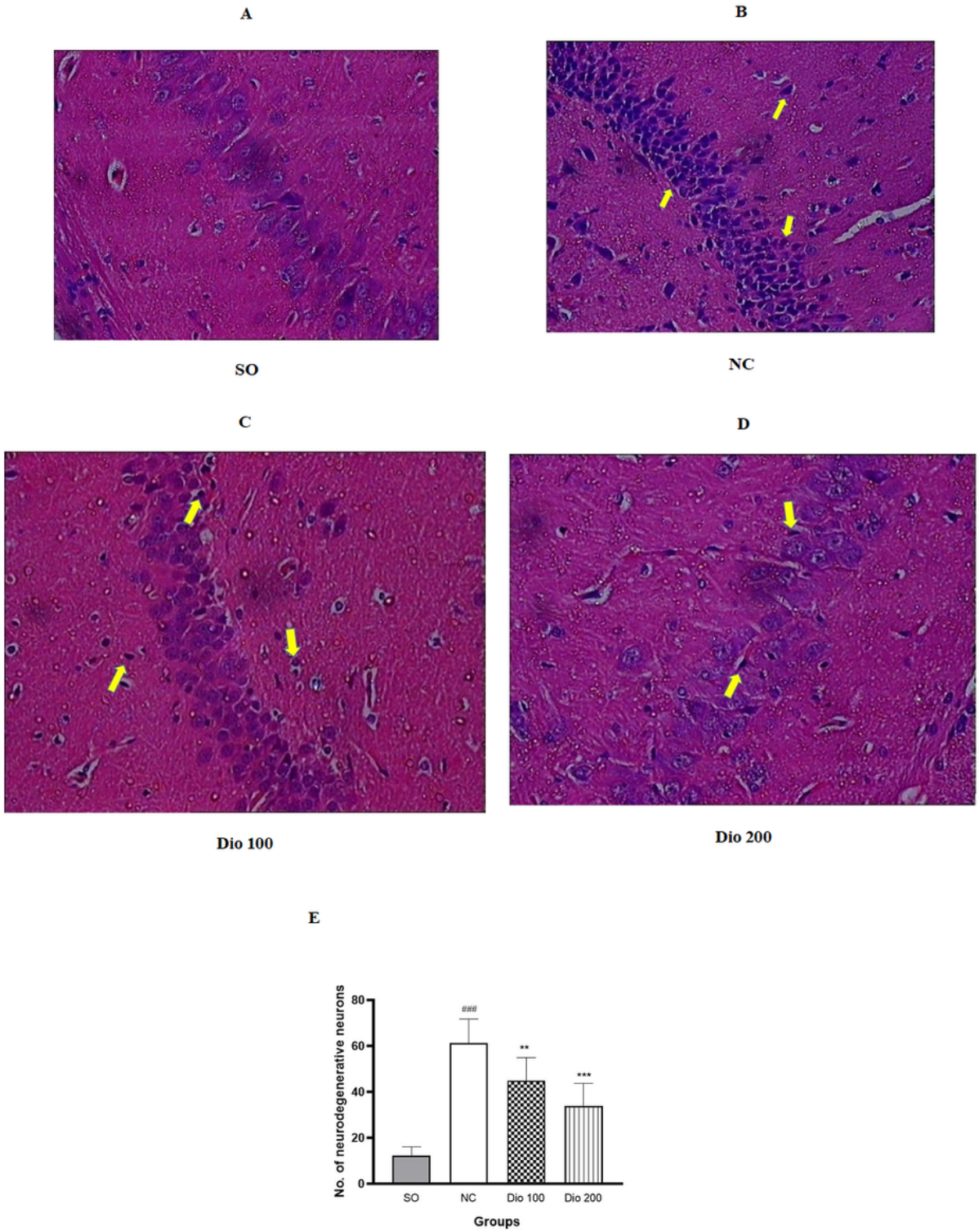

Figure 4

Histopathological examination of CA1 region of hippocampus stained with H & E (40X). (A) SO represents healthy neurons with prominent nuclei, (B) NC group representing neuronal damage, eosinophilic stained cytoplasm, vacuolization and neuronal shrinkage, (C) diosgenin (100 mg/kg) and (D) diosgenin (200 mg/kg) treated groups representing mild neuronal injury with lesser number of eosinophilic stained neurons, (E) quantitative assessment of number of degenerated neurons in CA1 hippocampal section of rat brain. The values were expressed as mean ± SD (n=10) and were tested by one-way ANOVA (df1 = 3, df2 = 36) followed by Tukey post hoc test. Significant change was reported at ###p < 0.001 vs. SO, **p < 0.01 and ***p < 0.001 vs. NC group.

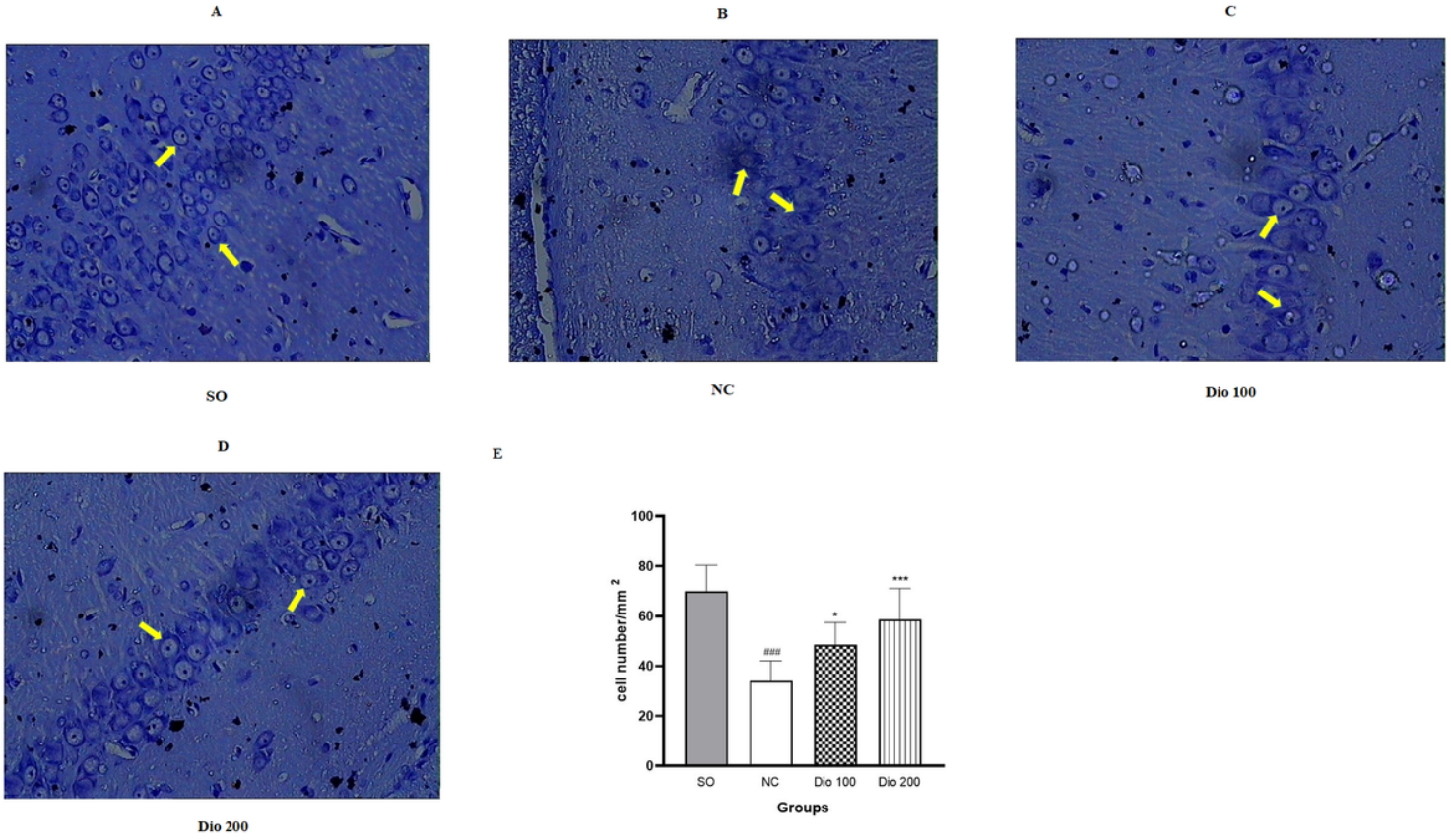

Figure 5

Histopathological examination of CA1 region of hippocampus stained with Cresyl Violet (40X). (A) SO represents healthy neurons with prominent nuclei and densely stained, (B) NC infused group representing neuronal damage with lesser number of stained neuron indicating less viable neurons (C) diosgenin (100 mg/kg) and (D) diosgenin (200 mg/kg) treated groups representing mild neuronal injury with higher number of stained neurons, (E) quantitative assessment of number of healthy neurons in hippocampal sections of rats brain. The values were expressed as mean ± SD (n=10) and were tested by one-way ANOVA (df1 = 3, df2 = 36) followed by Tukey post hoc test. Significant change was reported at ###p < 0.001 vs. SO, *p < 0.05 and ***p < 0.001 vs. NC group.