

Neuroprotective Effect of Ceftriaxone and Selegiline on Scopolamine Induced Cognitive Impairment in Mice

Sravanthi Akina, Mamatha Thati and Goverdhan Puchchakayala

Department of Pharmacology, Vaagdevi College of Pharmacy,
Ramnagar, Hanamkonda, Warangal-506001 andhra Pradesh, India

Abstract: Glutamate is the principal excitatory neurotransmitter in the nervous system. Inactivation of synaptic glutamate is handled by the glutamate transporter GLT1 (Excitatory Amino Acid Transporter EAAT), the physiologically dominant astroglial protein, while its dysfunction may contribute to this neurodegenerative disorders like Alzheimer's disease (AD). It is characterized by a gradual decline in memory associated with shrinkage of brain tissue, with localized loss of neurons mainly in the hippocampus and basal forebrain, with glutamate induced excitotoxicity related oxidative stress in association with diminished level of central cholinergic neurotransmitter-Acetylcholine. Glutamate transporters are important in preventing glutamate oxidative neurotoxicity. In the present study, the effects of ceftriaxone, selegiline and co-administration of these drugs on scopolamine induced learning and memory impairments in mice were studied. Using Rectangular maze test, Morris water maze test, Locomotor activity, Pole climbing tests and Various biochemical parameters such as acetylcholinesterase, TBARS assay, catalase activity and DPPH assay, we discovered that co-administration of ceftriaxone (β -lactam antibiotics which are potent stimulators of GLT1 expression) and selegiline (antioxidant) in mice exerts synergistic potent cognitive-enhancing activity than individually through both anti-acetylcholinesterase and antioxidant mechanisms. The memory enhancing capacity of the drugs were very significant when compared to negative control ($p < 0.001$). Donepezil was used as standard drug.

Key words: Ceftriaxone • Glutamate Transporter 1 • Synergism • Cognitive-Enhancing Activity • Antioxidant Activity • Acetylcholinesterase • Donepezil • Neuroprotection

INTRODUCTION

The memory is the most important function of the brain. Memory is the process by which organisms are able to record their experiences and use this information to adapt their responses to the environment [1]. Hence it is vital for survival. Central cholinergic system and glutamate transportation are considered as the most important neurotransmitters involved in regulation of cognitive functions. Impaired cognitive functions are the major features of Alzheimer disease (AD) [2]. Recent evidence suggests that the interaction of these two neurotransmitters may be important for some forms of memory and that acetylcholine, in particular, may function to facilitate glutamate activity by coordinating states of acquisition and recall in the cortex and hippocampus [3].

Scopolamine, a nonselective muscarinic cholinergic antagonist, causes impairment in learning and memory by

reducing cholinergic activity [4]. Loss of cholinergic cells particularly in the basal forebrain is accompanied by loss of the neurotransmitter acetylcholine and increase in glutamate at the synaptic cleft leading to excitotoxicity [5]. In addition to excitotoxicity, oxidative stress plays a role in the disease and neuronal cell death because of oxidative stress can be studied *in vitro* using oxidative glutamate toxicity (by estimating levels of malondialdehyde), a form of glutamate-induced cell death [6].

It was recently reported that excitatory amino acid transporters (EAATs) mediate the re-uptake of glutamate released by synaptic activity in the central nervous system [7] and thus protecting against oxidative glutamate toxicity. It was clear that Glutamate transporter is important in preventing glutamate neurotoxicity [8].

It was recently reported that ceftriaxone (a β -lactam antibiotic) is a potent stimulator of GLT1 expression.

Ceftriaxone is neuroprotective *in vitro* when used in models of neurodegenerative disorders [9]. Selegiline (L-deprenyl), an irreversible inhibitor of monoamine oxidase-B (MAO-B), a therapeutic agent of Parkinson's disease could alleviate the oxidative stress and prevent degenerative changes [10]. Eight of 11 controlled trials showed that selegiline has a positive effect on cognition (e.g., word fluency, delayed recall, total recall). Two of 5 controlled trials evaluating selegiline's effect on behavior (e.g., anxiety, tension, excitement, depression) showed a positive effect [11].

Therefore, we investigated whether co-administration of ceftriaxone and selegiline *in vivo* has synergistic protecting action against oxidative glutamate toxicity. For that, the activities of acetylcholinesterase (AChE), antioxidant enzymes such as catalase and malondialdehyde (MDA) levels were analyzed.

MATERIALS AND METHODS

Experimental Animals: Swiss albino mice (24.6 ± 0.9 g) were procured from Mahaveer Enterprises, Hyderabad. They (36) were housed into groups of six mice per cage and maintained at $22 \pm 1^\circ\text{C}$ with relative humidity 45-55% and 12:12 hours' dark/light cycle. The animals had free access to food (standard chew pellets) and water *ad libitum*. They were acclimatized to laboratory conditions for 2 days before behavioral studies. The Institution of Animals Ethics Committee (IAEC) had approved the experimental protocol (VCOP/2011/10/1/06) and care of animals was taken as per guidelines of CPCSEA, Department of Animal Welfare and Government of India (1047/ac/07/CPCSEA).

Drugs and Treatment: Ceftriaxone (Aristo Laboratories PVT.Ltd, Mumbai), selegiline (Intas Pharmaceuticals Ltd), scopolamine (Cadila Health Care Ltd) and donepezil (Alkem Laboratories Ltd) were used. Other chemical and reagents were of analytical grade. Memory impairment was induced by scopolamine (1.4ml/kg i.p.) and given

30 min after administration of test drug. Scopolamine and ceftriaxone was dissolved in sterile water for injection. Donepezil, selegiline were dissolved in 0.1% CMC solution. All drugs were prepared freshly and given once daily in the morning and followed the same regimen. The readings of behavioral studies were taken after 1 hr of drug administration. Doses were given according to the respective mice weights (Table 1). The doses of ceftriaxone and selegiline were selected based on those reported in the literature [12].

Assessment of Cognitive Performance: All the animals were trained for 3 days before drugs administration.

Rectangular Maze Test: The maze consists of completely closed rectangular box with an entry and reward chamber partitioned with wooden slats into blind passages leaving just twisting corridor leading from the entry to the reward chamber. All the mice were familiarized with rectangular maze for a period of 10 min for 2h. Well trained animals were taken for the experiment. The time taken for the mouse to reach the reward chamber was taken as the latency time. Four readings are taken and average of reading gives learning score. Lower scores indicate efficient learning and higher scores indicates poor learning in animals. The time taken by the animals to reach the reward chamber from the entry chamber was noted on day 1, 3, 5, 7 and 9 [13, 14].

Morris Water Maze Test: Method was carried out in a circular pool (30 cm in diameter and 40 cm in height) of water with a featureless inner surface [15, 16]. In the water maze experiments, the day prior to the experiment was dedicated to swim training for 60 s in the absence of the platform. In the days following, the mice were given the trial session each day for four consecutive days. During each trial, the escape latencies of mice were recorded. The point of entry of the mouse into the pool and the location of the platform [17] for escape between trials changed each day thereafter. The decrease in escape latency from

Table 1: Experimental Design:

Groups	Treatment
Group-I Normal Control	Vehicle (0.1% CMC).
Group-II Drug induced memory impairment control group	Vehicle + Scopolamine (1.4 ml/kg)i.p.
Group-III Standard	Donepezil (5 mg/kg)oral + Scopolamine (1.4 ml/kg)i.p.
Group-IV Test-I	Ceftriaxone (206mg/kg)i.p.+ Scopolamine (1.4ml/kg)i.p.
Group-V Test-II	Selegiline (0.49mg/kg)oral+ Scopolamine (1.4 ml/kg)i.p.
Group-VI Test-III	Ceftriaxone(206mg/kg) i.p.+ Selegiline(0.49mg/kg)oral+ Scopolamine (1.4 ml/kg)i.p.

day to day in trials represents long-term memory or reference memory. Six mice were used per treatment. Mice were treated with CMC or test compounds given before the training trial, respectively. After 90 min, amnesia was induced in mice with scopolamine (1.4 ml/kg)i.p body weight. All mice were tested for spatial memory 30 min after the scopolamine treatment [18].

Pole Climbing Test in Rodents: This test was used to study the neuroprotective and nootropic effect of test drugs. The mice were trained for conditioned avoidance response (CAR) by using pole climbing apparatus. Each mouse was allowed to acclimatize for 2 min and then exposed to a buzzer noise. After 10 sec of putting on the buzzer, mild electric shocks were given through the grid floor. The magnitude of the voltage was adequate (30v) to stimulate the mice to escape from the floor and climb the pole. As soon as the mice climbed the pole, both the buzzer and foot-shock were switched off. At least 10 such trails were given to each mouse at an interval of 1 min. per day for 3 days. After training schedule, most of the mice learned to climb the pole within 10 sec of starting the buzzer to avoid the electric foot shocks. On the test days, after 2 min of acclimatization period, each mouse was exposed to the buzzer for 10 sec; ten such trails were given at an interval of 1 min, without giving any shock. Mice, responding by climbing the pole when exposed to the buzzer noise, were considered to have retained the conditional avoidance response [19].

Measurement of Locomotor Activity: Most of the CNS drugs influence the locomotor activities in man and animals. The locomotor activity of drug can be studied using actophotometer which operates on photoelectric cells which are connected in circuit with a counter when the beam of light falling on photo cell is cut off by the animal a count is recorded [20]. Animals were placed individually in the activity cage for 10 min and the activity was monitored. The test was done after 1hour of drug administration. The photo cell count was noted and decrease or increase in locomotor activity was calculated [21].

Histopathological Studies: After 8 days treatment, the brains of different groups were perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and postfixed in the same fixative overnight at 48°C. The brains were then routinely embedded in paraffin and stained with Hematoxylin-Eosin. The hippocampal lesions were assessed microscopically at 40X magnification [22].

Dissection and Homogenization: Following the behavioral testing, animals were sacrificed and the brain tissues were quickly removed, cleaned with ice-cold saline and stored at -20°C for bio chemical estimation. Brain tissue samples were thawed and homogenized 10 times (w/v) with ice cold 0.1M phosphate buffer (pH 7.4). Aliquots of homogenates from mice brains were separated and centrifuged at 3,000 rpm for 30 min and the supernatant was then used for biochemical estimation [2].

Biochemical Tests

Estimation of Cholinergic Status in the Mice Brain: The cholinergic marker, acetylcholinesterase was estimated in the whole brain according to the method of Ellman[23]. Ellman's reagent is 5, 5'-dithiobis(2-nitrobenzoate) and it is also abbreviated as DTNB. 0.1 ml of Brain tissue homogenate was incubated for 5 min with 2.7 ml of phosphate buffer and 0.1 ml of DTNB. Then, 0.1 ml of freshly prepared acetylthiocholine iodide (pH 8) was added and the absorbance was read at 412 nm [2, 24].

DPPH Radical Scavenging Assay: The free radical scavenging activity of the test drug was measured *in vitro* by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay [25]. Measurement was made from the bleaching of purple colour. About 0.3mM solution of DPPH was dissolved in 100ml ethanol and 1ml of the brain tissue homogenate supernatant dissolved in ethanol was added to 3ml of the DPPH solution. The mixture was shaken and allowed to keep at room temperature for 30 min and the absorbance was measured at 517nm using a spectrophotometer. The percentage of scavenging activity was determined as follows:

$$\% = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$$

A_{blank} -absorbance of control reaction

A_{sample} -absorbance of test sample [26].

Catalase Activity: Catalase activity was assessed by the method of Luck, wherein the breakdown of hydrogen peroxide is measured. Hydrogen peroxide (H₂O₂) solution (2mM/L) was prepared with standard phosphate buffer (pH 7.4). The brain tissue homogenate supernatant was added to 0.6ml of H₂O₂ solution. Absorbance was determined at 230nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging activity was determined [2, 27].

TBARS (Thiobarbituric Acid Reactive Substance) Assay:

The tissue homogenate(0.5ml) was supplemented with 0.5ml of phosphate buffer and then with 1ml of 10%trichloroacetic acid. The mixture was centrifuged at 3000rpm at 4°C for 10 min. The supernatants of the tissue homogenates were incubated with 1 ml of 0.8% w/v of the thiobarbituric acid at 100°C for 15 min. After a cooling period, TBARS concentration was spectrophotometrically determined at 532nm. The levels of lipid peroxides were expressed as nano moles of TBARS [28].

Statistical Analysis: All data were expressed as mean \pm SD. The data were analyzed using one way analysis of variance (ANOVA), followed by Dunnett-test where readings of the control, standard and test drug induced groups were compared against scopolamine induced group. The value $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Excessive activation of glutamate receptors by excitatory amino acids leads to a number of deleterious consequences, including impairment of calcium buffering, generation of free radicals by lipid peroxidation, secondary excitotoxicity and promote oxidative damage leading to a rise in tissue peroxide levels [29]. Thus, understanding the pathways involved in excitotoxicity is of critical importance for the future clinical treatment of many neurodegenerative diseases. Oxidative stress is the cytotoxic consequence of oxyradical and oxidant formation and the reaction with cellular constituents [30]. Reactive oxidative species (ROS) are generated continuously in nervous system during normal metabolism and neuronal activity [31]. Increased MDA level as one of the ROS has been shown to be an important marker for *in vivo*.

β -lactam antibiotics are potent stimulators of GLT1 expression. The β -lactam ceftriaxone increases both brain expression of GLT1 and its biochemical and functional activity. Glutamate transporters are important in preventing glutamate oxidative neurotoxicity [9]. In addition, selegiline may act as an antioxidant in neurons and protect against glutamate-receptor-mediated toxicity. Administering selegiline to aged male laboratory animals slows their cognitive and behavioural deterioration and significantly prolongs their average life span in comparison with control animals [32].

Scopolamine, acetylcholine receptor antagonist, was reported to impair cognitive performances especially

spatial learning and memory [21]. Therefore, scopolamine is considered as reliable tool to study neuroprotective effects of candidate molecules [33].

Salient findings of this study were that pre and Post scopolamine treatment with ceftriaxone and selegiline improved cognition, decreased malondialdehyde and increased activities of catalase and antioxidant activity [2]. This illustrates that intraperitoneal administration of scopolamine was characterized by progressive deterioration of learning and memory [17], oxidative stress and decrease in acetylcholine turnover [34] which correlates with AD [35].

In the present study, the co-administration of ceftriaxone and selegiline (CCS) was evaluated to demonstrate its synergistic action in cognitive enhancing effects on spatial memory and learning function of mice against scopolamine-induced amnesic deficits via Rectangular maze, Morris water maze [20] and Conditioned avoidance tests. In addition, the neuroprotective activity was evaluated by measuring Cholinergic Status and antioxidant levels in mice brain.

Ceftriaxone and Selegiline Improved of Behavioral Alteration in Disease Mice

Rectangular Maze Test: In this test, co-administration therapy showed significant ($p < 0.001$) decrease in latency time on day 7 and on day 9 and standard showed significant ($p < 0.001$) decrease in latency time with mice compared to scopolamine (drug induced memory impairment) group (Fig. 1). Moreover the higher latency time induced by scopolamine was significantly reversed by treatment group compared with scopolamine group and co-administration therapy showed slightly higher efficacy than standard donepezil which was used as the positive control.

Morris Water Maze Method: The efficacy of ceftriaxone and selegiline in enhancing cognition after impairment of spatial memory via scopolamine was evaluated through the Morris water maze test. Over the 4 days of water maze testing, on day 5, mice in drug induced memory impairment group exhibited longer swim latency time in comparison with other groups receiving test and standard drugs. Swim latencies exhibited by all groups of mice except drug induced memory impairment group were gradually decreased from day 1 to day 5. Significant ($p < 0.01$) reduction was observed with co-administration. Standard drug group has shown significant ($p < 0.001$) reduction in latency time (Fig. 2).

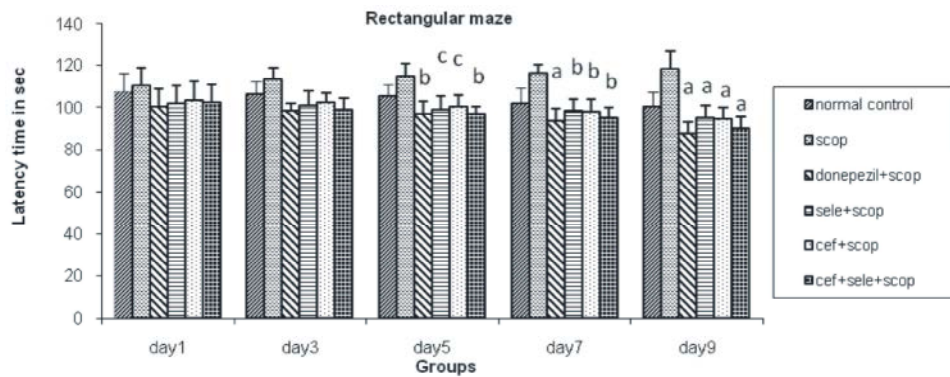


Fig. 1: Effect of Ceftriaxone and Selegiline on latency time in Rectangular maze test compared to the drug induced memory impairment group (Mean \pm SD, n = 6).

Values are expressed as Mean \pm SD; c(*p<0.05), b(**p<0.01), a(***p<0.001) different letters (a, b and c) are significant (p<0.05) as compared to disease group. Note: scop=scopolamine; cef=ceftriaxone; sele=selegiline.

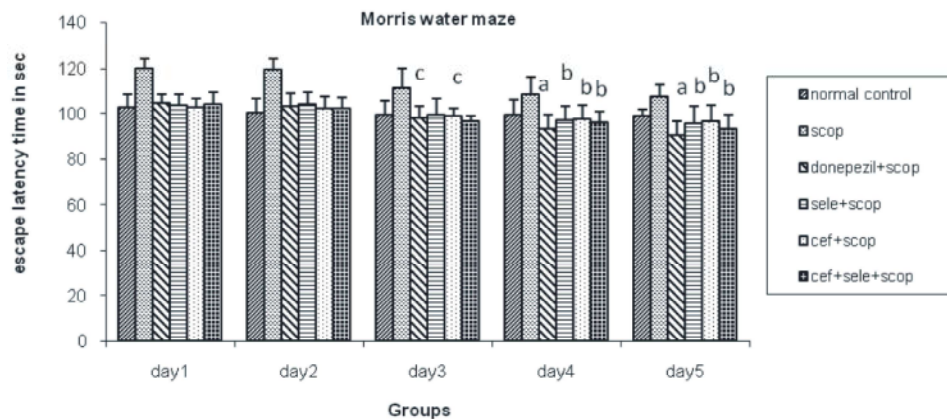


Fig. 2: Effect of Ceftriaxone and Selegiline on latency time in Morris water maze test compared to the drug induced memory impairment group (Mean \pm SD, n = 6).

Values are expressed as Mean \pm SD; c(*p<0.05), b(**p<0.01), a(***p<0.001) different letters (a, b and c) are significant (p<0.05) as compared to disease group. Note: scop=scopolamine; cef=ceftriaxone; sele=selegiline.

Pole Climbing Test: Ceftriaxone and selegiline showed significant (p<0.05) conditioned avoidance response and co-administration therapy showed significant (p< 0.01) increase in response on day 7 and on day 9, co-administration therapy showed significant (p< 0.001) increase in CAR with mice compared to drug induced memory impairment group (Fig. 3). Moreover the lower CAR induced by scopolamine was significantly reversed by treatment group compared with scopolamine group.

Effect of Ceftriaxone and Selegiline on Locomotor Activity: In the present series of experiments, the mean scores of locomotor activity for each mouse were relatively stable and showed no significant variation

among different groups. The mean scores in control, disease groups remain unchanged. The test drugs individually and in co-administration also did not cause any significant alteration in the locomotor activity as compared to disease mice (Fig. 4).

Acetylcholinesterase Inhibitory Effects of Ceftriaxone and Selegiline: Scopolamine treatment increased brain Acetylcholinesterase (AChE) significantly. Attenuation of enhanced AChE was observed in standard and test drugs treated groups compared to disease group. Co-administration of ceftriaxone and selegiline has shown synergistic action in inhibiting AChE than ceftriaxone and selegiline individually (Fig. 5).

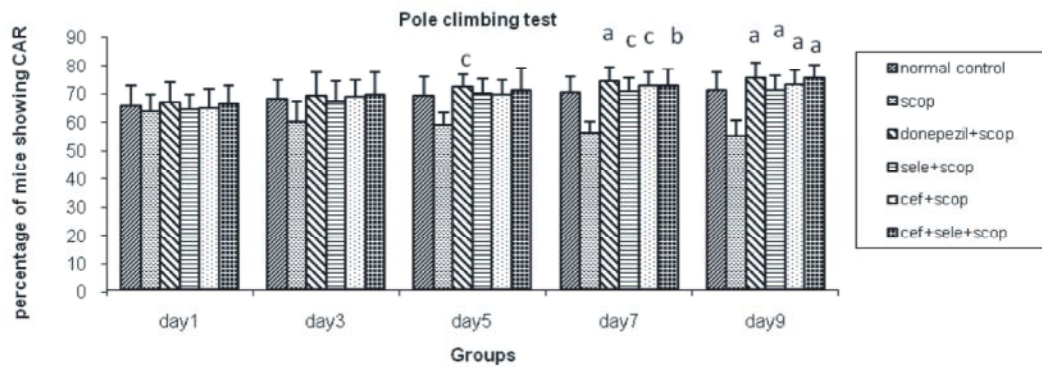


Fig. 3: Effect of Ceftriaxone and Selegiline on CAR in Pole climbing test compared to the drug induced memory impairment group (Mean \pm SD, n = 6).

Values are expressed as Mean \pm SD; different letters (a, b and c) are significant ($p < 0.05$) as compared to disease group. Note: scop=scopolamine; cef=ceftriaxone; sele=selegiline.

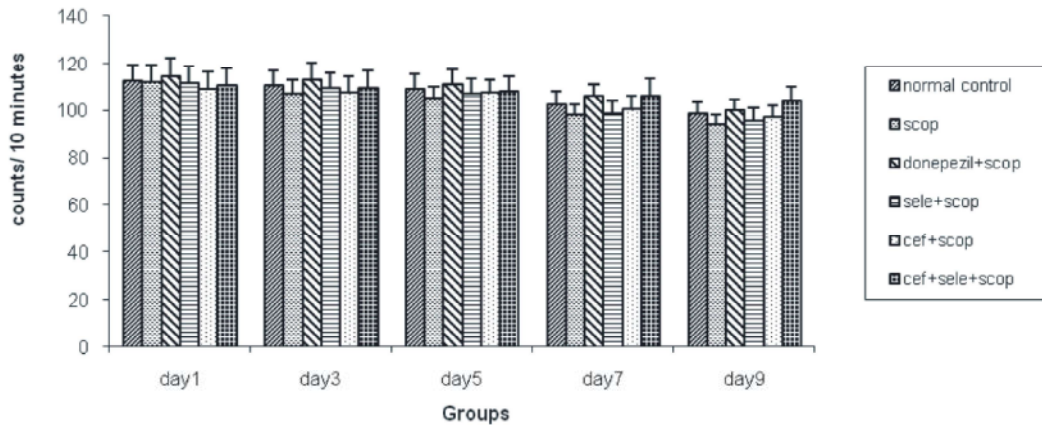


Fig. 4: Effect of Ceftriaxone and Selegiline on Locomotor activity compared to the drug induced memory impairment group (Mean \pm SD, n = 6).

Values are expressed as Mean \pm SD as compared to disease group. Note: scop=scopolamine; cef=ceftriaxone; sele=selegiline.

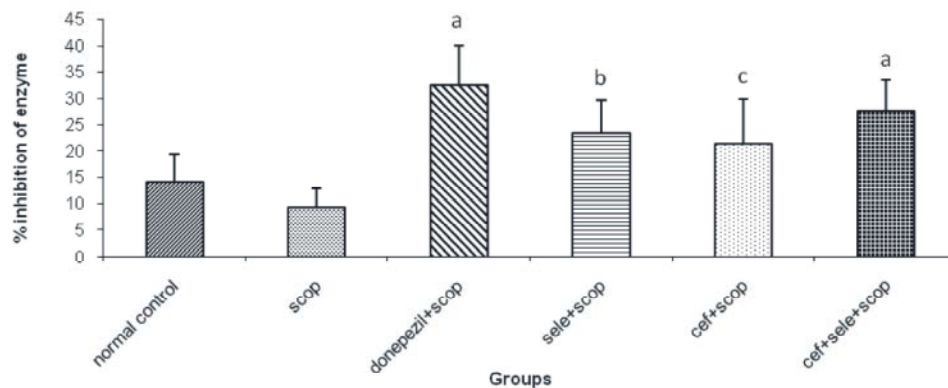


Fig. 5: Effect of Ceftriaxone and Selegiline on % inhibition of AChE enzyme compared to the drug induced memory impairment group (Mean \pm SD, n = 6).

Values are expressed as Mean \pm SD; different letters (a, b and c) are significant ($p < 0.05$) as compared to disease group. Note: scop=scopolamine; cef=ceftriaxone; sele=selegiline.

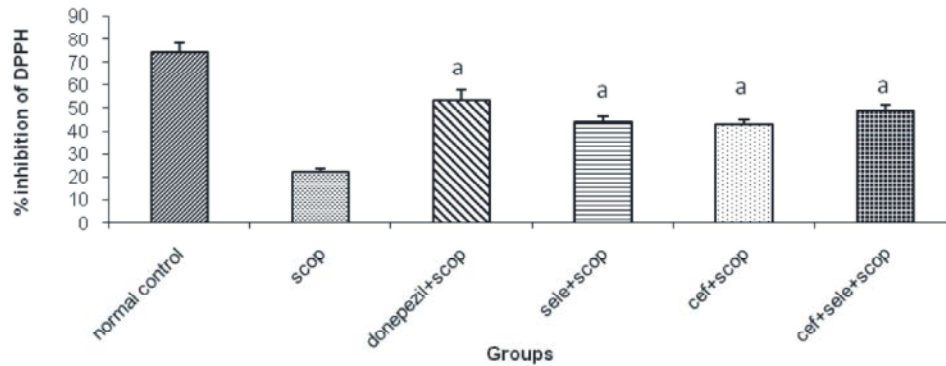


Fig. 6: Effect of Ceftriaxone and Selegiline on % inhibition of DPPH compared to the drug induced memory impairment group (Mean \pm SD, n = 6).

Values are expressed as Mean \pm SD; a(p<0.001) as compared to disease group. Note: scop=scopolamine; cef=ceftriaxone; sele=selegiline.

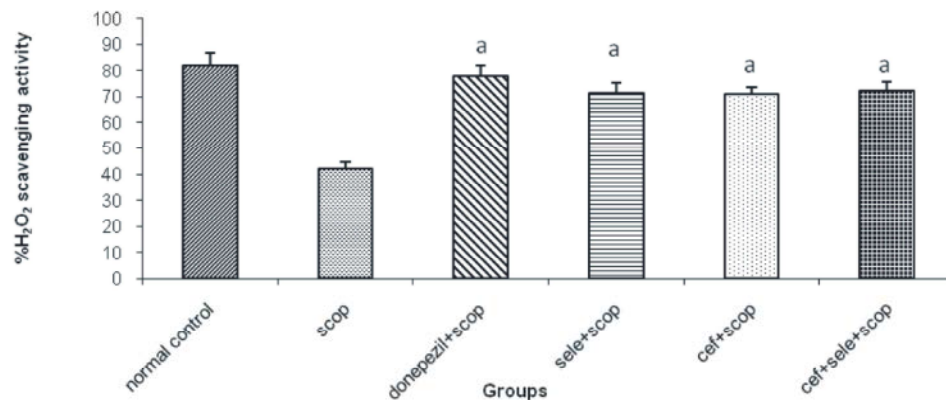


Fig. 7: Effect of Ceftriaxone and Selegiline on % of H₂O₂ scavenging activity compared to the drug induced memory impairment group (Mean \pm SD, n = 6). Values are expressed as Mean \pm SD; a(p<0.001) as compared to disease group. Note: scop=scopolamine; cef=ceftriaxone; sele=selegiline.

Antioxidant Effect of Ceftriaxone and Selegiline in Disease Mice

DPPH Method: Antioxidant activity has been expressed in percentage inhibition of DPPH activity. Scopolamine treatment decreased brain antioxidant activity significantly. Improvement of antioxidant activity was observed in standard and test drugs treated groups compared to disease group. Co-administration of ceftriaxone and selegiline has shown synergistic action in increasing antioxidant activity than ceftriaxone and selegiline individually (Fig. 6).

Catalase Activity: Catalase activity has been expressed in percentage hydrogen peroxide (H₂O₂) scavenging activity. Scopolamine treatment decreased brain catalase activity significantly.

Improvement of catalase activity was observed in standard and test drugs treated groups compared to disease group. Co-administration of ceftriaxone and selegiline has shown synergistic action in increasing catalase activity than ceftriaxone and selegiline individually (Fig. 7).

TBARS Assay: Scopolamine treatment significantly increased the brain malondialdehyde (MDA) levels compared to control group. Standard drug donepezil and test drugs (ceftriaxone and selegiline) treatment significantly decreased brain MDA levels compared to disease group. Co-administration of ceftriaxone and selegiline has shown synergistic action in reducing MDA levels than ceftriaxone and selegiline individually (Fig. 8).

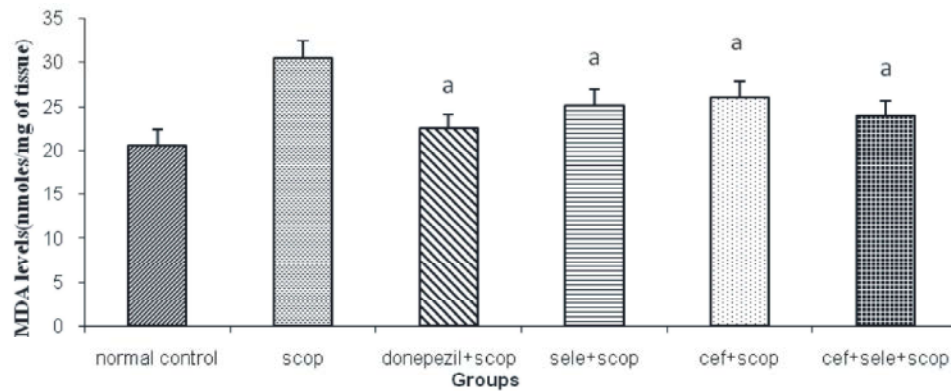


Fig. 8: Effect of Ceftriaxone and Selegiline on MDA levels in TBARS assay compared to the drug induced memory impairment group (Mean \pm SD, n = 6).

Values are expressed as Mean \pm SD; a(p<0.001) as compared to disease group. Note: scop=scopolamine; cef=ceftriaxone; sele=selegiline.

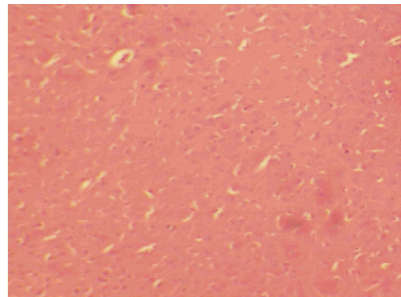


Fig. 9.1: Normal control

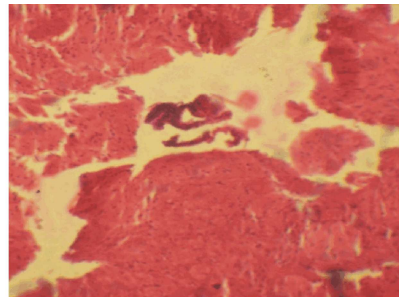


Fig. 9.2: Scopolamine

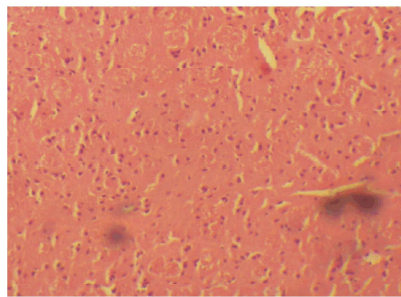


Fig. 9.3: Donepezil

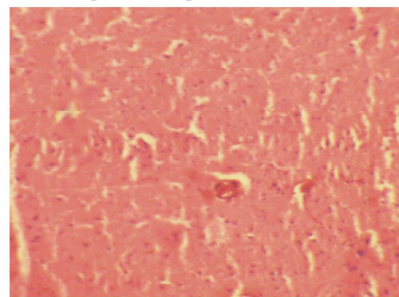


Fig. 9.4: Ceftriaxone

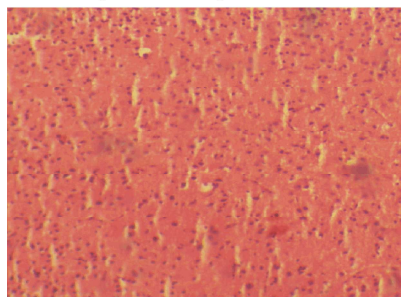


Fig. 9.5: Selegiline

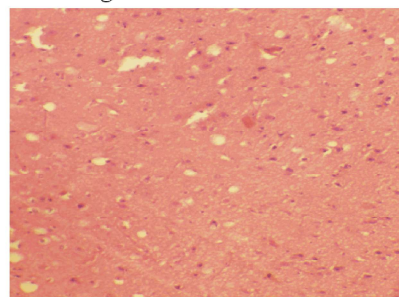


Fig. 9.6: Ceftriaxone+Selegiline

Fig. 9: 9.1, 9.2, 9.3, 9.4, 9.5 and 9.6 are normal control, scopolamine (drug induced memory impairment), donepezil (standard), ceftriaxone, selegiline and ceftriaxone+ selegiline respectively representing the histological sections of the brain tissue showing neurological lesions.

Histopathological Studies: From the Fig. 9, it is clearly visible that in drug induced memory impairment group the degenerated cells are more compared to other groups. This is indicated by the gaps in slides. The drug induced memory impairment group has shown more gaps than test drugs treated groups. The co-administration group was mostly near to the control group compared to the individual drug treated groups.

These results indicated that the memory and cognition-enhancing effects of ceftriaxone and selegiline might be related to factors such as antioxidant and anti-acetylcholinesterase activities.

The present study therefore demonstrated the probable mechanism by which ceftriaxone and selegiline showed neuroprotective activity by increasing the performance of learning and memory which was clear from behavioral test and biochemical results. It was generally assumed that the up-regulation of EAAT2 is responsible for ceftriaxone-mediated neuroprotection by virtue of its ability to reduce extracellular glutamate levels and subsequent excitotoxicity related oxidative stress [7]. Selegiline, antioxidant drug had attenuated oxidizing enzymes.

In conclusion, the present study demonstrated that co-administration of these test drugs, ceftriaxone and selegiline has potential therapeutic effects on improving the anti-amnesic activity in mice through inhibiting lipid peroxidation, augmenting endogenous antioxidant enzymes and decreasing acetylcholinesterase (AChE) activity in brain. It showed co-administration of ceftriaxone with selegiline had a synergistic cognition enhancement effect against scopolamine induced memory impairment and oxidative stress.

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