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Levosimendan ameliorates the scopolamine induced cognitive impairment via modulating pCREB/BDNF expression, cholinergic neurotransmission and neuroinflammation

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Abstract

Levosimendan, an inotropic drug that potentiates ATP-dependent K^+ channel and sensitise troponin-C for calcium. It has positive effects in neurodegenerative diseases like Parkinson's disease and mild septic encephalopathy. However, its effect on Alzheimer's disease remains unclear. Hence, in this study, we have investigated the effect of levosimendan against scopolamine induced cognitive impairment in rats via behavioural tests (Y maze, water maze and novel object recognition test), cholinesterase enzymes, oxidative stress markers, amyloid-β plaques, inflammatory mediators, and neurotrophic factors analysis. In this study, rats were divided into five groups. The first and second groups were given with vehicle or levosimendan (100 μg/kg/day i.p.) throughout the study. The remaining groups were pre-treated with vehicle/Donepezil (3mg/kg/day i.p.)/Levosimendan (100 μg/kg/day i.p.) respectively. From day – 8 these groups were given with their respective drugs and Scopolamine (3 mg/kg., i.p.) with 1 hour time interval. Behavioral analysis was carried out to assess the learning and memory functions of rats after 30 min of scopolamine administration from day 8 to day 17. Levosimendan treated group significantly improves in number of spontaneous alterations and recognition ability in Y-maze and NOR test respectively. In addition, levosimendan inhibited scopolamine induced pathological changes such as increased levels of cholinesterase enzymes, Aβ accumulation, neuro inflammation and reduced levels of BDNF. In summary, these findings indicate that pre-treatment with levosimendan mitigates the scopolamine induced behavioural and biochemical changes.

Highlights

- Protective role of Levosimendan against scopolamine induced cognitive deficits was investigated.
- Scopolamine decreases the expression of BDNF, and enhances Aβ accumulation and neuro inflammation.
- Levosimendan ameliorated all the behavioural changes caused by scopolamine in the rats.
- Levosimendan inhibited scopolamine induced pathological changes such as increased levels of cholinesterase enzymes, Aβ accumulation, neuro inflammation and reduced levels of BDNF.

Introduction

Neurodegenerative disorders are wide-ranging medical complications associated with the gradual loss of functional neurons in the brain. In these, Alzheimer's Disease (AD) is an age-related neurodegenerative disorder characterized by cognitive impairment and memory loss. The Alzheimer's disease Facts and Figs. 2020 reported that the number of patients above 65 years with AD will greatly increase to about 5.8 million to 13.8 million by 2050 in Western countries. Several pathological expressions were influenced to play an important role in progression of AD *viz.*, abnormal amyloid beta accumulation, hyperphosphorylated tau proteins, cholinergic neuronal dysfunction, oxidative stress and neuroinflammation. All these conditions together can subsequently lead to neuronal apoptosis and cognitive dysfunction [1, 2]

Acetylcholine (Ach) governs signal conduction and memory in the brain [3]. Any degeneration of cholinergic neurons in the brain and hippocampus limits acetylcholine transferase. As a result, choline reuptake gets diminished, and thereby the synthesis of ACh is restrained. On the other hand, cholinesterase enzymes in the brain metabolises the Ach and diminishes the availability of Ach in synaptic cleft results in cholinergic dysfunction. So, acetylcholinesterase inhibitors *viz.*, Donepezil, Rivastigmine, Tacrine, and Galantamine and NMDA receptor antagonists like memantine were prescribed for mild to moderate AD like symptoms. However, these drugs are more likely to produce dose-dependent adverse effects than producing transient and beneficial effects to alleviate AD [4].

Amyloid beta (Aβ) accumulation and hyper phosphorylated tau proteins are the major hallmarks of AD. However, in early stages of AD, Tau oligomers are detected as a pathogenic cascade and increased levels correlates with the neuronal loss and synaptic dysfunction. Besides, Amyloid precursor protein (APP), the precursor for Aβ deposition undergo breakdown by α, β and γ-secretase enzymes. α- secretase prevents the formation of Aβ by generating αAPPs, that is neurotrophic and neuroprotective. On the other hand, APP cleaved by β-secretase (BACE-1) releases the sAPPβ that can be further cleaved by γ-secretase to form Aβ intracellularly. Aβ oligomers migrates to extracellular milieu and triggers the cascade of events leading to neurotoxicity and neuroinflammation [4, 5]. Moreover, over expression of Aβ down regulate the expression of critical neurotrophic factors like Brain-derived neurotrophic factor (BDNF) in the brain [6].

BDNF, a member of the neurotrophin family, is active in the cerebral cortex, amygdala, and hippocampus. It plays a crucial role in growth of certain neuronal populations, and acts as an effector of neuronal plasticity both during development and in the adult. Further, it plays a part in long term potentiation and up-regulated in the hippocampus during learning and memory. A deficit in the hippocampal BDNF levels contribute to the development of cognitive impairment in AD.

Cyclic adenosine monophosphate response element binding protein (CREB) is a transcriptional regulator of BDNF and plays an important role in memory processes. Further, decreased levels of phosphorylated CREB were observed in the post-mortem AD brain. It has been evidenced that amyloid plaques suppress the activity of CREB and then BDNF [7]. In support to this several studies described the role of pCREB/BDNF in acquisition of long-term potentiation and synaptic plasticity [8]. Further, Aβ deposition and BDNF decline in cortex and hippocampus results in production of Reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydroxyl radical (-OH), Peroxynitrite (ONOO-) can oxidises the cellular components and causes neuronal cell death [9].

Aβ deposition, oxidative stress and neuroinflammation in AD can promotes depolarization of neuronal membrane leads to over activation of glutamate receptor which stimulates the excitatory neurotransmitter “glutamate” results in Ca^{2+} influx through the NMDA receptor channel. The excessive intracellular Ca^{2+} ions in the neurons can promote tau hyperphosphorylation in hippocampal neurons, impairs synaptic function and causes neuronal damage in AD [10].

Ion channels are the proteins situated across the cell membrane and facilitates the ion transport in the cells. Among them, ATP-dependent K^+ channels are the most diverse and abundantly present in the brain

[11]. Some studies investigated and reported that chronic activation of ATP-dependent K^+ channels in the brain cells (microglia, neurons, and lymphocytes) play a crucial role in reducing the Aβ and tau pathologies by NMDA receptor-mediated cellular Ca^{2+} overload (excitotoxicity) and improves cognitive function by hyperpolarizing the plasma membrane and [10].

Levosimendan, a positive inotropic drug approved to increase cardiac contractility and management of decompensated heart failure. Indeed, earlier research has reported the ability of levosimendan against Parkinson's disease [12], mild septic encephalopathy [13], ototoxicity [14], nephrotoxicity [15], tau-oligomerisation inhibition in brain cells of Tau^{P301L}-BiFC mice [16] and other attributes include anti-oxidant and anti-inflammatory effects [17–19]. The cardioprotective effects and vasodilatory actions Levosimendan are mediated by calcium sensitization of contractile proteins and opening of ATP-dependent K^+ channels in vascular smooth muscle cells and cardiac mitochondria [20–24]. Further, levosimendan as a phosphodiesterase III inhibitor, increases the levels of cAMP and activates protein kinase – A which phosphorylates the downstream substrates (CREB) that promotes neurogenesis and increases synaptic plasticity [25, 26]. Hence, the one of the plausible ways to curb the AD progression is to find the novel therapeutic approach which activates the CREB/BDNF pathway, ATP-dependent K + channels and modulate the levels of Aβ, BDNF, ROS, and proinflammatory cytokines in the brain.

However, no studies have addressed the anti-amnesic effect of levosimendan on learning and memory in rats. Hence, the present study aims to investigate the neuroprotective effect of levosimendan against scopolamine induced cognitive impairment and elucidate the underlying mechanisms.

Materials and methods

Animals

Male albino wistar rats of weight 250–300 g each were used in this study. Animals were purchased from Vyas labs, Hyderabad. Animals allowed to acclimatize for one week in the animal house (439/PO/S/01/CPCSEA) of Shri Vishnu College of Pharmacy, Bhimavaram. After adaptation, the animals were randomly divided into 5 groups. All the animals were fed with a standard pellet diet and water *ad libitum*. The temperature of 23 ± 1°C with 12 h light/dark cycles and relative humidity of about 60% was maintained throughout the study. Behavioural tests present in the study were performed between 8:00 to 16:00 hrs. The study protocol (05/IAEC/SVCP/20–21) was approved by the Institutional Animal Ethics Committee (IAEC).

Drugs and Chemicals

Scopolamine hydrobromide, donepezil HCL were purchased from yarrow chem products (Mumbai). Levosimendan (simenda®), acetylcholine thiocholine iodide (ACTCI), butyryl thiocholine iodide (BuTCI), 2,2-Diphenyl-2-picrylhydrazyl (DPPH), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), bovine serum albumin, Bradford's reagent were purchased from sigma aldrich, thiobarbituric acid, reduced glutathione, pyrogallol, hydrogen peroxide, cacodylic acid were purchased from loba chemie (Mumbai), tetra methoxy propane

was purchased from Tokyo chemical industries and ELISA kits for β-amyloid plaques, BDNF (brain derived neurotropic factor), NF-κB are purchased from krishgen biosystems (Mumbai)

Experimental design

Rats were randomly divided into 5 groups, containing 6 in each; the control group [Veh + Veh] only received normal saline (1 ml/kg, i.p.), Per se group [Veh + Levo] received Levosimendan (100 μg/kg, i.p.), Disease group [Veh + Sco] was injected with Scopolamine (3 mg/kg, i.p.), Standard group [DPZ + SCO] was pre-treated with donepezil (3 mg/kg, i.p.) followed by scopolamine (3 mg/kg, i.p.) administration till the end of the study. The treatment group [Levo + SCO] rats were pre-treated with Levosimendan (100 μg/kg, i.p.) thereafter scopolamine (3 mg/kg, i.p.) was administered till the end of the study. Therapeutic doses of all the drugs are selected based on the previous studies with few modifications [27–29]. All the drugs used were dissolved in normal saline (Veh). The pre-treatment was given to per se, standard and treatment groups respectively. Scopolamine was given from Day 8 to Day 17 after one hour of drug administration and behavioural studies were carried out after 30 minutes of scopolamine injection.

Study timeline

Details of the experiment protocol and treatment schedule were represented in Fig:1

Fig:1 - Scheme of experimental schedule and intervals for estimation of various parameters

Behavioral tests

Open field test

Test apparatus consists of a square field (100 × 100) which is enclosed by black plexiglass wall of 35 cm height. The maze is virtually divided into 16 squares of equal size (4 × 4). 12 squares which are adjacent to the wall are named as peripheral squares representing field periphery and remaining 4 squares are named as central squares representing the exposing field. Animals were subjected to an open field test to assess the locomotor activity of the animal by placing it at the centre of the arena and allowed to move freely for 8 min.

In the open field, behaviour of animals like number of crossings, distance travelled by the animal were observed [30].

Novel object recognition test

Novel object recognition test is an experimental tool for assessing short-term memory impairment caused by neurological changes in the brain and hippocampus. This test uses the natural behaviour of the animal that prefers to expose the novel object. It helps to assess the cognitive deficits in rodent models related to neurodegenerative disorders induced by drugs or chemicals [31]. The test was done in an open arena measuring 50×50 cm closed field surrounded by 50 cm walls and the whole apparatus is made of black polyester plastic. All the objects placed in the arena were with similar structure, colour, and size but different in shape. This test includes 3 phases namely 1) Habituation 2) Training and 3) Testing. In the

habituation phase animals were allowed to explore the open field arena freely for 10 min to habituate the maze environment. After twenty-four hours of habituation phase, the training phase is carried out by placing two identical objects (A_1 and A_2) at opposite corners 10 cm away from the wall. The training phase was performed for 5 min in which animals were allowed to explore the objects for at least 30 sec. Testing phase was initiated after 60 min of delay time. In this phase we have replaced one of the old objects (A_1 and A_2) with a novel object (B_1) and observed the exploration of each animal for 5 min. Exploration is defined as touching or sniffing the object with nose or forepaws and sitting on the object is not considered as exploration [3]. The total time spent with the two identical objects (A_1 and A_2) in the training phase and two different objects (A_1 or A_2 and B_1) in the testing phase was noted [32]. All the objects and arena were cleaned using 70% ethanol between each trail [33]. Cognitive ability of each animal is evaluated by discrimination index and it is calculated by using the formula (DI) is B-A/B + A where, A is the time spent with old object (A_1 or A_2) and B is the time spent with novel object in testing phase [34]. All the movements and animal behaviour were observed with a video tracking system.

Y Maze – Spontaneous alterations test

Y-maze test was performed to assess the working memory index by spontaneous alternation behaviour. Spontaneous alternation behaviour was considered as a replicate to spatial working memory which is a form of short-term memory index. The maze is made of three identical arms of equal dimensions (40 cm long, 20 cm height and 10 cm width) which is symmetrically separated at 120° and labelled as A, B and C. It can be identified by using visual cues. Rats were allowed to explore freely throughout the maze for 8 min by placing it at the end of one arm (e.g., A) and calculated the % spontaneous alterations. An arm entry was considered when all the four paws of rat were completely inside the arm. Spontaneous alternation behaviour is defined as successive entry of the animal into all the three arms (i.e., ABC, BAC, CBA but not BAB) [35]. Total number of arm entries were recorded as locomotor index and percentage spontaneous alterations were calculated as [36].

$$\% spontaneousalterations = \frac{numberofalterations}{totalnumberofarmentries - 2} \times 100$$

The maze is cleaned after each trail with 70% ethanol to clear the residual odour and dried with cloth before testing another animal [37].

Morris Water Maze (MWM) Test

The spatial learning and long-term memory of the animals were tested by using MWM. The apparatus consists of a black circular water pool of 250 cm in diameter, 50 cm in height, and filled with water up to 40 cm. The water tank was divided into four equal quadrants and each quadrant is marked with an identification sign using visual cues. The platform of 10 cm diameter is placed at a fixed position (centre of south-west quadrant) 1 cm beneath the surface of the water and remains in the same position throughout the experiment. All the trails were recorded using a video tracking system by placing the camera above the circular tank and visual movements of the animal was analysed by using MAZE MASTER software (V. J INSTRUMENTS). The test was divided into three phases 1) Training 2) Evaluation

phase 3) Probe analysis. In the training phase, animals were familiarized with the water maze as four trails per day by starting at four different points (North, East, West & South) for two days to find the platform using visual cues. If the animal does not find the platform, we guide them carefully to the platform and remain on it for 30 s before starting the next trail. Evaluation phase was carried out on day 13 for three consecutive days. In this phase, water in the maze was made opaque using milk powder to hide the platform and the escape latency (time to reach the hidden platform) was measured. Probe analysis was conducted after 24 hours of the completion of the evaluation phase. In this analysis we removed the platform and the animal was allowed to swim freely for 60 s and time spent in the target quadrant was recorded [38, 39].

Estimation of biochemical parameters

On the next day of behavioral analysis, rats were anaesthetized by using ketamine (80 mg/kg i.p.)[40] to collect blood in a heparinised tubes through retro-orbital route. Animals were sacrificed by decapitation, brain tissues were harvested and washed with ice-cold PBS. Hippocampus was isolated from the whole brain and 20% homogenate was prepared using ice-cold PBS (pH – 7.4) by using a tissue homogenizer. The homogenate/blood was subjected to centrifugation at 10000g at 4°C for 15 min and supernatant/plasma was collected and stored at -80°C until the analysis. Hippocampus for histopathological examination were placed in 10% formalin immediately after washing.

Total Protein estimation

Total protein concentration in the plasma and homogenate samples were estimated as reported earlier by using bovine serum albumin as standard [41].

Acetylcholinesterase (AChE) activity

AChE activity in plasma and tissue homogenate were carried out as previously described by Ellman *et.al.*, [42]. with small modifications. In this method acetylthiocholine iodide (ACTCI) was used as a substrate. The increase in yellow colour by reacting thiocholine with DTNB (dithio*bis*nitrobenzoate) was measured spectrophotometrically. We have performed the enzyme kinetics periodically at standard time points in the Human ELISA reader at 405 nm. All the samples were run in triplicate and the activity of AChE is expressed as nmol ACTCI hydrolysed min^{-1} mg^{-1} of protein [43].

Butyrylcholinesterase (BuChE) activity

BuChE activity was also analysed in plasma and tissue homogenate since, BuChE is also a cholinesterase enzyme that hydrolyses acetylcholinesterase and promotes AD. Butyryl thiocholine iodide (BuTCI) is used as substrate and the same spectrophotometric method using DTNB was used and absorbance at 405 nm was measured by using Human ELISA reader. All the samples were run in triplicate and the activity of BuChE is expressed as nmol BuCTCI hydrolysed min^{-1} mg^{-1} of protein [44].

Lipid peroxidation

The rate of lipid peroxidation was measured by estimating the level of malondialdehyde (MDA). MDA is a lipid peroxidation by-product that occurs by the breakdown of polyunsaturated fatty acids. Hence, MDA estimation was a good indicator of lipid peroxidation. Briefly, 100 µl of sample, 750 µl of 0.8% Thiobarbituric acid, 750 µl of 20% glacial acetic acid (pH – 4.0) and 100 µl of 8.1% sodium dodecyl sulphate were added in a micro centrifuge tube. The final resultant mixture is incubated at 95^0 C for 60 mins. The absorbance of pink colour produced is measured spectrophotometrically at 535 nm. The amount of MDA is expressed as nmol mg^{-1} of protein [44].

Nitric oxide (NO)

NO content in the brain homogenate was measured as total nitrate/nitrite by using Griess reagent. Griess reaction involves the formation of azo dye by the diazotization of sulfanilamide when react with nitrites followed by conjugation with N-(1-napthyl) ethylenediamine to form a pink colour chromophore which can be measured spectrophotometrically at 540 nm [45].

Reduced glutathione (GSH)

Glutathione is a most important antioxidant present in the cells that participates in the detoxification of oxidative free radicals and inhibits lipid peroxidation. Measurement of reduced glutathione was performed as per the method described by Ellman et.al., using 5,5-Dithio-bis-(2-nitrobenzoic acid) (DTNB). The glutathione standards (0, 0.5, 1.0, 2.0, 4.0, 8.0, 12, and 16.0 mM) and samples were prepared by diluting with phosphate buffer pH7.4, from this 80 µl of standards / sample were added to 60 µl of DTNB and measured the absorbance at 412 nm [46].

Superoxide dismutase (SOD)

SOD is an enzyme that helps to break down the harmful superoxide free radicals in the cells which might prevent damage to tissues. By using the pyrogallol auto-oxidise technique, SOD levels in samples were calculated. In this method, pyrogallol self-oxidizes in alkaline solutions to form superoxide anion radicals, which are then oxidized by SOD in the sample to produce oxygen and hydrogen peroxide. Briefly, 50mM Tris-cacodylate buffer (pH 8.2), 1mM EDTA, 100 µl of a sample, 2.6 mM pyrogallol, 40µg catalase and the necessary volume of distilled water were taken in a 2 ml reaction tube. The blank and sample wells were filled with all the reagents except pyrogallol. As pyrogallol acts as reaction initiating reagent. It was added just before reading the absorbance. Absorbance of samples were taken at 450 nm for 4 minutes at every 30 seconds using Human ELISA reader. The activity of SOD is expressed as U mg^{-1} of protein [47].

Enzyme-Linked Immunosorbent Assay (ELISA)

To estimate the BDNF, NF-κB, TNF-α, Amyloid-β levels in the tissue homogenate was performed according to the user guide provided in the respective ELISA kits (Krishgen Biosystems) using sandwich ELISA technique. In brief, 50 µl Standards and 40 µl Samples were added to respective wells. Immediately, 10 µl biotinylated antibody were added to sample wells respectively. Then, 50 µl Streptavidin: HRP Conjugate solution was added to each well containing standards and samples and subjected to incubation for 60 mins at 37^0 C. Aspirate and washed the plate for 4 times. Then, we added 50 µl TMB Substrate A and B

and incubated for 10 mins at 37^0 C. After incubation, 50 µl of stop solution was added and read the absorbance at 450 nm by using an ELISA reader (Human Reader HS). Both the standards and samples were processed for each assay and the concentration of samples were analysed by interpolating with standard. The levels of BDNF, NF-κB were expressed as ng/mg of protein and TNF-α, amyloid-β levels were expressed as pg/mg of protein.

Western blotting

The hippocampus was homogenized using RIPA buffer loaded with protease and phosphatase inhibitors. The supernatant was collected by centrifugation at 8,000 rpm for 20 min at 4°C followed by protein quantification using the Bradford method. Equal quantities of protein (40 µg) were placed on each well in 10% SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane, followed by probing with primary antibodies (BACE-1, pCREB, and CREB Cell Signaling) with a 1:1000 dilution for overnight. Later, membranes were washed and probed with a secondary antibody (1:10,000 dilution) and visualized by chemiluminescence. The band densities were computed by image analysis software, Image J (NIH, Bethesda, MD, USA). β-Actin was cast off as an internal control for equal loading.

Histopathology

The Hippocampus samples were fixed in 10% formalin and further processed and fixed in paraffin blocks to obtain 200µm sections. Using the technique outlined by [48] the general histological appearance of the hippocampus was displayed in this approach. The glass slides were labelled and placed in a slide stand to de-waxed the slide by immersing in two changes of xylene. The sections were hydrated by immersing in descending grades of alcohol 100%, 70% and 50% and washed off the excess fluids in running tap water. Stain the slide with 200 µl of haematoxylin and wash thoroughly with PBS. The sections were differentiated by using 1% acid alcohol, washed and dried. Then counter staining with 200 µl of eosin was applied, washed, and removed the excess stain. The samples were again dehydrated by ascending grades of alcohol 50%, 70% and absolute for 2 min each. Finally clear the slides with xylene and covered by microscopic cover slides to observe under electron light microscope.

Statistical analysis

The data analysis was performed by using Graph pad prism software version – 8.4.2. All the values were expressed as Mean ± S.E.M. Statistical difference between and within the groups was analysed by ANOVA followed by Dunnett's *post-hoc* test. The p – value less than 0.05 was considered as statistically significant.

Results

Effect of Levosimendan on locomotor activity

The Open Field test was used to assess locomotor activity. Figure 2A illustrates that there were no significant variations in the number of crossings when compared with the scopolamine-alone treated group *vis-á-vis* the normal control group ($F_{(2, 15)} = 0.1678$, $P = 0.8471$) and disease group *vis-á-vis* the

Levosimendan treatment group ($F_{(2, 15)} = 0.1022$, $P = 0.9035$). Figure 2B depicted that there was no significant variation across the groups in distance travelled (cm) vis-a-vis the scopolamine-alone treated group to the normal control group ($F_{(2, 15)} = 0.2854$, $P = 0.7557$) and disease group to the Levosimendan treatment group ($F_{(2, 15)} = 0.06920$, $P = 0.9334$), this indicates that all the drug treatments does not show any locomotor decline between the groups.

Effect of Levosimendan on Discrimination index

Novel Object Recognition (NOR) test was conducted to assess the impact of Levosimendan treatment on the discrimination index in scopolamine-induced cognitive impairment. One-way ANOVA found overall significant differences across groups for the discrimination index in the NOR test (Fig. 3). The findings from the study implied that scopolamine induced rats were failed to discriminate between two objects *vis-á-vis* the normal control group ($F_{(2, 15)} = 7.602$) ($^{**}P < 0.01$). Levosimendan pre-treatment to scopolamine induced rats showed a beneficial effect in protecting the discriminative decline ($F_{(2, 15)} = 7.598$) ($^{##} p < 0.01$) when compared to scopolamine alone treated group.

Effect of Levosimendan on short-term memory

Y-maze task is used to examine short term spatial working memory. % spontaneous alterations were evaluated between the groups (Fig. 4A). scopolamine injected group showed a significant decline in % spontaneous alterations when compared to normal control group ($F_{(2, 15)} = 70.31$, $^{***}P < 0.001$). Whereas, levosimendan pre-treatment protected the scopolamine induced short-term memory impairment significantly ($F_{(2, 15)} = 20.89$, $^{###}P < 0.001$). Number of arm entries (Fig. 4B) does not show any significant difference across the groups which indicates no locomotor decline ($F_{(2, 15)} = 2.809$, $P = 0.0920$) versus normal control group, ($F (2, 15) = 1.919$, $P = 0.1811$) versus scopolamine alone treated group.

Effect of Levosimendan on Long-term memory using MWM

MWM test was conducted in order to assess spatial learning and memory. in this test escape latency is used as a parameter to assess the learning ability of the animal. Scopolamine showed a significant loss of learning ability from day – 1 with (day – 2, $^{@}p < 0.05$), (day – 3, $^{\$\$\$}p < 0.001$). whereas, intraday variations between the groups showed significant increase in escape latency when compared to normal control group in all the three days ($^{*}p < 0.05$, $^{**}p < 0.01$). However, pre-treatment with Levosimendan significantly attenuated the loss of learning ability in all the three days ($^{#}p < 0.05$, $^{##}p < 0.01$, $^{###}p < 0.001$) when compared to scopolamine alone treated group (Fig. 5A). On day 4 probe trail test was performed to calculate time spent in target quadrant. Spatial reference memory was significantly affected by scopolamine injection when compared to normal control group ($F_{(2, 15)} = 10.79$, $^{***}p < 0.001$)). Levosimendan prophylactic therapy protects the spatial memory depletion significantly when compared to scopolamine injected group ($F_{(2, 15)} = 9.181$, $^{#}p < 0.05$, $^{##}p < 0.01$) (Fig. 5B)

Effect of Levosimendan on Acetylcholinesterase activity

The activity of acetylcholinesterase rat (**A**) Hippocampus and (**B**) Plasma was shown in Fig. 6. In hippocampus, AChE activity in the Scopolamine treatment group was considerably greater than normal control group ($F_{(2, 15)} = 6.923$, $^{**}P < 0.01$). However, protective therapy with Donepezil and Levosimendan greatly reduced this activity compared with the disease group (F (2, 15) = 7.909, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$) (Fig. 6A). In plasma, scopolamine injection significantly increased the activity of AChE when compared to normal control group ($F_{(2, 15)} = 10.77$, $^{**}P < 0.01$). levosimendan pre-treatment decreased the AChE activity when compared to scopolamine alone treated group ($F_{(2, 15)} = 12.01$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$) (Fig. 6B).

Effect of Levosimendan on Butyrylcholinesterase activity

The results of butyrylcholinesterase activity in rat (**A**) Hippocampus and (**B**) Plasma were shown in Fig. 7. In hippocampus, BuChE activity in the scopolamine treatment group was considerably greater than normal control group ($F_{(2, 15)} = 3.246$, $^{**}P < 0.01$). However, treatment with Donepezil and Levosimendan significantly reduced this activity compared with the disease group (F (2, 15) = 8.954, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$) (Fig. 7A). In plasma, scopolamine injection significantly increased the activity of AChE when compared to normal control group $F_{(2, 15)} = 7.026$, $^{**}P < 0.01$). levosimendan pre-treatment decreased the AChE activity when compared to scopolamine treated group ($F_{(2, 15)} = 6.974$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$,) (Fig. 7B).

Effect of levosimendan on lipid peroxidation and NO

To ascribe the antioxidant effect of levosimendan in hippocampal cells, the level of MDA (Fig. 8A) and Nitric oxide was analysed (Fig. 8B). Statistical analysis between the group showed a significant difference between scopolamine injected and control group in MDA ($F_{(2, 15)} = 5.032$, $^{*}P < 0.05$) and Nitrates ($F_{(2, 15)} = 10.97$, $^{***}P < 0.001$). when treated with levosimendan it protected significantly (MDA, $F_{(2, 15)} = 8.487$, $^{\#\#}P < 0.01$), (Nitrates, $F_{(2, 15)} = 107.0$, $^{\#\#\#}P < 0.001$) against scopolamine induced oxidative stress in hippocampal cells.

Effect of levosimendan on reduced glutathione (GSH) and Superoxide dismutase

As shown in Fig. 9A the level of reduced glutathione is reduced significantly by the scopolamine injection when compared to the normal control group (F (2, 15) = 8.463, P < 0.05). whereas, levosimendan pre-treatment attenuates the decrease in GSH and protects against oxidative stress ($F_{(2, 15)} = 13.87$, $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$, respectively). In Fig. 9B scopolamine injection decreases the activity of SOD significantly ($F_{(2, 15)} = 0.852$, P = 0.4461).

Effect of Levosimendan on β-Amyloid Levels

The quantification of β -Amyloid (A β) levels in the hippocampus is estimated by using ELISA kits supplied by KRISHGEN biosystems. Figure 10A shows a significant difference in A β levels between normal control and scopolamine treated groups ($F_{(2,15)} = 7.169$, $^{**}P < 0.01$). Levosimendan pre-treatment attenuated the deposition of A β plaques induced by scopolamine significantly ($F_{(2,15)} = 7.117$, $^{\#\#}P < 0.01$).

Effect of Levosimendan on BDNF levels

BDNF plays an important role in neuronal generation and maintenance of synaptic plasticity. Its decreased levels may affect the neuronal growth and survival. The scopolamine injection significantly decreased the levels of BDNF in the hippocampus ($F_{(2,15)} = 7.635$, $^{**}P < 0.01$) when compared with the control group. However, Donepezil and Levosimendan pre-treatment inhibited the scopolamine induced changes in the BDNF levels ($F_{(2,15)} = 13.75$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$) in the hippocampus cells compared with the disease group (Fig. 10B).

Effect of Levosimendan NF-κB and TNF-α levels

As shown in Fig. 10C and 10D higher levels of NF-κB and TNF-α levels were identified in the hippocampal homogenates of rats injected with scopolamine when compared with normal control group ($F_{(2, 15)}$ = 27.03, 124.0 $^{***}P < 0.001$). It indicates that scopolamine induces neuroinflammation. while pre-treatment with Donepezil and Levosimendan significantly inhibited this neuroinflammation ($F_{(2, 15)} = 5.976$, $^{\#}P < 0.05$) and ($F_{(2, 15)} = 58.97$, $^{\#\#\#}P < 0.001$) caused by NF-κB and TNF-α when compared with the scopolamine alone treated group.

Western blotting analysis

To assess the underlying mechanisms of levosimendan in AD, we estimated the protein level of BACE-1, and p-CREB/CREB ratio in hippocampus (n = 3) by using western blotting technique represented in Fig. 11A. BACE-1 is an APP cleaving enzyme and contributes to the development and accumulation of Aβ plaques. The level of BACE-1 is significantly higher in scopolamine alone treated group (Fig. 13C). Whereas, levosimendan administration significantly decreases the BACE-1 protein expression and protects the hippocampus against amyloidogenic response. On the other hand, phospho-CREB plays a fundamental role in the management of AD. In general, p-CREB activates the transcription process and enhances the production of neurotropic factors i.e., BDNF in the hippocampus and helps for neuronal growth and survival. The ratio of pCREB/CREB in the hippocampus was significantly reduced by scopolamine. Whereas, levosimendan enhances the CREB phosphorylation and significantly increases the p-CREB levels (Fig. 13B).

Histopathology

Discussion

The purpose of this study is to investigate the protective effect of levosimendan *vis-á-vis* scopolamine induced cognitive impairment. To confirm this hypothesis, a predominantly used scopolamine model for the screening of drugs to treat Alzheimer's disease was adopted [36, 49]. Scopolamine, a post-synaptic muscarinic acetylcholine receptor (mAChR) antagonist, down regulates the cholinergic transmission. It leads to decreased acetylcholine release and results in cognitive dysfunction [50]. Further, scopolamine at high doses impairs the cognitive function via activation of BACE-1 enzyme, Amyloid-β accumulation, oxidative stress and neuroinflammation [51].

In this study, open field test was performed to address the self-independent exploring property and general analysis of rats' locomotor behaviour. As shown in **(Fig.-2)**, there were no change in the parameters analysed among all the groups. In light of this evidence, we can state that scopolamine administration does not impact the locomotor activity and the behavioural deficits observed in NORT, Y maze and water maze were solely due to its impairment of cognitive function [52, 53]. Novel object recognition (NOR) test was carried out to assess the visual recognition of animal and exploration of novel object in a familiar environment. In this test, non-spatial recognition ability termed as discrimination index was measured to evaluate the cognitive performance. In the present study **(**Fig. 3**)**, we demonstrated that scopolamine injection decreases the discrimination index of the animal. Whereas, levosimendan countered this result and ameliorated the decrease in discrimination index caused by scopolamine and the results are in accordance with the previous studies [54, 55]. On the other hand, Y-maze test is used predominantly to measure the short-term spatial working memory [56]. In this % spontaneous alternation was used as an indicator for short-term working memory [51]. Previous studies reported that scopolamine administration decreases the short-term memory and it is evidenced by decrease in % spontaneous alterations [27, 57]. Results from **(**Fig. 4**)** this study were in line with previous studies and further, levosimendan pretreatment protected short-term memory decline [58].

MWM test was performed to measure the deficits of learning and memory associated with hippocampal dysfunction. In this test, non-spatial learning and memory was postulated by gauging the latency time to identify the hidden platform. In this study, scopolamine administered rats were observed with increase in latency time to find the platform when compared with the normal control group. It indicates the impairment in learning and memory. [59]. The animals treated with levosimendan had shown decrease in the escape latency time while comparing with the scopolamine alone treated group, represents its protective ability against cognitive impairment **(Fig. – 5A)**. Further, in the probe trial test, scopolamine administered rats spends less time in target quadrant. However, levosimendan treated rats spent more time in target quadrant comparing to the disease group signifying its role in improving spatial learning and memory. All the results in MWM test were familiar with earlier reported studies **(Fig.- 5B)** [60]. In this context, the results of behavioural tests suggests that levosimendan treatment could ameliorates long-term and reference memory impairment induced by scopolamine.

The cholinergic neurotransmission is involved in many physiological processes, including synaptic plasticity, learning and memory. Dysfunction of cholinergic neurons in the brain decreases the release of Ach and results in cognitive impairment. Further, cholinergic agonists like donepezil, rivastigmine facilitate

the memory and cholinergic antagonists like scopolamine impair the memory [61]. In that sense, molecules that modulate cholinergic hypoactivity by slowing the rate of acetylcholine degradation have potential clinical use. In order to elucidate the effect of levosimendan on cholinergic function, we have estimated the activity of AChE and BuChE [62]. According to the previous studies, scopolamine administration produced severe cholinergic deficits evidenced by increase in AChE and BuChE activity and decreases synaptic plasticity [43, 63]. However, levosimendan pre-treatment significantly decreased the enzyme activity and loss of cholinergic neurons induced by scopolamine (Fig. 6). Our results in this study are relevant to the previous studies [64, 65]. These findings agree with the behavioral data and together suggest that ameliorative effect of levosimendan against scopolamine induced memory impairment.

Furthermore, many devastating pieces of evidence suggest that oxidative stress is another causative factor of Alzheimer pathology [66]. In experimental animals, amnesia induced by scopolamine has been reported to be associated with increase free radicals in the brain, especially hippocampus. In agreement with the previous studies, our study results showed that scopolamine administration resulted in remarkable oxidative damage to the hippocampus and it is evidenced by increased levels of MDA and NO, a valid index of lipid peroxidation and significant decrease in antioxidant defense systems such as GSH and SOD [9, 67]. In contrast, levosimendan administration showed its beneficial role against oxidative stress by increasing the activity of GSH, SOD and by decreasing the lipid peroxidation and No levels when compared to scopolamine alone treated group. All the results from oxidative stress parameters were in line with the previous studies (Fig. 7,8) [67]. These data demonstrated that levosimendan has free radical scavenging activity and increases the levels of anti-oxidants. Hence, it suggested that the reduction of oxidative stress could be related to the cognitive effect of levosimendan in scopolamine treated rats [12, 19, 26].

Microglia, an inhabitant immune cells of the brain, is regularly inspect the microenvironment under biological conditions. In AD, activation of microglia mediated by amyloid-β deposition and oxidative stress initiates cerebral neuroinflammation. Microglial polarization in the brain plays a detrimental role by provoking the expression of pro-inflammatory cytokines. The transcription factor, NF-κB, involved in various gene regulations in inflammation [68]. It has been reported scopolamine injection initiates the production of inflammatory cascade NF-κB and helps production of pro-inflammatory cytokines like TNF-α, IL- 6 [69, 70]. Here, we demonstrated that NF-κB and TNF- α levels were elevated significantly in scopolamine alone administered rats. While, pre-treatment with levosimendan significantly attenuated the upregulation of NF-κB and in response decreased the inflammatory mediators caused by the scopolamine administration showing its anti-inflammatory property (Fig. 10C, 10D). These results were in line with the previous studies stating that levosimendan ameliorated the scopolamine induced neuroinflammation by inhibiting NF-κB expression [71].

Cerebral vasculature is crucial for neuronal function as it is responsible for delivery of nutrients and removal of metabolites. Over production of amyloid beta plaques in the cerebral blood vessels worsens the disease pathology and contributes to cognitive decline [72]. According to the previous studies, muscarinic receptor blockage by scopolamine injection activates beta secretase activating cleavage enzyme – 1

(BACE-1). It cleaves amyloid precursor protein (APP) and causes Aβ over production [73]. In this study, the scopolamine alone administered group has shown a significant increase in Aβ deposition in the hippocampus which is similar to the previous studies [10, 25]. Whereas, our experiments in the present study proved the same trend that pretreatment with levosimendan effect on APP and BACE1 repression confirmed that its anti-AD effects are related to the inhibition of Aβ production. In addition, cilostazol does not affect LRP, which is a key Aβ clearance receptor and NEP and IDE involved in the degradation of the Aβ peptide [74, 75] (Fig. 10A, 11C). Previous studies demonstrated the neuroprotective effect of levosimendan i.e., Levijoki et al, 2015 reported that, levosimendan increases the cerebral blood volume and protects the brain against primary and secondary stroke [76]. Roehl et al, 2010 reported the neuroprotective effect of levosimendan in an in vitro model of traumatic brain injury [77]. These results are in line with previous studies supporting the beneficial effects of levosimendan as PDE inhibitor through an increase in Aβ aggregation by inhibiting BACE-1 and Aβ clearance.

The neurotrophin family, BDNF plays a pivotal role in the differentiation, survival, and functional maintenance of nerve cells. It is expressed all over the brain including astrocytes, glial cells and protects against disease causing pathologies by promoting neuronal survival. Aβ oligomers in the brain blocks the activation of CREB pathway and decreased the levels of BDNF. This reduced BDNF levels in the brain causes neurodegeneration that leads to many neurological diseases like AD [27]. In this study, scopolamine injection decreases the expression of BDNF that affects the neurogenesis and synaptic plasticity which is similar to previous studies [78]. Meanwhile, pre-treatment with levosimendan significantly increased the expression of BDNF. The possible protective action of levosimendan might be due to its inhibitory action on phosphodiesterase III enzyme. The inhibition of PDE III by levosimendan, increases the levels of cAMP. The cAMP binds to the regulatory subunit of protein kinase A (PKA) and causes the dissociation of PKA from the regulatory subunit. These dissociated PKA enters into the nucleus and phosphorylate the CREB. Further, the phosphorylated CREB bind with the cAMP responsive element in the promotor region of DNA and promotes BDNF expression [79, 80]. These results are consistent with previous studies that inhibition of PDE III enzyme stimulates pCREB/BDNF signalling and enhance neurogenesis [81].

Hence, based on these findings from behavioural and biochemical analysis, we demonstrate that levosimendan exerted its neuroprotective role against scopolamine induced cognitive dysfunction, oxidative stress, amyloid beta accumulation, BDNF decline and neuroinflammation.

Conclusion

In summary, this study clearly demonstrates that pretreatment with levosimendan improves the cognitive deficits and improved cholinergic transmission in the hippocampus induced by scopolamine. The protective mechanisms are likely due to the attenuation of Amyloid beta in the brain by inhibiting the BACE-1 and enhancing Aβ degradation. Inhibition of inflammatory responses via suppression of TNF-α and NF-κB activation. Moreover, inhibition of PDE mediates the protective effects through the increase in

pCREB/BDNF expression. These results provide the evidence that levosimendan might be a promising drug that can be used as adjuvant in the treatment of AD.

Abbreviations

Alzheimer’s Disease (AD), Amyloid Precursor Protein (APP), Amyloid-β (Aβ), Acetylcholine (ACh), N-methyl-D-aspartate (NMDA), Nuclear factor kappa (NF-κB), Brain derived neurotropic factor (BDNF), Phosphodiesterase inhibitors (PDEI), Cyclic AMP reacting element binding protein (CREB), acetylcholine thiocholine iodide (ACTCI), butyryl thiocholine iodide (BuTCI), 2,2-Diphenyl-2-picrylhydrazyl (DPPH), 5,5’-dithiobis (2-nitrobenzoic acid) (DTNB), Vehicle (Veh), Scopolamine (Sco), Donepezil (DPZ), Levosimendan (Levo), Open field test (OFT), Novel object recognition test (NORT), Discrimination index (DI), Morris water maze (MWM), Acetylcholinesterase (AChE), Butyrylcholinesterase (BuChE), Reduced glutathione (GSH), Nitric oxide (NO), Superoxide dismutase (SOD), Enzyme-linked immunosorbent assay (ELISA).

Declarations

Author contributions

A.S.K, G.S.G, P.K.S, K.V.S.N contributed to the study's conceptualization and methodology. A.S.K, G.S.G, M.N conducted the experiments. P.K.S, K.V.S.N, P.K investigated and supervised the study. A.S.K, G.S.G, M.N performed formal analysis of the data. A.S.K, M.N wrote the original draft paper. P.K.S, K.V.S.N, R.M.M reviewed and edited the paper.

Conflict of Interest:

The authors declare no conflicts of interest

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Author Contribution

A.S.K, G.S.G, P.K.S, K.V.S.N contributed to the study's conceptualization and methodology. A.S.K, G.S.G, M.N conducted the experiments. P.K.S, K.V.S.N, P.K investigated and supervised the study. A.S.K, G.S.G, M.N performed formal analysis of the data. A.S.K, M.N wrote the original draft paper. P.K.S, K.V.S.N, R.M.M reviewed and edited the paper.

References

1. Karthivashan G, Park SY, Kweon MH, et al (2018) Ameliorative potential of desalted Salicornia europaea L. extract in multifaceted Alzheimer's-like scopolamine-induced amnesic mice model. Scientific Reports 2018 8:1 8:1–16. https://doi.org/10.1038/s41598-018-25381-0
2. Simunkova M, Alwasel SH, Alhazza IM, et al (2019) Management of oxidative stress and other pathologies in Alzheimer's disease. Arch Toxicol 93:2491–2513. https://doi.org/10.1007/S00204-019-02538-Y
3. Bhuvanendran S, Kumari Y, Othman I, Shaikh MF (2018) Amelioration ofc ognitive deficit by embelin in a scopolamine-induced Alzheimer's disease-like condition in a rat model. Front Pharmacol 9:665. https://doi.org/10.3389/FPHAR.2018.00665/BIBTEX
4. Fisher A (2012) Cholinergic modulation of amyloid precursor protein processing with emphasis on M1 muscarinic receptor: perspectives and challenges in treatment of Alzheimer's disease. J Neurochem 120:22–33. https://doi.org/10.1111/J.1471-4159.2011.07507.X
5. Choi SJ, Jeong CH, Choi SG, et al (2009) Zeatin prevents amyloid beta-induced neurotoxicity and scopolamine-induced cognitive deficits. J Med Food 12:271–277. https://doi.org/10.1089/JMF.2007.0678
6. Mansouri F, Ghanbari H, Marefati N, et al (2021) Protective effects of vitamin D on learning and memory deficit induced by scopolamine in male rats: the roles of brain-derived neurotrophic factor and oxidative stress. Naunyn Schmiedebergs Arch Pharmacol 394:1451–1466. https://doi.org/10.1007/S00210-021-02062-W
7. Rosa E, Fahnestock M (2015) CREB expression mediates amyloid β-induced basal BDNF downregulation. Neurobiol Aging 36:2406–2413. https://doi.org/10.1016/J.NEUROBIOLAGING.2015.04.014
8. Gonzalez MC, Radiske A, Cammarota M (2019) On the Involvement of BDNF Signaling in Memory Reconsolidation. Front Cell Neurosci 13:383. https://doi.org/10.3389/FNCEL.2019.00383/BIBTEX
9. Kim YJ, Kim JH, He MT, et al (2021) Apigenin Ameliorates Scopolamine-Induced Cognitive Dysfunction and Neuronal Damage in Mice. Molecules 2021, Vol 26, Page 5192 26:5192. https://doi.org/10.3390/MOLECULES26175192
10. Liu D, Pitta M, Lee J-H, et al (2010) The K ATP Channel Activator Diazoxide Ameliorates Aβ and Tau Pathologies and Improves Memory in the 3xTgAD Mouse Model of Alzheimer's Disease NIH Public Access. J Alzheimers Dis 22:443–457. https://doi.org/10.3233/JAD-2010-101017
11. Villa C, Suphesiz H, Combi R, Akyuz E (2020) Potassium channels in the neuronal homeostasis and neurodegenerative pathways underlying Alzheimer's disease: An update. Mech Ageing Dev 185:111197. https://doi.org/10.1016/J.MAD.2019.111197
12. Abuirmeileh AN, Alzoubi KH, Rababa'h AM (2020) The Effect of Levosimendan on Two Distinct Rodent Models of Parkinson's Disease. Curr Alzheimer Res 17:1043–1051. https://doi.org/10.2174/1567205017666201218102724

13. Plaschke K, Bent F, Wagner S, et al (2014) In contrast to its anti-inflammatory and anti-apoptotic peripheral effect, levosimendan failed to induce a long-term neuroprotective effect in a rat model of mild septic encephalopathy: A pilot study. Neurosci Lett 560:117–121. https://doi.org/10.1016/J.NEULET.2013.12.021
14. Gozeler MS, Nur F, Akdemir E, et al (2019) International Journal of Pediatric Otorhinolaryngology Levosimendan ameliorates cisplatin-induced ototoxicity : Rat model. Int J Pediatr Otorhinolaryngol 122:70–75. https://doi.org/10.1016/j.ijporl.2019.04.004
15. Zhao L, Tian L, Wang S, et al (2021) Levosimendan in rats decreases acute kidney injury after cardiopulmonary resuscitation by improving mitochondrial dysfunction. 10:3010–3020. https://doi.org/10.21037/tau-21-443
16. Kim YK, Lee G Levosimendan prevents tau pathology by inhibiting disulfide-linked tau oligomerization posing as a promising anti-tau therapeutics. 1–35
17. Abdelrahman AM, Salam S Al, Sulemani Y Al, et al (2020) Effect of levosimendan, an inodilator, on streptozotocin-induced diabetic nephropathy in rats. Eur J Pharmacol 172960. https://doi.org/10.1016/j.ejphar.2020.172960
18. Abdelrahman AM, Al Y, Shalaby A, et al (2019) E ff ect of levosimendan , a calcium sensitizer , on cisplatin-induced nephrotoxicity in rats. Toxicol Rep 6:232–238. https://doi.org/10.1016/j.toxrep.2019.02.006
19. Liu T, Li G, Xu G (2009) Levosimendan May Prevent Postoperative Atrial Fibrillation Through Anti-inflammatory and Antioxidant Modulation. J Cardiothorac Vasc Anesth 23:757–758. https://doi.org/10.1053/j.jvca.2008.09.022
20. Pathak A, Lebrin M, Vaccaro A, et al (2013) Pharmacology of levosimendan: inotropic, vasodilatory and cardioprotective effects. J Clin Pharm Ther 38:341–349. https://doi.org/10.1111/JCPT.12067
21. Levosimendan | C14H12N6O - PubChem. https://pubchem.ncbi.nlm.nih.gov/compound/Levosimendan. Accessed 4 Aug 2022
22. Lehmann A, Boldt J, Kirchner J (2003) The role of Ca++-sensitizers for the treatment of heart failure. Curr Opin Crit Care 9:337–344. https://doi.org/10.1097/00075198-200310000-00002
23. Matsumoto N, Komiyama S, Akaike N (2002) Pre- and postsynaptic ATP-sensitive potassium channels during metabolic inhibition of rat hippocampal CA1 neurons. Journal of Physiology 541:511–520. https://doi.org/10.1113/JPHYSIOL.2002.018267
24. Yamada K, Juan Juan Ji, Yuan H, et al (2001) Protective role of ATP-sensitive potassium channels in hypoxia-induced generalized seizure. Science (1979) 292:1543–1546. https://doi.org/10.1126/SCIENCE.1059829/SUPPL_FILE/1059829S1_THUMB.GIF
25. Liu L, Xu H, Ding S, et al (2019) Phosphodiesterase 5 inhibitors as novel agents for the treatment of Alzheimer's disease. Brain Res Bull 153:223–231. https://doi.org/10.1016/j.brainresbull.2019.09.001
26. Rababa AM, Alzoubi KH, Atmeh A (2017) Levosimendan enhances memory through antioxidant effect in rat model : behavioral and molecular study. 1–7. https://doi.org/10.1097/FBP.0000000000000362

27. Ishola IO, Jacinta AA, Adeyemi OO (2019) Cortico-hippocampal memory enhancing activity of hesperetin on scopolamine-induced amnesia in mice: role of antioxidant defense system, cholinergic neurotransmission and expression of BDNF. Metabolic Brain Disease 2019 34:4 34:979–989. https://doi.org/10.1007/S11011-019-00409-0
28. Shabani S, Mirshekar MA (2018) Diosmin is neuroprotective in a rat model of scopolamine-induced cognitive impairment. Biomed Pharmacother 108:1376–1383. https://doi.org/10.1016/J.BIOPHA.2018.09.127
29. Sandeep Ganesh G, Konduri P, Kolusu AS, et al (2023) Neuroprotective Effect of Saroglitazar on Scopolamine-Induced Alzheimer's in Rats: Insights into the Underlying Mechanisms. ACS Chem Neurosci 14:3444–3459. https://doi.org/10.1021/ACSCHEMNEURO.3C00320/ASSET/IMAGES/MEDIUM/CN3C00320_0018.GIF
30. Hira S, Saleem U, Anwar F, et al (2019) β-Carotene: A Natural Compound Improves Cognitive Impairment and Oxidative Stress in a Mouse Model of Streptozotocin-Induced Alzheimer's Disease. Biomolecules 9:. https://doi.org/10.3390/BIOM9090441
31. Pardo Andreu GL, Maurmann N, Reolon GK, et al (2010) Mangiferin, a naturally occurring glucoxilxanthone improves long-term object recognition memory in rats. Eur J Pharmacol 635:124–128. https://doi.org/10.1016/J.EJPHAR.2010.03.011
32. Reolon GK, Braga LME, Camassola M, et al (2006) Long-term memory for aversive training is impaired in Idua(-/-) mice, a genetic model of mucopolysaccharidosis type I. Brain Res 1076:225–230. https://doi.org/10.1016/J.BRAINRES.2006.01.008
33. Foyet HS, Asongalem AE, Oben EK, et al (2016) Effects of the Methanolic Extract of Vitellaria paradoxa Stem Bark Against Scopolamine-Induced Cognitive Dysfunction and Oxidative Stress in the Rat Hippocampus. Cell Mol Neurobiol 36:1139–1149. https://doi.org/10.1007/S10571-015-0310-7
34. Lu C, Lv J, Dong L, et al (2018) Neuroprotective effects of 20(S)-protopanaxatriol (PPT) on scopolamine-induced cognitive deficits in mice. Phytotherapy Research 32:1056–1063. https://doi.org/10.1002/PTR.6044
35. Ishola IO, Adamson FM, Adeyemi OO (2016) Ameliorative effect of kolaviron, a biflavonoid complex from Garcinia kola seeds against scopolamine-induced memory impairment in rats: role of antioxidant defense system. Metabolic Brain Disease 2016 32:1 32:235–245. https://doi.org/10.1007/S11011-016-9902-2
36. Bagci E, Aydin E, Mihasan M, et al (2016) Anxiolytic and antidepressant-like effects of Ferulago angulata essential oil in the scopolamine rat model of Alzheimer's disease. Flavour Fragr J 31:70–80. https://doi.org/10.1002/FFJ.3289
37. Abd-El-Fattah MA, Abdelakader NF, Zaki HF (2014) Pyrrolidine dithiocarbamate protects against scopolamine-induced cognitive impairment in rats. Eur J Pharmacol 723:330–338. https://doi.org/10.1016/J.EJPHAR.2013.11.008
38. Ishrat T, Parveen K, Khan MM, et al (2009) Selenium prevents cognitive decline and oxidative damage in rat model of streptozotocin-induced experimental dementia of Alzheimer's type. Brain Res 1281:117–127. https://doi.org/10.1016/J.BRAINRES.2009.04.010

39. Gupta R, Gupta LK, Mediratta PK, Bhattacharya SK (2012) Effect of resveratrol on scopolamine-induced cognitive impairment in mice. Pharmacol Rep 64:438–444. https://doi.org/10.1016/S1734-1140(12)70785-5
40. Anesthesia for Laboratory Animals | Research and Innovation. https://research.uoregon.edu/manage/integrity-compliance/animal-research/anesthesia-laboratory-animals. Accessed 7 Aug 2022
41. MM B (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. https://doi.org/10.1006/ABIO.1976.9999
42. Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:. https://doi.org/10.1016/0006-2952(61)90145-9
43. Berté TE, Dalmagro AP, Zimath PL, et al (2018) Taraxerol as a possible therapeutic agent on memory impairments and Alzheimer's disease: Effects against scopolamine and streptozotocin-induced cognitive dysfunctions. Steroids 132:5–11. https://doi.org/10.1016/J.STEROIDS.2018.01.002
44. Barai P, Raval N, Acharya S, et al (2019) Neuroprotective effects of bergenin in Alzheimer's disease: Investigation through molecular docking, in vitro and in vivo studies. Behavioural brain research 356:18–40. https://doi.org/10.1016/J.BBR.2018.08.010
45. Zaki HF, Abd-El-Fattah MA, Attia AS (2014) Naringenin protects against scopolamine-induced dementia in rats. Bulletin of Faculty of Pharmacy, Cairo University 52:15–25. https://doi.org/10.1016/J.BFOPCU.2013.11.001
46. Owen JB, Allan Butterfiel D (2010) Measurement of oxidized/reduced glutathione ratio. Methods Mol Biol 648:269–277. https://doi.org/10.1007/978-1-60761-756-3_18
47. Nandi A, Chatterjee IB (1988) Assay of superoxide dismutase activity in animal tissues. Journal of Biosciences 1988 13:3 13:305–315. https://doi.org/10.1007/BF02712155
48. Memudu AE, Adewumi AE (2021) Alpha lipoic acid ameliorates scopolamine induced memory deficit and neurodegeneration in the cerebello-hippocampal cortex. Metab Brain Dis 36:1729–1745. https://doi.org/10.1007/S11011-021-00720-9/METRICS
49. Park SJ, Kim DH, Jung JM, et al (2012) The ameliorating effects of stigmasterol on scopolamine-induced memory impairments in mice. Eur J Pharmacol 676:64–70. https://doi.org/10.1016/J.EJPHAR.2011.11.050
50. Chen BH, Park JH, Lee TK, et al (2018) Melatonin attenuates scopolamine-induced cognitive impairment via protecting against demyelination through BDNF-TrkB signaling in the mouse dentate gyrus. Chem Biol Interact 285:8–13. https://doi.org/10.1016/J.CBI.2018.02.023
51. Park HS, Hwang ES, Choi GY, et al (2021) Sulforaphane enhances long-term potentiation and ameliorate scopolamine-induced memory impairment. Physiol Behav 238:. https://doi.org/10.1016/J.PHYSBEH.2021.113467

52. Mansouri MT, Farbood Y, Naghizadeh B, et al (2016) Beneficial effects of ellagic acid against animal models of scopolamine- and diazepam-induced cognitive impairments. Pharm Biol 54:1947–1953. https://doi.org/10.3109/13880209.2015.1137601
53. Gutierres JM, Carvalho FB, Schetinger MRC, et al (2012) Protective effects of anthocyanins on the ectonucleotidase activity in the impairment of memory induced by scopolamine in adult rats. Life Sci 91:1221–1228. https://doi.org/10.1016/J.LFS.2012.09.013
54. Wang J, Wang X, Lv B, et al (2014) Effects of Fructus Akebiae on learning and memory impairment in a scopolamine-induced animal model of dementia. Exp Ther Med 8:671. https://doi.org/10.3892/ETM.2014.1775
55. Aykac A, Ozbeyli D, Uncu M, et al (2019) Evaluation of the protective effect of Myrtus communis in scopolamine-induced Alzheimer model through cholinergic receptors. Gene 689:194–201. https://doi.org/10.1016/J.GENE.2018.12.007
56. Ionita R, Postu PA, Mihasan M, et al (2018) Ameliorative effects of Matricaria chamomilla L. hydroalcoholic extract on scopolamine-induced memory impairment in rats: A behavioral and molecular study. Phytomedicine 47:113–120. https://doi.org/10.1016/J.PHYMED.2018.04.049
57. Kwon SH, Lee HK, Kim JA, et al (2010) Neuroprotective effects of chlorogenic acid on scopolamine-induced amnesia via anti-acetylcholinesterase and anti-oxidative activities in mice. Eur J Pharmacol 649:210–217. https://doi.org/10.1016/J.EJPHAR.2010.09.001
58. Kwon SH, Kim HC, Lee SY, Jang CG (2009) Loganin improves learning and memory impairments induced by scopolamine in mice. Eur J Pharmacol 619:44–49. https://doi.org/10.1016/J.EJPHAR.2009.06.062
59. Gacar N, Mutlu O, Utkan T, et al (2011) Beneficial effects of resveratrol on scopolamine but not mecamylamine induced memory impairment in the passive avoidance and Morris water maze tests in rats. Pharmacol Biochem Behav 99:316–323. https://doi.org/10.1016/J.PBB.2011.05.017
60. Hosseini M, Mohammadpour T, Karami R, et al (2015) Effects of the hydro-alcoholic extract of Nigella sativa on scopolamine-induced spatial memory impairment in rats and its possible mechanism. Chin J Integr Med 21:438–444. https://doi.org/10.1007/S11655-014-1742-5
61. Blake MG, Krawczyk MC, Baratti CM, Boccia MM (2014) Neuropharmacology of memory consolidation and reconsolidation: Insights on central cholinergic mechanisms. Journal of Physiology-Paris 108:286–291. https://doi.org/10.1016/J.JPHYSPARIS.2014.04.005
62. Ohba T, Yoshino Y, Ishisaka M, et al (2015) Japanese Huperzia serrata extract and the constituent, huperzine A, ameliorate the scopolamine-induced cognitive impairment in mice. Biosci Biotechnol Biochem 79:1838–1844. https://doi.org/10.1080/09168451.2015.1052773/SUPPL_FILE/TBBB_A_1052773_SM4141.ZIP
63. Hafez HS, Ghareeb DA, Saleh SR, et al (2017) Neuroprotective effect of ipriflavone against scopolamine-induced memory impairment in rats. Psychopharmacology (Berl) 234:3037–3053. https://doi.org/10.1007/S00213-017-4690-X

64. Sun K, Bai Y, Zhao R, et al (2019) Neuroprotective effects of matrine on scopolamine-induced amnesia via inhibition of AChE/BuChE and oxidative stress. Metab Brain Dis 34:173–181. https://doi.org/10.1007/S11011-018-0335-Y
65. Rajashri K, Mudhol S, Serva Peddha M, Borse BB (2020) Neuroprotective Effect of Spice Oleoresins on Memory and Cognitive Impairment Associated with Scopolamine-Induced Alzheimer's Disease in Rats. ACS Omega 5:30898–30905. https://doi.org/10.1021/ACSOMEGA.0C03689
66. Melo JB, Agostinho P, Oliveira CR (2003) Involvement of oxidative stress in the enhancement of acetylcholinesterase activity induced by amyloid beta-peptide. Neurosci Res 45:117–127. https://doi.org/10.1016/S0168-0102(02)00201-8
67. Beheshti F, Akbari HR, Baghcheghi Y, et al (2021) Beneficial effects of angiotensin converting enzyme inhibition on scopolamine-induced learning and memory impairment in rats, the roles of brain-derived neurotrophic factor, nitric oxide and neuroinflammation. https://doi.org/101080/1064196320211901112 43:505–515. https://doi.org/10.1080/10641963.2021.1901112
68. Demirci K, Nazıroğlu M, Övey İS, Balaban H (2016) Selenium attenuates apoptosis, inflammation and oxidative stress in the blood and brain of aged rats with scopolamine-induced dementia. Metabolic Brain Disease 2016 32:2 32:321–329. https://doi.org/10.1007/S11011-016-9903-1
69. Iqbal S, Shah FA, Naeem K, et al (2020) Succinamide Derivatives Ameliorate Neuroinflammation and Oxidative Stress in Scopolamine-Induced Neurodegeneration. Biomolecules 10:443. https://doi.org/10.3390/BIOM10030443
70. Muhammad T, Ali T, Ikram M, et al (2019) Melatonin Rescue Oxidative Stress-Mediated Neuroinflammation/ Neurodegeneration and Memory Impairment in Scopolamine-Induced Amnesia Mice Model. J Neuroimmune Pharmacol 14:278–294. https://doi.org/10.1007/S11481-018-9824-3
71. Yerraguravagari B, Penchikala NP, Kolusu A sai, et al (2023) Montelukast Ameliorates Scopolamine-induced Alzheimer's Disease: Role on Cholinergic Neurotransmission, Antioxidant Defence System, Neuroinflammation and Expression of BDNF. CNS Neurol Disord Drug Targets 23:. https://doi.org/10.2174/0118715273258337230925040049
72. Maki T, Okamoto Y, Carare RO, et al (2014) Phosphodiesterase III inhibitor promotes drainage of cerebrovascular β-amyloid. Ann Clin Transl Neurol 1:519–533. https://doi.org/10.1002/ACN3.79
73. Hernández-Rodríguez M, Arciniega-Martínez IM, García-Marín ID, et al (2020) Chronic Administration of Scopolamine Increased GSK3βP9, Beta Secretase, Amyloid Beta, and Oxidative Stress in the Hippocampus of Wistar Rats. Mol Neurobiol 57:3979–3988. https://doi.org/10.1007/S12035-020-02009-X
74. Yin C, Deng Y, Gao J, et al (2016) Icariside II, a novel phosphodiesterase-5 inhibitor, attenuates streptozotocin-induced cognitive deficits in rats. Neuroscience 328:69–79. https://doi.org/10.1016/J.NEUROSCIENCE.2016.04.022
75. Park SH, Kim JH, Bae SS, et al (2011) Protective effect of the phosphodiesterase III inhibitor cilostazol on amyloid β-induced cognitive deficits associated with decreased amyloid β accumulation. Biochem Biophys Res Commun 408:602–608. https://doi.org/10.1016/J.BBRC.2011.04.068

76. Levijoki J, Kivikko M, Pollesello P, et al (2015) Levosimendan alone and in combination with valsartan prevents stroke in Dahl salt-sensitive rats. Eur J Pharmacol 750:132–140. https://doi.org/10.1016/J.EJPHAR.2015.01.037
77. Roehl AB, Hein M, Loetscher PD, et al (2010) Neuroprotective properties of levosimendan in an in vitro model of traumatic brain injury. BMC Neurol 10:1–4. https://doi.org/10.1186/1471-2377-10-97/FIGURES/3
78. Min AY, Doo CN, Son EJ, et al (2015) N-palmitoyl serotonin alleviates scopolamine-induced memory impairment via regulation of cholinergic and antioxidant systems, and expression of BDNF and p-CREB in mice. Chem Biol Interact 242:153–162. https://doi.org/10.1016/J.CBI.2015.09.016
79. Lu C, Wang Y, Xu T, et al (2018) Genistein ameliorates scopolamine-induced amnesia in mice through the regulation of the cholinergic neurotransmission, antioxidant system and the ERK/CREB/BDNF signaling. Front Pharmacol 9:1153. https://doi.org/10.3389/FPHAR.2018.01153/XML/NLM
80. Chen Z, Huang C, Ding W (2016) Z-Guggulsterone Improves the Scopolamine-Induced Memory Impairments Through Enhancement of the BDNF Signal in C57BL/6J Mice. Neurochemical Research 2016 41:12 41:3322–3332. https://doi.org/10.1007/S11064-016-2064-0
81. Itoh T, Tokumura M, Abe K (2004) Effects of rolipram, a phosphodiesterase 4 inhibitor, in combination with imipramine on depressive behavior, CRE-binding activity and BDNF level in learned helplessness rats. Eur J Pharmacol 498:135–142. https://doi.org/10.1016/J.EJPHAR.2004.07.084

Figures

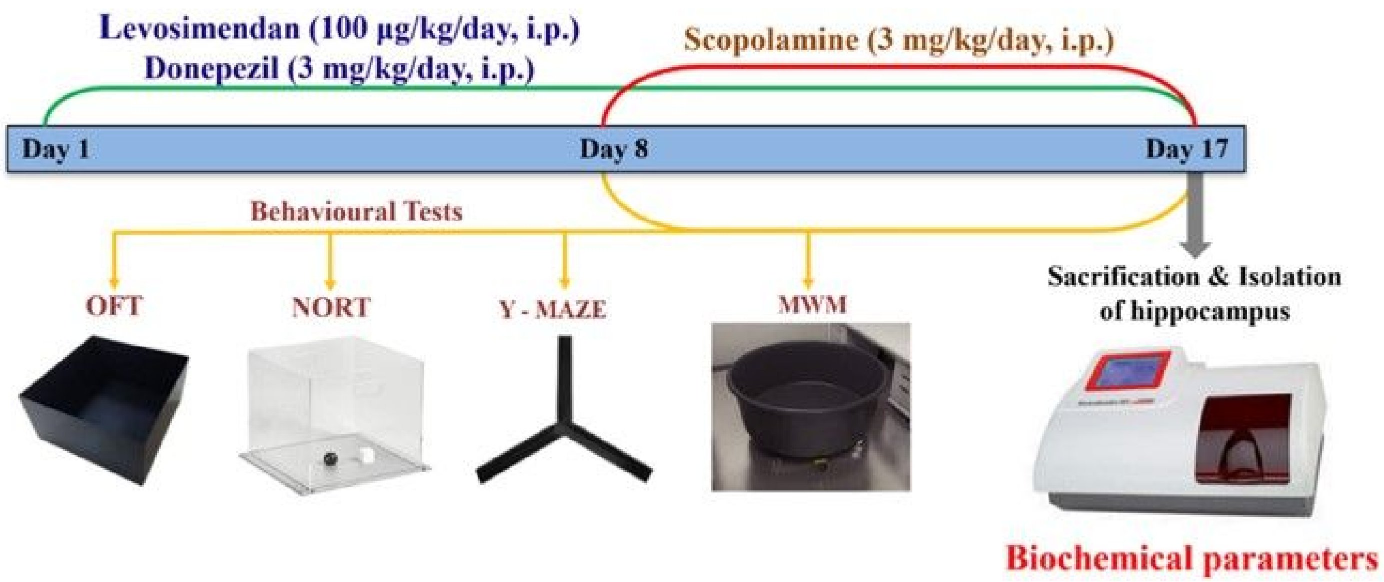
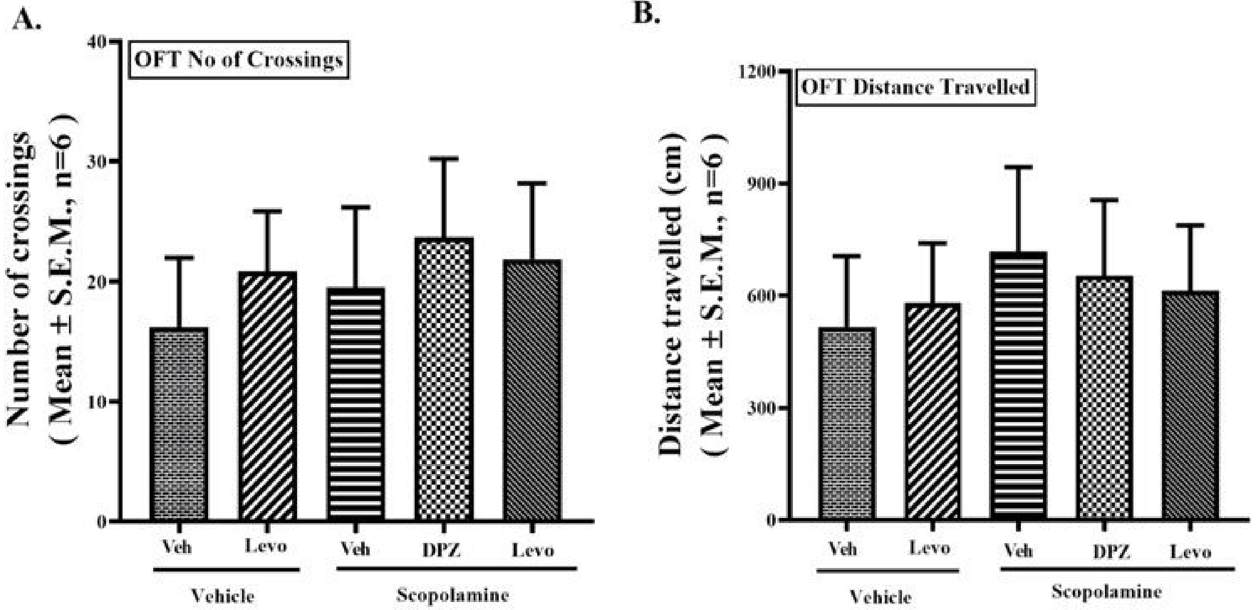


Figure 1

Scheme of experimental schedule and intervals for estimation of various parameters



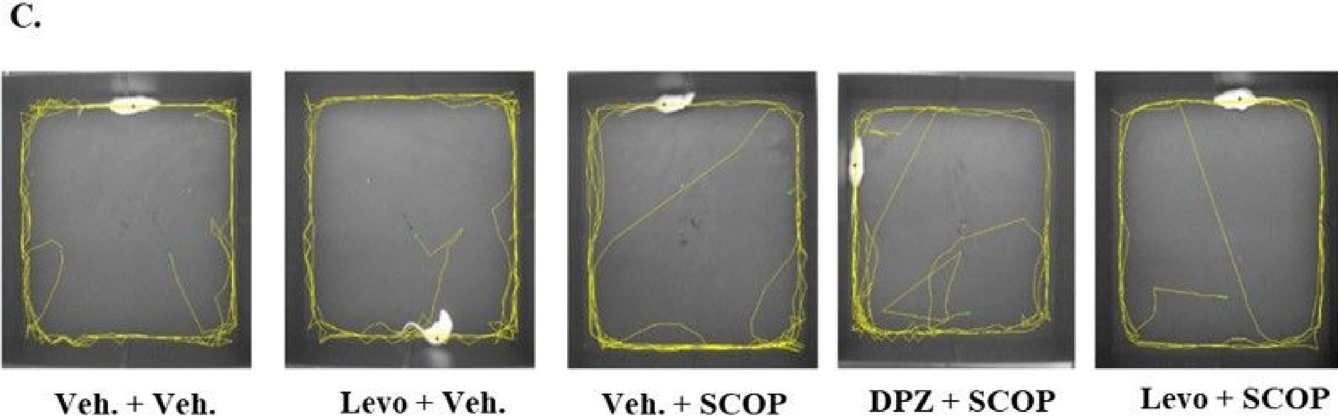


Figure 2

Effect of Levosimendan on locomotor activity (A) No of crossings (B) Distance travelled (C) Tracking of animals represents the locomotor activity in OFT. All the values were expressed as Mean ± S.E.M, n=6.

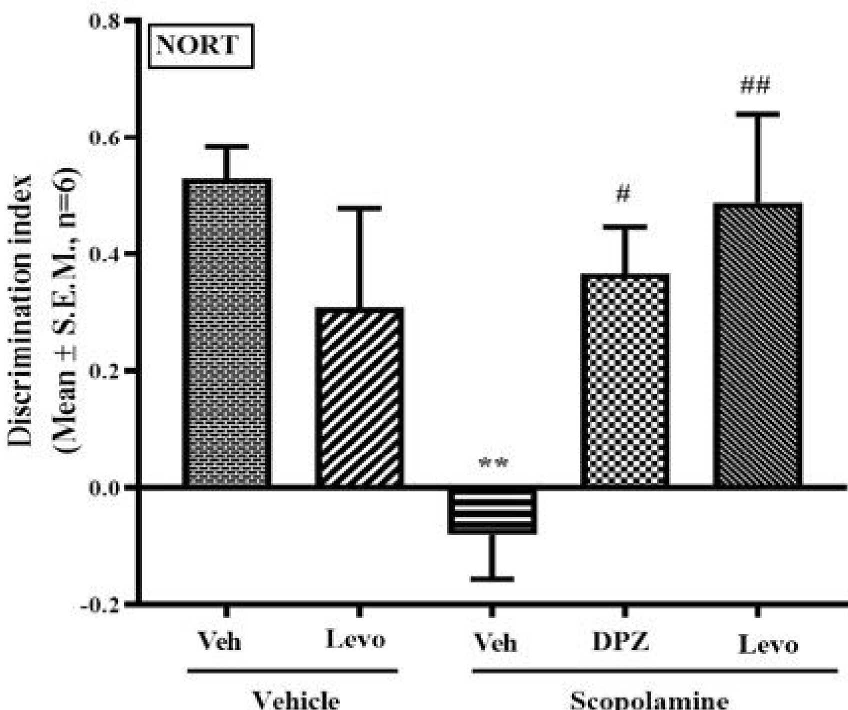


Figure 3

Effect of Levosimendan on Discrimination index in NORT. All the values were expressed as Mean ± S.E.M, n=6. $^{**}P < 0.01$ versus normal control group, $^{\#}P< 0.05$, $^{\#\#}P < 0.01$ versus scopolamine alone treated group.

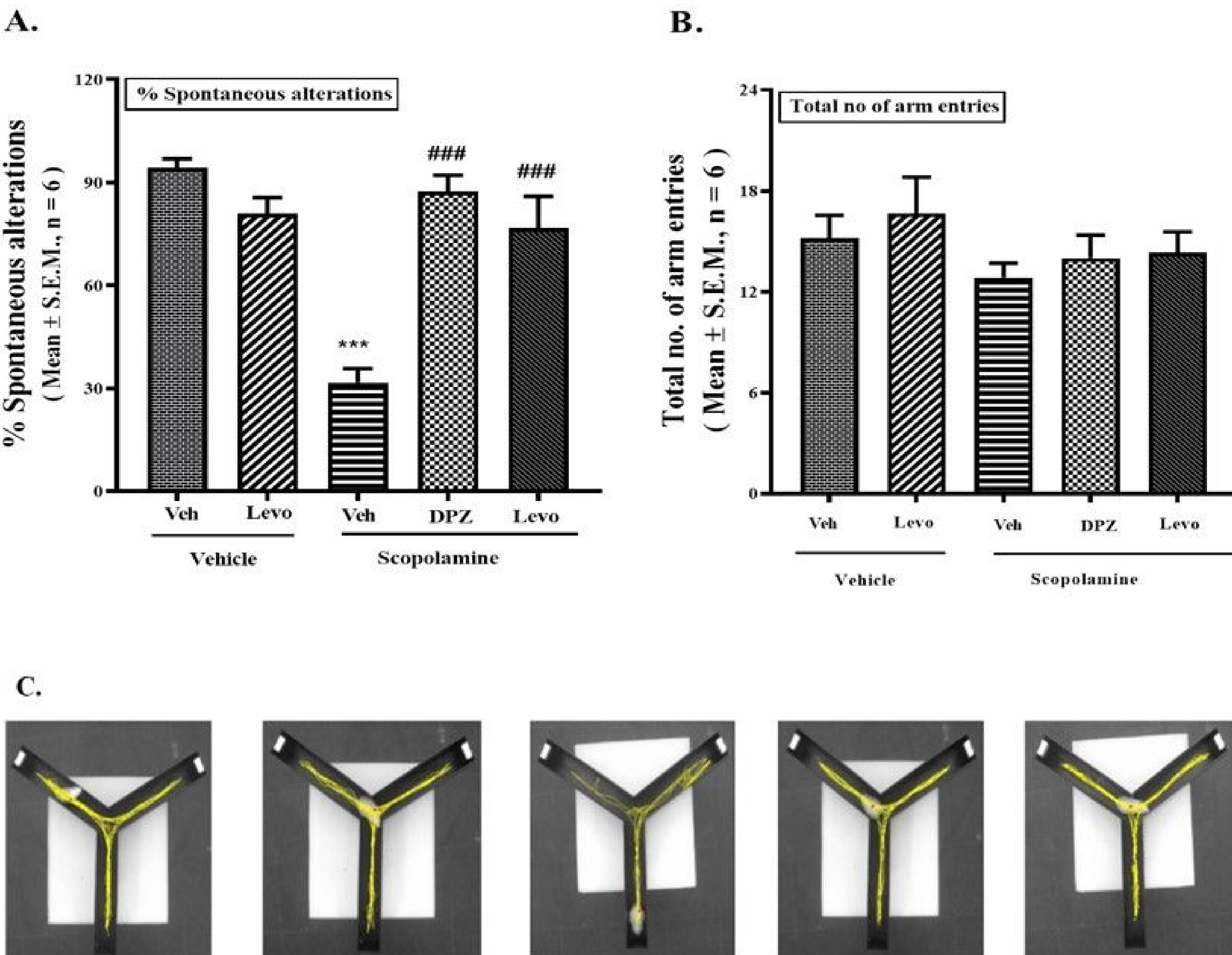
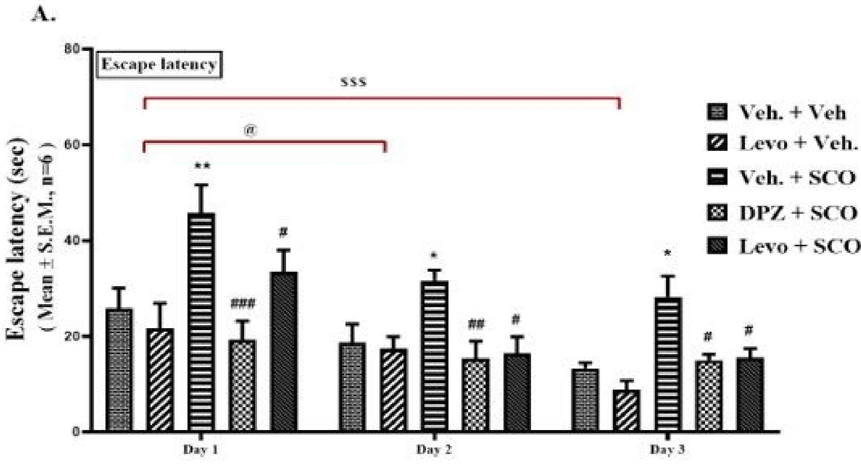
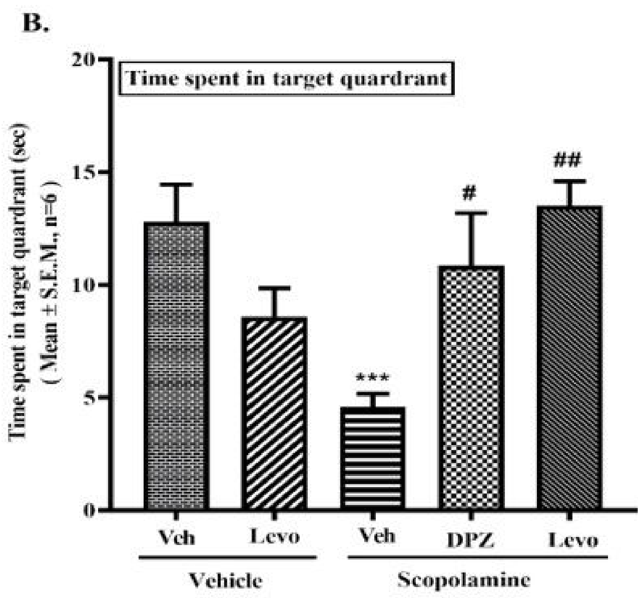


Figure 4

Effect of Levosimendan on short-term memory. (A) % Spontaneous alterations, (B) Total no of arm entries, (C) Tracking of the animals represents spontaneous alterations in Y-maze. All the values were expressed as Mean ± S.E.M, n=6. ***P < 0.01 versus normal control group, ###P < 0.001 versus scopolamine alone treated group.





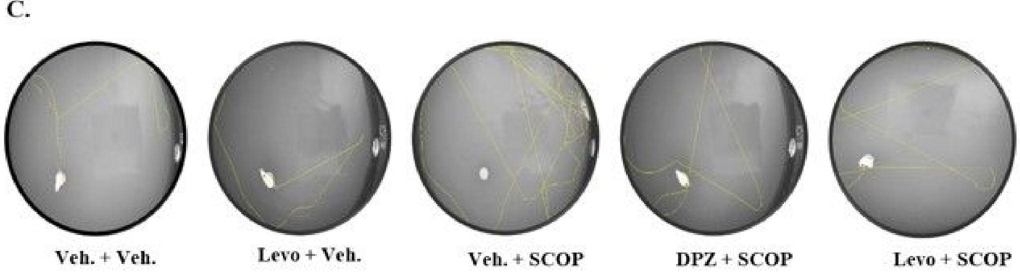


Figure 5

Effect of Levosimendan on Long-term memory in MWM test A) Escape latency, B) Probe analysis C) Tracking of the animals. All the values were expressed as Mean ± S.E.M, *P < 0.05, ***P < 0.001 compared with the control group, #P< 0.05, ##P < 0.01 compared with the scopolamine treated group. @p < 0.05 versus day-2, $$$p < 0.001 versus day-3

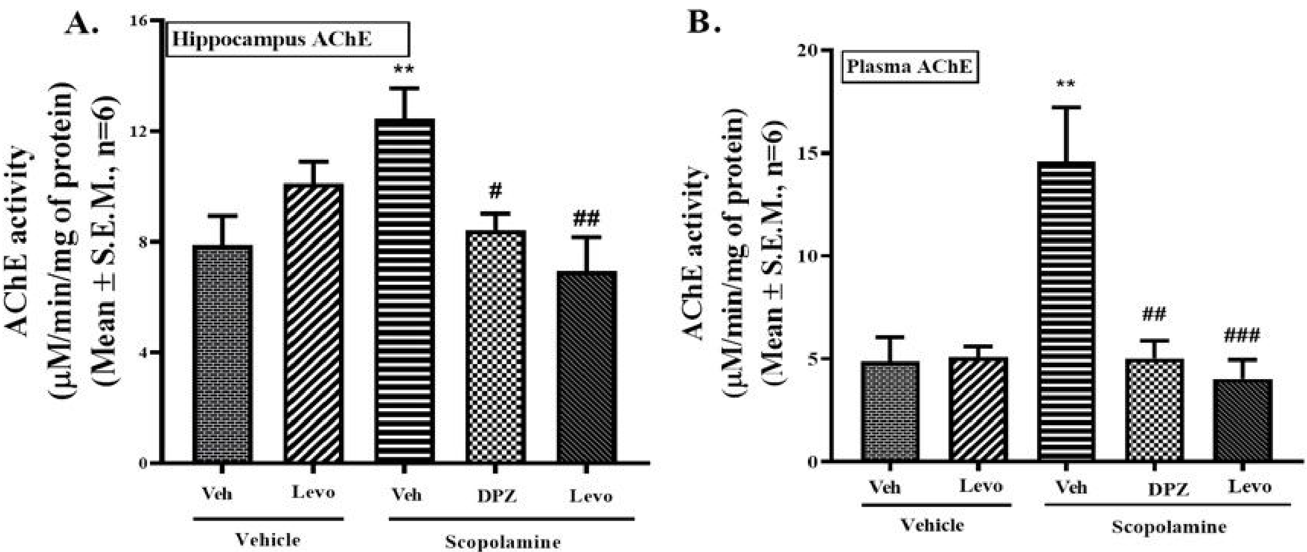


Figure 6

Effect of Levosimendan on AChE activity of (A) Hippocampus and (B) Plasma. All the values were expressed as Mean ± S.E.M, **P < 0.01 versus normal control group, $^{\#}$P< 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 versus scopolamine alone treated group.

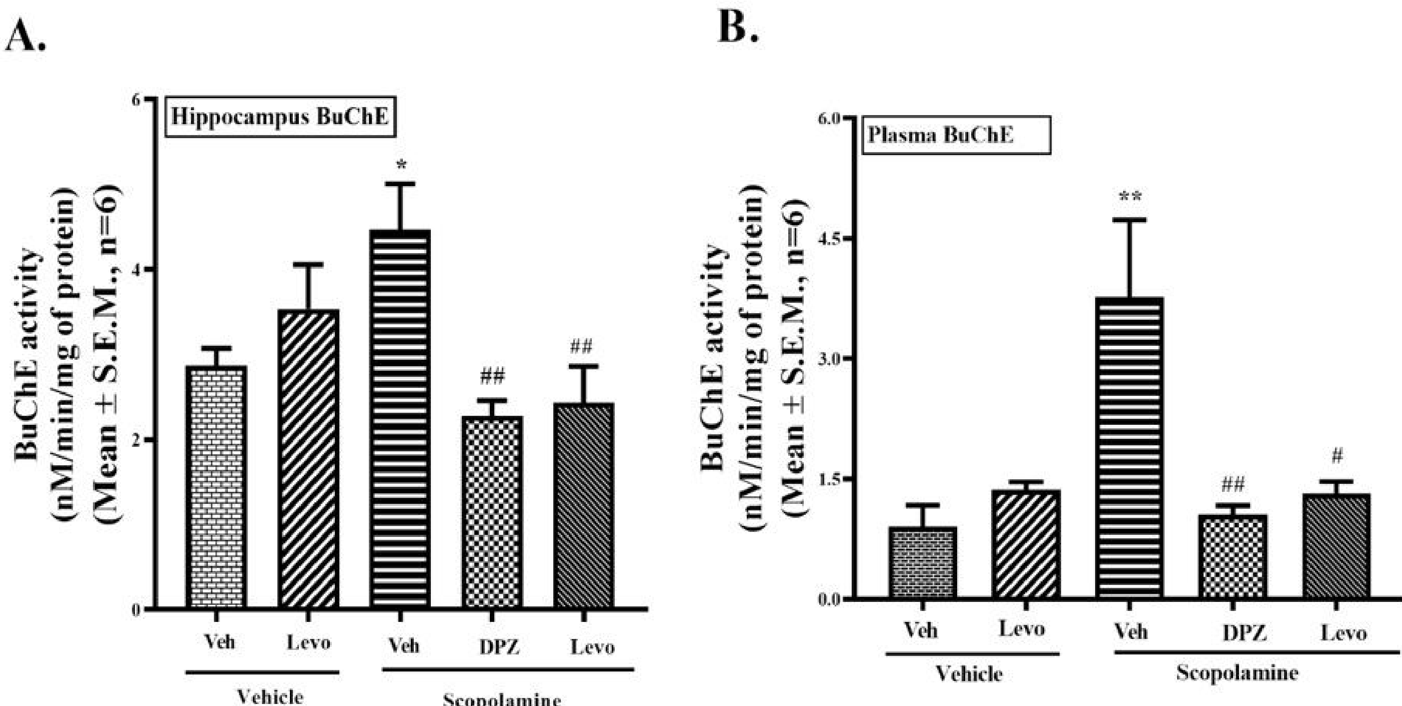


Figure 7

Effect of Levosimendan on BuChE activity of (**A**) Hippocampus and (**B**) Plasma. All the values were expressed as Mean ± S.E.M, $^{*}P < 0.05$, $^{**}P < 0.01$ versus normal control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus scopolamine alone treated group.

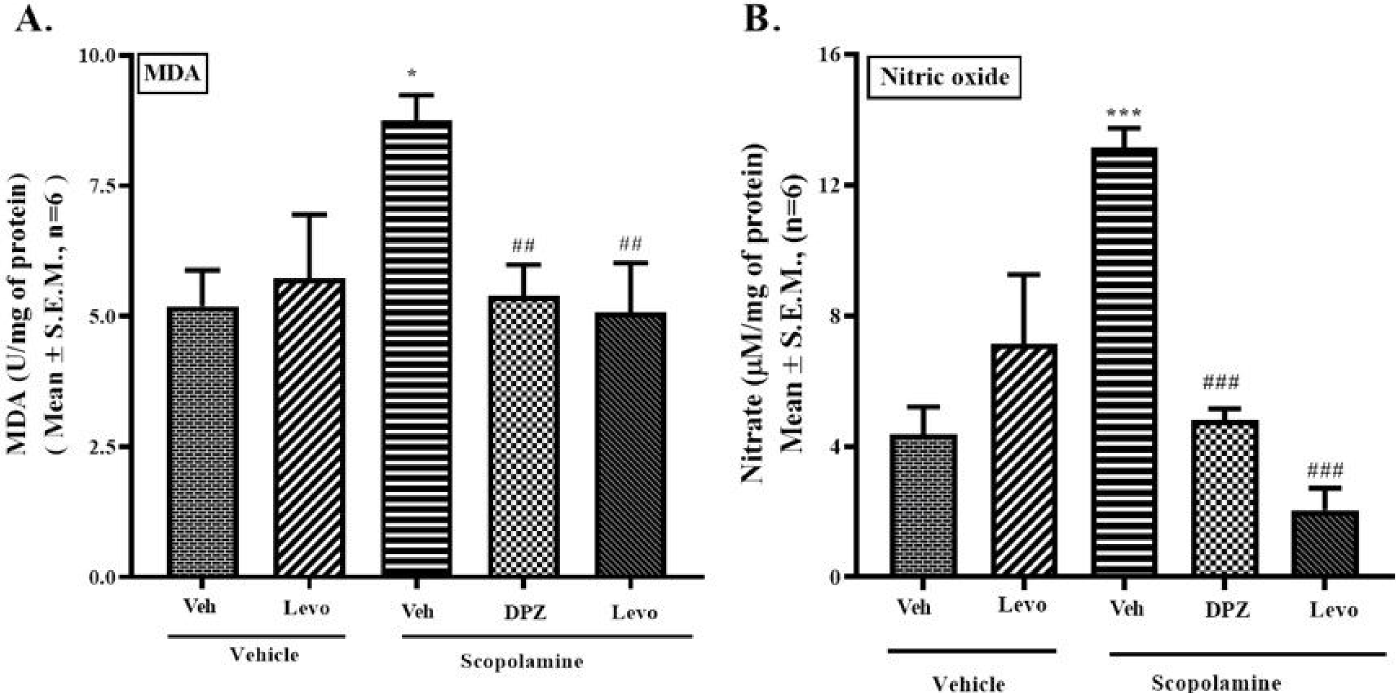


Figure 8

Effect of Levosimendan on lipid peroxidation and nitric oxide activity (**A**) MDA levels (**B**) Nitrate levels. All the values were expressed as Mean ± S.E.M, $^{*}P < 0.05$, $^{***}P < 0.001$ versus normal control group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$versus scopolamine alone treated group.

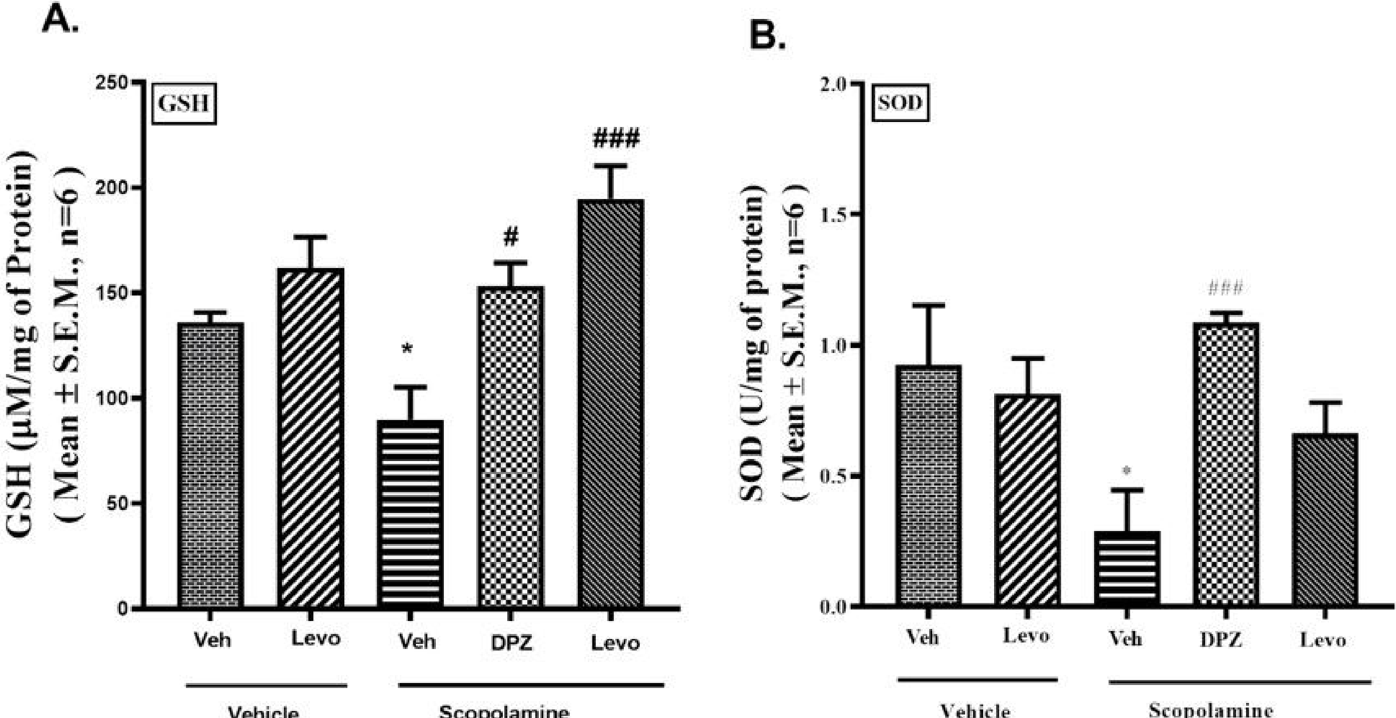


Figure 9

Effect of levosimendan on reduced glutathione (GSH) and Superoxide dismutase (**A**) Reduced glutathione (**B**) SOD activity. All the values were expressed as Mean ± S.E.M, $^{*}P < 0.05$ compared with the control group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the scopolamine alone treated group.

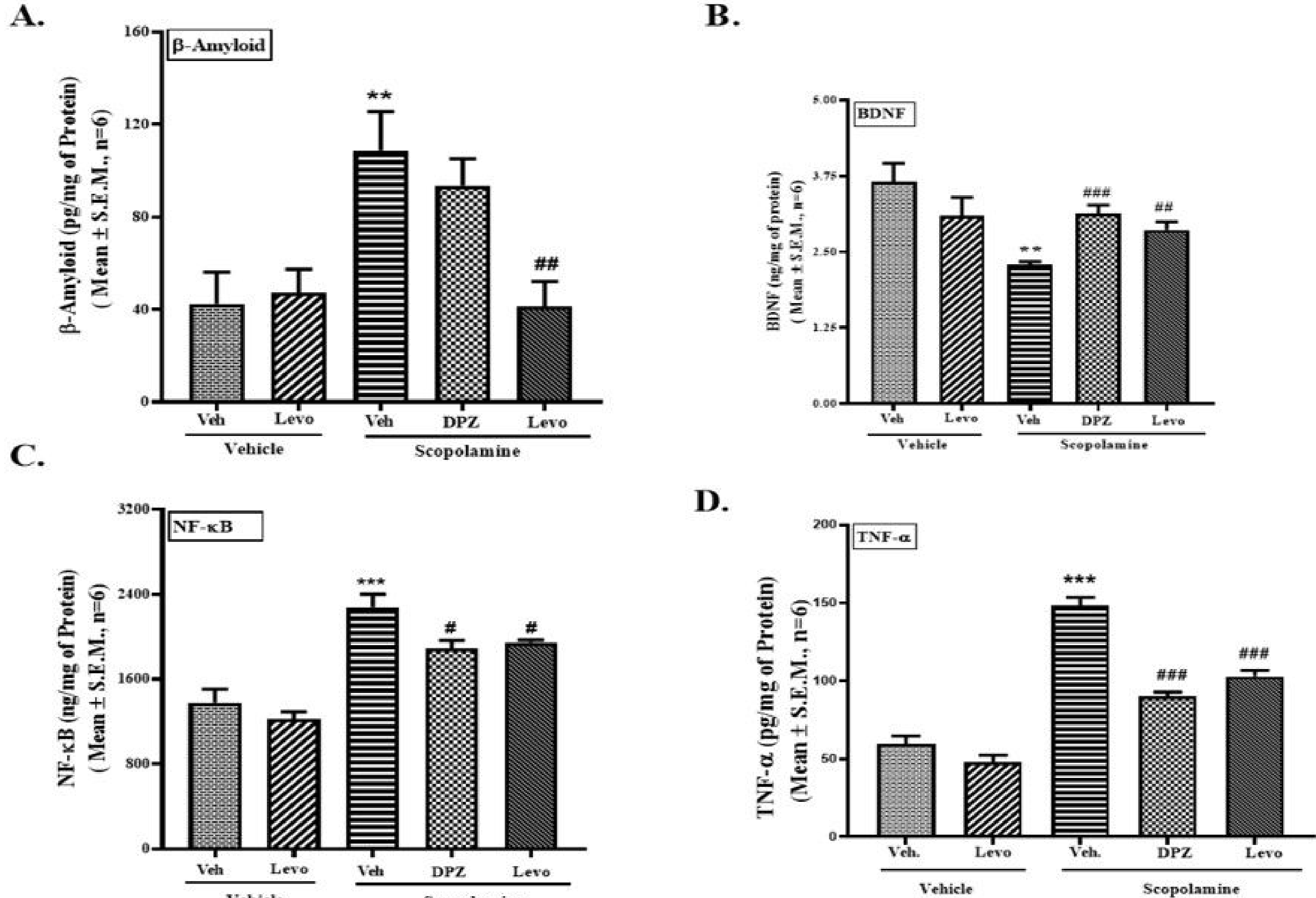
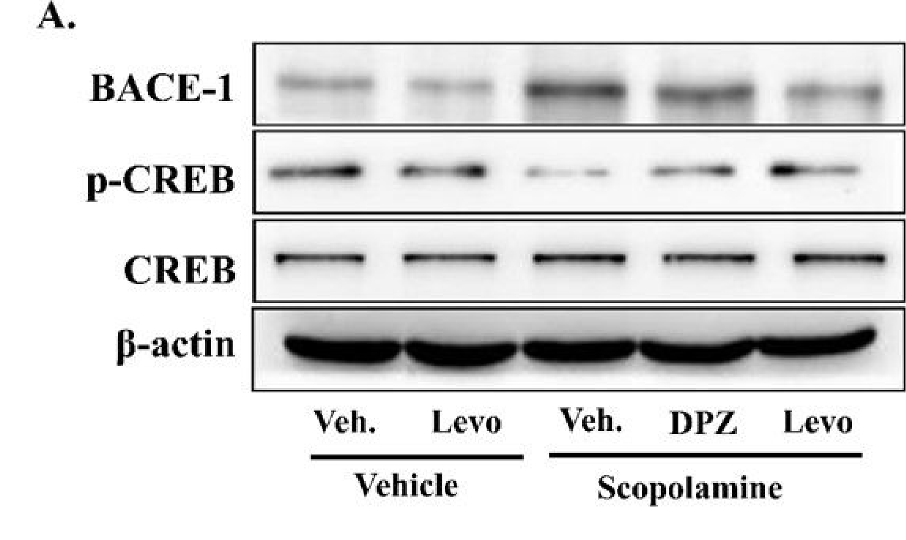


Figure 10

Effect of Levosimendan on A) β-Amyloid levels, B) BDNF, C) NF-κB, D) TNF-α. All the values were expressed as Mean ± S.E.M, $^{**}P < 0.01$, $^{***}P < 0.001$ versus normal control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus scopolamine alone treated group.



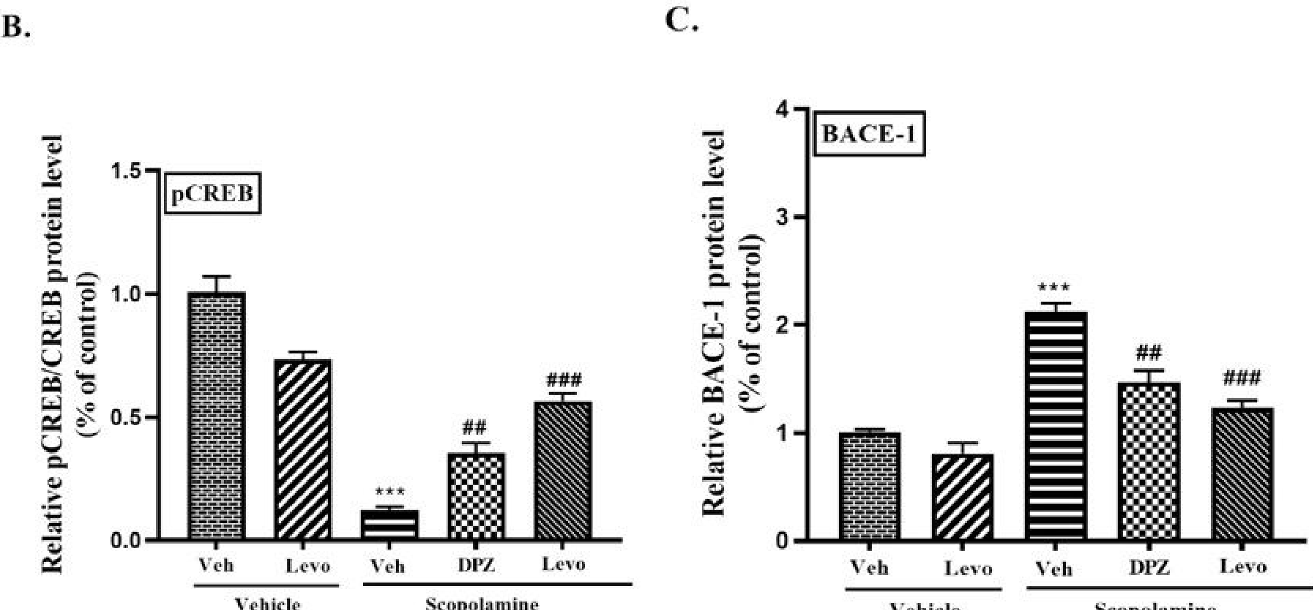


Figure 11

Effect of saroglitazar on protein expression levels of A) BACE-1, p-CREB and CREB in hippocampus B) Relative fold change of p-CREB, C) Relative fold change of BACE-1.All values are expressed as mean ± S.E.M (n=3). *$p < 0.05$, ***$p < 0.001$ vs. control group; #$p < 0.05$, ###$p < 0.001$ vs. scopolamine treated group.

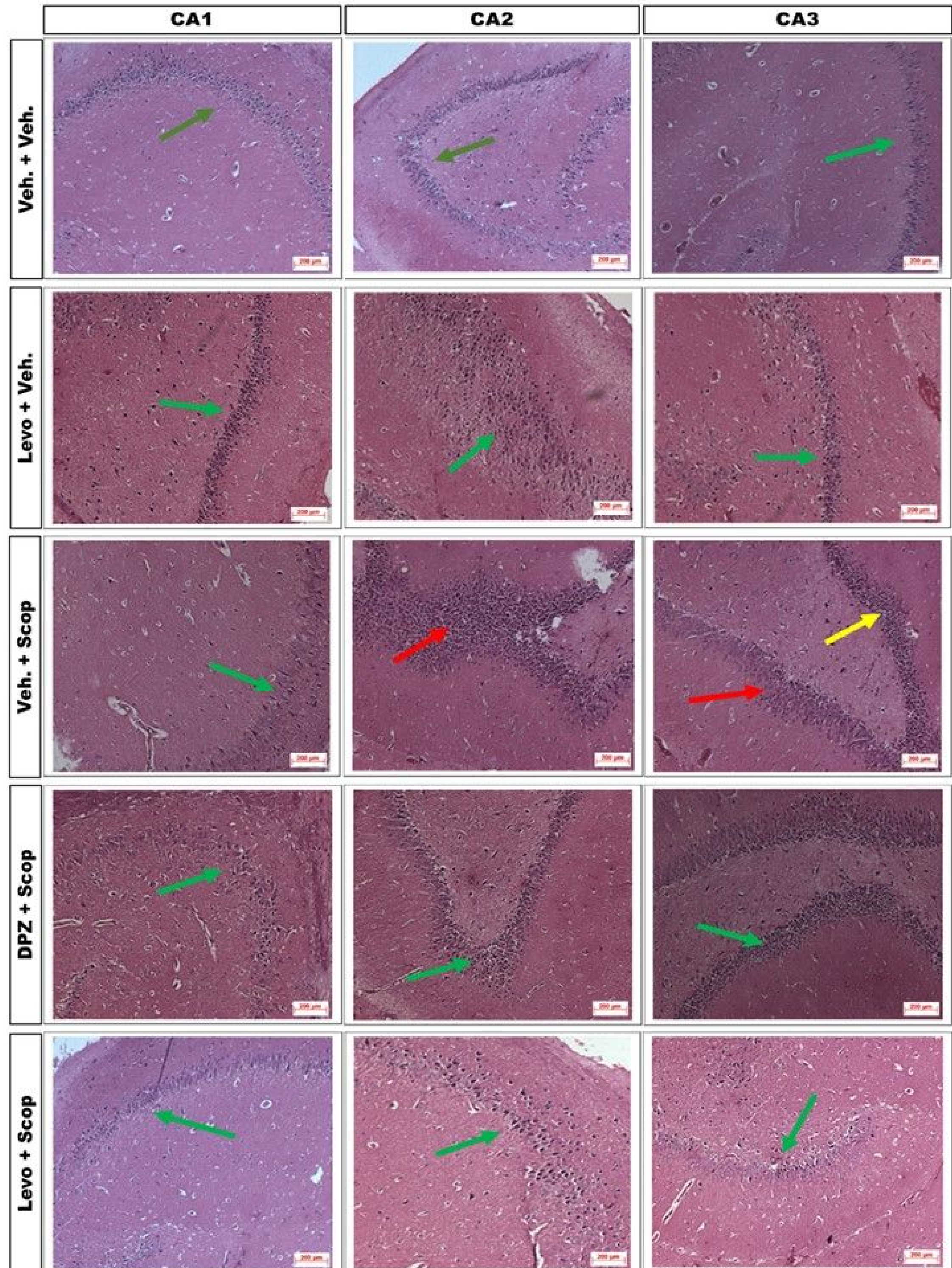


Figure 12

Showing the microscopic morphology of hippocampal regions (CA1, CA2 &CA3) in all groups: Control group **(A-C)**, Perse group **(D-F)**, Diseased group **(G-I)**, Standard group **(J-L)**, Treatment group **(M-O).** Green arrow indicates normal/mild changes in morphology, red arrow indicates severe hypertrophy, yellow arrow indicates hyperplasia

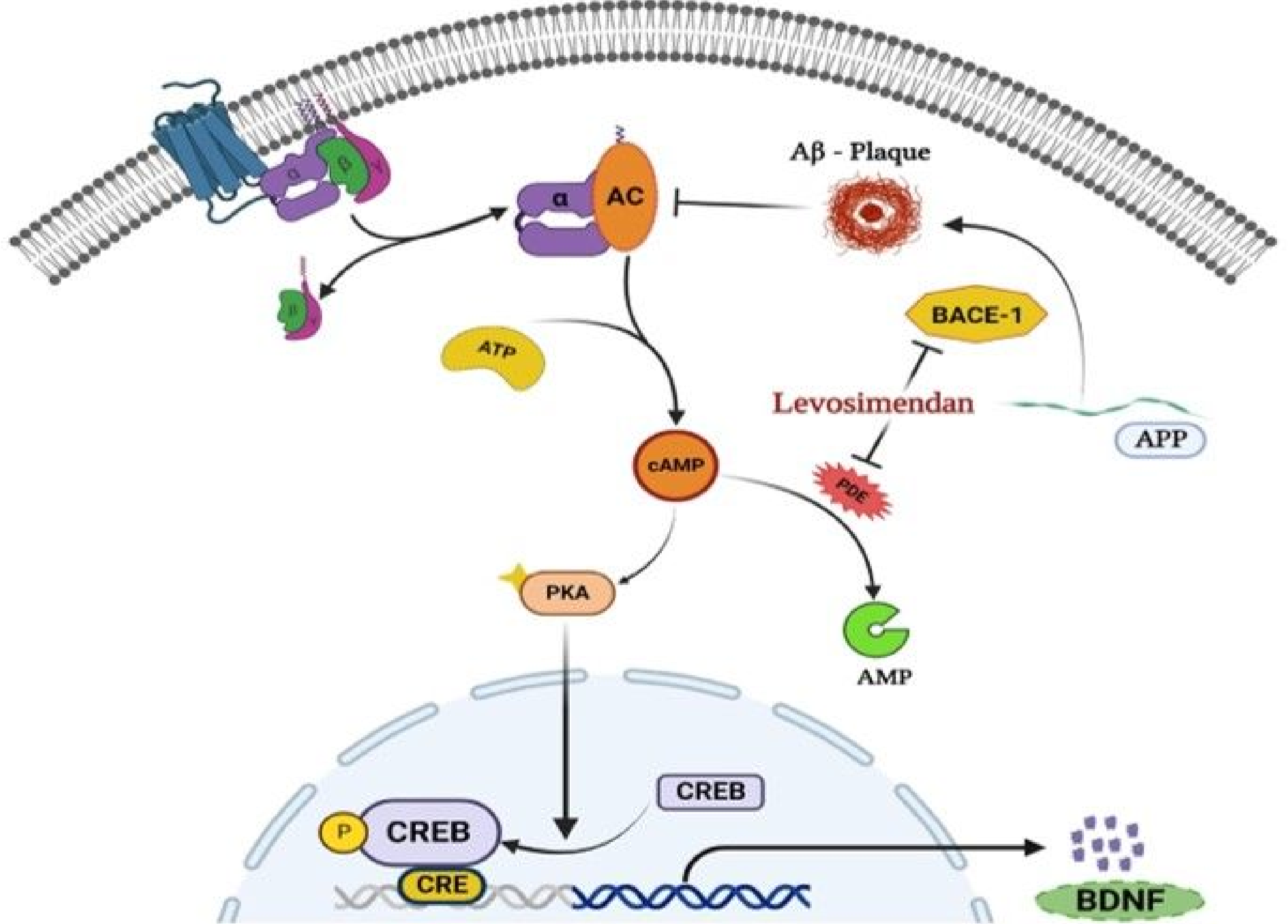


Figure 13

Pleotropic effects of levosimendan against the progression of AD