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Research Article
Neuroprotective Effects of Co-Administration of Selegiline with Piracetam on Cognitive Impairment: Involvement of NR2B, NR1 and Bax Signaling Pathway

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Abstract

Background and Objective: Alzheimer's Disease (AD) is a progressive neurodegenerative disorder characterized by loss of memory due to phosphorylation of tau protein, neurofibrillary lesions composed of the β-amyloid peptide and aberrant oxidative stress. The objective is to study the combined neuroprotective effect of Selegiline with Piracetam against scopolamine and streptozotocin (STZ)-induced memory impairment in animals. **Materials and Methods:** This study was designed to investigate the synergistic neuroprotective effect of Selegiline with Piracetam by use of scopolamine and STZ model of memory impairment using the Morris water maze for the assessment of learning and memory. Various biochemical parameters such as acetylcholinesterase activity (AChE) as a marker of cholinergic activity, malondialdehyde (MDA) a marker of lipid peroxidation and glutathione (GSH) a marker of oxidative stress were estimated. Gene expression was also performed by RT-PCR and western blot test. **Results:** There were a significant decrease in MDA and AChE level and increase in GSH level as compared to the disease control group in Selegiline with Piracetam. In the present study, Bax and Bak mRNA (proapoptotic) were down-regulated, while Bcl-2 mRNA and protein which are antiapoptotic were up-regulated in Selegiline with Piracetam group opposite to the STZ group. Also, p53 expression in the given experiment was appreciably reduced in Selegiline with Piracetam group. **Conclusion:** The present study indicates that the administration of scopolamine and STZ cause a decline in memory function which is significantly improved by the treatment of selegiline with piracetam. The combined effect of selegiline with piracetam was found to be therapeutically more effective than the individual administration.

Key words: Selegiline, piracetam, behavioral studies, scopolamine, streptozotocin, RT-PCR, western blot test

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Memory is one of the most complex functions that our brain is capable of performing. It is an endless reel of events and processing that involves various neurotransmitters and related neuronal pathways. The impairment can be mild to severe depending upon the person's age, sex, state of mind, previous events and more. We can conveniently term it as a "loss of one's intellectual ability". It can further lead to occupational dysfunction, loss of motor ability, social inactiveness, clouded consciousness and difficulty in recognizing the relationships[1].

Approximately, 10% of adults older than 65 years and 50% of adults older than 90 years have dementia[2]. The most common cause of dementia is Alzheimer's Disease (AD). It is a progressive neurodegenerative disorder, associated with loss of neurons in distinct brain areas. Factors that directly contribute to the advancement of memory impairments or more specifically Schizophrenia and Alzheimer's disease are age, stress and emotions[3]. Degeneration of cerebral neurons is one of the leading causes of dementia in elderly people which not only hampers their day to day life but also destroys their ability to perform their tasks by themselves. Constant physical, emotional and economic pressure is experienced by family members and caregivers as a result of this deteriorating condition. In elderly people, oxygen-free radicals are responsible for the process of age-related decline in cognitive performance, which may further lead to the development of AD[4].

Some positive approaches for the management of AD can be: reducing oxidative stress by antioxidants, protecting brain inflammatory lesions using anti-inflammatory drugs and facilitating cholinergic transmission with anticholinesterase[5,6]. Memory impairment can be induced by many methods in experimental animals. Using scopolamine, which is a muscarinic antagonist and streptozotocin, which depletes energy metabolism in the brain can be two of the well-established models in biomedical research.

A wide variety of drugs are available in the market to cure the disease, still, there is a dire need of finding new medication for the treatment of the disease. Elderly people suffer from more than one illness which puts them under tremendous physical and emotional pain. Along with that, it renders a heavy medical debt on their shoulders. Combination medication will help them take off a little load by engendering lower costs of treatment.

Selegiline, a selective monoamine oxidase (MAO)-B inhibitor, is the most accepted form of therapy in treating Parkinson's disease. Studies conclude that selegiline also improves episodic memory and learning in patients with Alzheimer's disease[7]. The piracetam like nootropic compounds, that exhibit cognition-enhancing properties are also capable of achieving the reversal of amnesia. Unfortunately, this drug has no defined mechanism of action. The present study was therefore designed to investigate the combined neuroprotective effect of selegiline with piracetam on memory impairment induced by scopolamine and STZ in rats and mice in the MWM test. The activity of AChE and oxidative stress parameters viz., MDA and GSH were also measured in the cerebral cortex and hippocampus of the animal brain. Gene expression was also performed by RT-PCR and western blot test.

MATERIALS AND METHODS

Study area: The study was carried out at the Hygia Institute of Pharmaceutical Education and Research, Lucknow, India from February, 2018-2019.

Chemicals: Scopolamine, streptozotocin, selegiline, donepezil, sodium chloride, potassium chloride, magnesium chloride, calcium chloride, chloral hydrate, phosphate buffer, o-phosphoric acid, n-butanol, EDTA, sodium dihydrogen phosphate, disodium hydrogen orthophosphate dehydrate, hydrochloric acid, TBA, TCA, DTNB and Folin-Ciocalteu's hydrochloric acid were purchased from Sigma-Aldrich (St. Louis, USA). Piracetam (Nootropil®) was purchased from UCB India Pvt. Ltd. (Mumbai, India). Trizol reagent, chemiluminescence (ECL) kit and cDNA Reverse Transcription Kit were purchased from Thermo Fischer Scientific (Mumbai, India).

Animals: Adult Swiss albino mice weighing 22-30 g and adult Wistar albino rats weighing 210- 240 g were used in the study. The animals were obtained from the Laboratory Animal Services Division of the Indian Institute of Toxicology Research (IITR), Lucknow, India. A polyacrylic cage of dimension 22.5×37.5 cm was used to contain the animals. Standard housing conditions were maintained by keeping the room temperature 24-27°C and humidity was 60-65%. A 12 hrs light and dark cycle were also maintained along with it. The food supply was limited to 1 h before the behavioral studies but was available for the rest of the time. The procedures described were reviewed and allowed by the Institutional Animal Ethics Committee (IAEC), Indian Institute of Toxicology Research, Lucknow, India.

Experimental design: Experimental Models of Memory Impairment: Albino rats and mice were taken up for the study. In albino rats (Wistar strain), the memory impairment was persuaded by intraperitoneal (i.p.) administration of scopolamine whereas, in the Swiss albino mice, it was induced by intracerebral (i.c.) injection of STZ.

Scopolamine-induced memory impairment in rats: After 30 min of the scopolamine injection, the rats were presented for behavioral studies. The solution was prepared by dissolving 1.4 mg mg kg^{-1} of scopolamine, a muscarinic receptor antagonist, in normal saline of strength 0.9% NaCl. The injection was given 1 mL mg kg^{-1} b.wt., i.p.[8].

Experimental protocol for drug administration for scopolamine-induced memory impairment in rats: Animals were randomly divided into six groups containing four animals each. Group 1 (control) rats were treated with vehicle for 7 days. Groups 2 and 3 rats were treated with donepezil (5 mg mg kg^{-1} b.wt., p.o.) and piracetam (100 mg mg kg^{-1} b. wt., i.p.), respectively, for 7 days. Group 4 rats were treated with selegiline (0.49 mg kg^{-1} b.wt., p.o.) for 7 days. Group 5 rats were treated with a combination of selegiline with piracetam for 7 days. On 7th-day scopolamine (i.p.) was administered in each group.

Intracerebral (i.c.) STZ-induced memory impairment in mice: The sub-diabetogenic dose of streptozotocin imparted centrally produces memory impairment in swiss albino mice[9]. STZ was dissolved in freshly prepared artificial CSF (aCSF) and administered slowly by i.c. route (0.5 mg mg kg^{-1}, 10 µL). To administer intracerebrally, the following procedure was implemented, chloral hydrate (300 mg mg kg^{-1}) was used to anesthetize the mice followed by a midline sagittal incision made through the scalp. Through the incision, a 100 µL Hamilton syringe was inserted (2.5 mm depth), affixed to a 27-gauge hypodermic needle. The site of injection was 2 mm from either side of the midline on a line drawn through the anterior base of the ears according to the method of Haley and McCormick[10], with a repeat dose after 48 hrs.

Experimental protocol for drug administration for STZ-induced memory impairment in mice: Animals were randomly divided into seven groups containing four animals each. Group 1 (control) mice were treated with vehicle for 21 days. Group 2 mice were injected with aCSF (i.c.) (the vehicle of STZ) on days 1 and 3 and treated with vehicle for 21 days. Group 3 mice were injected with STZ on days 1 and 3 and treated with vehicle for 21 days. Group 4 mice were injected with STZ on days 1 and 3 and treated with donepezil (5 mg mg kg^{-1} b.wt., p.o.) for 21 days. Group 5 and 6 mice were injected with STZ on days 1 and 3 and treated with piracetam (100 mg mg kg^{-1} b.wt., i.p.) and selegiline (0.49 mg mg kg^{-1} b. wt., p.o.) respectively, for 21 days. Group 7 mice were injected with STZ on days 1 and 3 and treated with a combination of selegiline with piracetam for 21 days.

Evaluation of memory function in animals

Morris water maze test: The Morris water maze is a test used to detect the behavioral task that is based on hippocampal-dependent learning. It contains a circular black pool that is 120 cm in diameter, 50 cm in height and 30-50 cm in depth. Water was filled in it till 30 cm and maintained at 26±2°C. Directions were marked on the edges namely, North (N), South (S), East (E) and West (W). The starting point was fixed to the SW quadrant in the pool for the entire trial. A round black colored platform was placed below the water tank at a distance of 1 cm, in the middle of the NE quadrant. The water inside of the pool was colored using a black dye (non-toxic) to hide the visibility of the platform. The trial was conducted for 5 days in a row for animals and 3 trials per day for mice, to train them in the maze. The total time given to rodents for finding the platform was 90 sec (Cut-off time) and the time for it to stay on it was 30 sec. The latency time to reach the hidden platform was noted after each trial and the mean latency time was calculated. A marked decrease in that from the 1st session can be regarded as a successful attempt[11].

Brain tissue preparation: The rats and mice were anesthetized using chloral hydrate (300 mg mg kg^{-1} b.wt.) upon which the decapitation was performed. The brain was extracted from the dorsal side of the skull but cutting it open. The extracted brain was then washed with cold normal saline on ice. The brain areas that were required, the hippocampus and the cerebral cortex, were isolated. An Ultra-Turrax T25 (USA) homogenizer was used to prepare the 10% w/v homogenized brain samples. The speed was maintained at 9500 rpm and a 0.03 M sodium phosphate buffer of pH 7.4 was used. The tissues thus extracted were used to measure the levels of MDA, GSH and AChE[12].

Preparation of aCSF: aCSF is prepared by dissolving 147 mM NaCl, 2.9 mM KCl, 1.6 mM $MgCl_2$, 1.7 mM $CaCl_2$ and 2.2 mM dextrose in distilled water[13].

Estimation of MDA level: Tissue homogenate was mixed with 30% Trichloroacetic Acid (TCA), 5N HCl followed by the addition of 2% Thiobarbituric Acid (TBA) in 0.5N NaOH. The

mixture was heated for 15 min at 90°C and centrifuged at 12,000×g for 10 min. The pink color of the supernatant was measured at 532 nm using an ELISA plate reader (BioTek, USA). MDA concentration was expressed as nmol mg^{-1} protein[14].

Estimation of GSH level: A yellow chromophore was obtained by the reaction of GSH and Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid). Spectrophotometric analysis was considered for the chromophore[15]. The brain tissue that was extracted earlier, was mixed with 10% TCA (equal amount). The mixture was centrifuged at 2,000×g for 10 min and maintained at 4°C. From the sample, 1 mL was taken and again mixed with 2 mL of phosphate buffer pH 8.4, 0.5 mL of DTNB and 0.4 mL of doubled-distilled water. The sample was vigorously shaken in a vortexer. An ELISA plate reader (BioTek, USA) was used to measure the absorbance at 412 nm and the concentration of GSH was expressed in µg mg^{-1} protein[14,16].

Estimation of AChE level: Ellman's method of estimation was used to measure the concentration of acetylcholinesterase, a cholinergic marker[17]. The homogenate of AChE was mixed with 0.1 mL DTNB (Ellman's reagent) along with 2.7 mL of phosphate buffer and was incubated for 5 min. To the above-incubated mixture, 0.1 mL of freshly prepared acetylcholine iodide was added (pH 8). An ELISA plate reader (BioTek, USA) was used to estimate the kinetic profile of enzymes activity at 412 nm at intervals of 15 sec. AChE activity was expressed as µmol min^{-1} (mg protein)[18].

RT-PCR (Reverse transcription-polymerase chain reaction) Analysis: Total RNA was extracted from hippocampal tissue using the Trizol reagent according to the manufacturer's instructions. For RT-PCR, a high-capacity cDNA Reverse Transcription Kit was used. The quantitative RT-PCR was performed using the Thermo Scientific Luminaris Color HiGreen qPCR Master Mix. Also, mRNA-specific primers for Bax, Bcl-2, Caspase-3 and β-actin as a housekeeping gene were done. Quantitative RT-PCR reactions were done in the Mastercycler® ep realplex and after data analysis, relative gene expression was calculated according to Livak and Schmittgen[19].

Western blot analysis: Brain samples were obtained from the hippocampal tissue of mice 24 hrs. Hippocampal tissue was homogenized with lysis buffer (50 nM L^{-1} NaCl, 1 mM L^{-1} EDTA, 1% Triton X-100, 0.5% SDS, 0.5% sodium deoxycholate and 20 mM L^{-1} Tris HCl, pH 7.5) and centrifuged at 15,000×g for 20 min. Protein samples (50 µg) per lane were run on a polyacrylamide gel, transferred to a PVDF membrane (Millipore, Billerica) and blocked with 5% milk solution (non-fat dry milk in PBST) for 2 hrs. The membrane was incubated at 4°C overnight with the following specific antibodies: rabbit polyclonal anti-ChAT (1:1000, Cell Signaling Technology, Boston, MA, United States), phospho-Akt (1:1000, Cell Signaling Technology), Akt (1:1000, Cell Signaling Technology), phosphoCREB (1:1000 dilution), CREB (1:1000 dilution), ERK1/2 (1:1000 dilution), phospho-ERK1/2 (Thr202/Thr204) (1:1000, dilution), BDNF (1:1000, dilution), NR1 (1:1000, dilution), NR2B (1:1000, dilution), GAP-43 (1:1000, dilution) and mouse monoclonal anti-β-actin (1:10000, dilution). After washing with TBST five times, the membranes were then incubated with the corresponding conjugated anti-rabbit IgG (1:10000, dilution) at room temperature for 1 h. Immunoreactive proteins were quantified using enhanced chemiluminescence (ECL) kit and the relative density of the protein bands was scanned using a LAS 4000 Fujifilm imaging system (SelectScience, Corston, Bath, UK) and analyzed by densitometric evaluation using Quantity-One software.

Statistical analysis: Results were expressed as mean±S.E.M. The statistical significance of difference among the different groups was determined by one-way ANOVA followed by Bonferroni's post hoc test using GraphPad Prism 5 software (GraphPad Inc., California, USA) for MWM test, MDA, AChE and GSH estimation. The significance level for Bonferroni's multiple comparison tests was set to 0.05 for 3 or more groups and $p<0.05$ was considered statistically significant. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison assay for RT-PCR analysis and western blot analysis.

RESULTS

Effect of combined administration of selegiline with piracetam on scopolamine-induced memory impairment in the mwm test: As shown in Fig. 1, the combined effects of selegiline with piracetam on spatial memory impairment were evaluated after the administration of scopolamine, using the Morris water maze test. In the control group, there is a significant decrease in escape latency time from session 1-5 which indicates successful learning. There is no significant decrease in latency time was found during all the session in scopolamine treated animals. On oral administration of selegiline showed a significant decrease in latency time in the

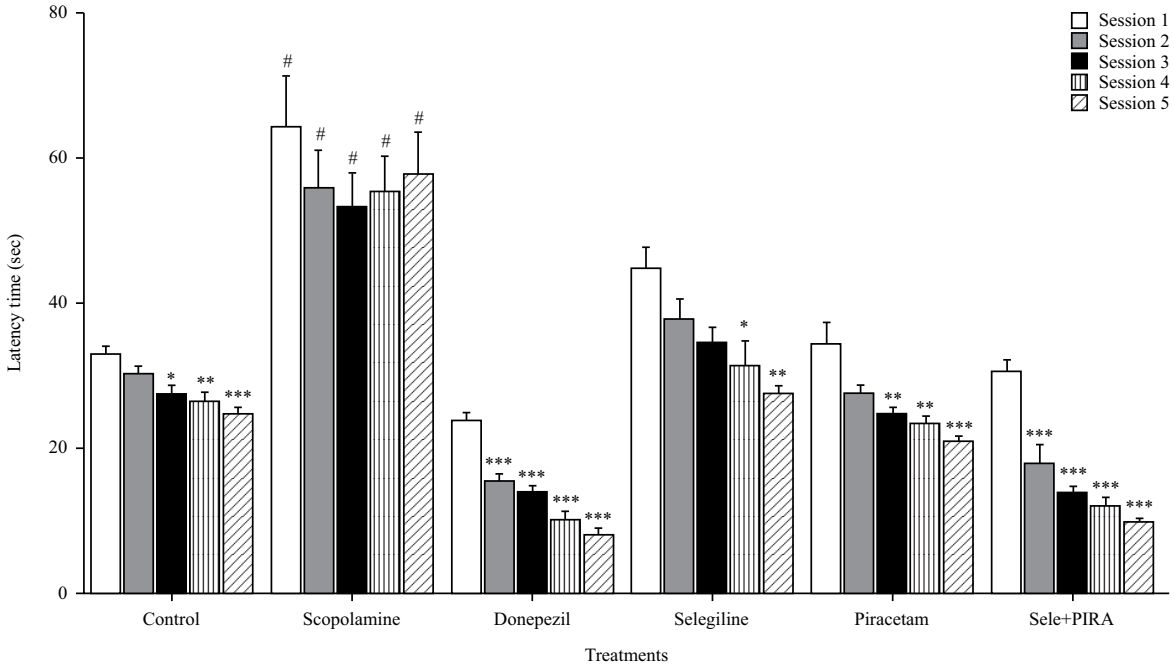

Fig. 1: Effect of combined administration of selegiline with piracetam on scopolamine-induced memory impairment in the MWM test

Data are expressed as mean escape latency time (s) ±S.E.M. (n = 4), *Significant difference (*p<0.05, **p<0.01 and ***p<0.001) in compare to the session 1, whereas, #p denotes no significant decrease in escape latency time

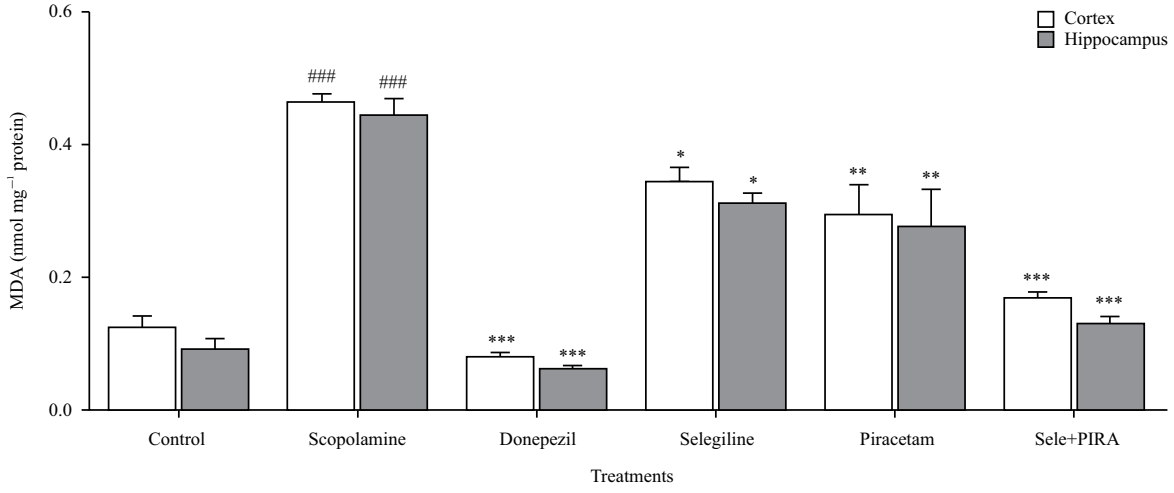

Fig. 2: Effect of combined administration of selegiline with piracetam on MDA level in scopolamine-induced memory impaired rat brain

Data are expressed as mean MDA level (nmol mg^{-1} protein) ±S.E.M. (n = 4), Significant difference ###p<0.001 scopolamine vs control group, **p<0.01 piracetam, *p<0.05 selegiline, ***p<0.001 selegiline+piracetam, ***p<0.001 donepezil vs scopolamine group

4th and 5th sessions as compared to session 1. Pretreatment with piracetam significantly decreased the latency time to reach the hidden platform in the MWM test. On combined administration of selegiline with piracetam significantly reduced the latency time from session 2 onwards. Treatment with donepezil caused a significant decrease in latency time during the 4th and 5th sessions in comparison to the first session.

Effect of combined administration of selegiline with piracetam on MDA level in scopolamine-induced memory impaired rat brain: As shown in Fig. 2, on the 5th day, MDA

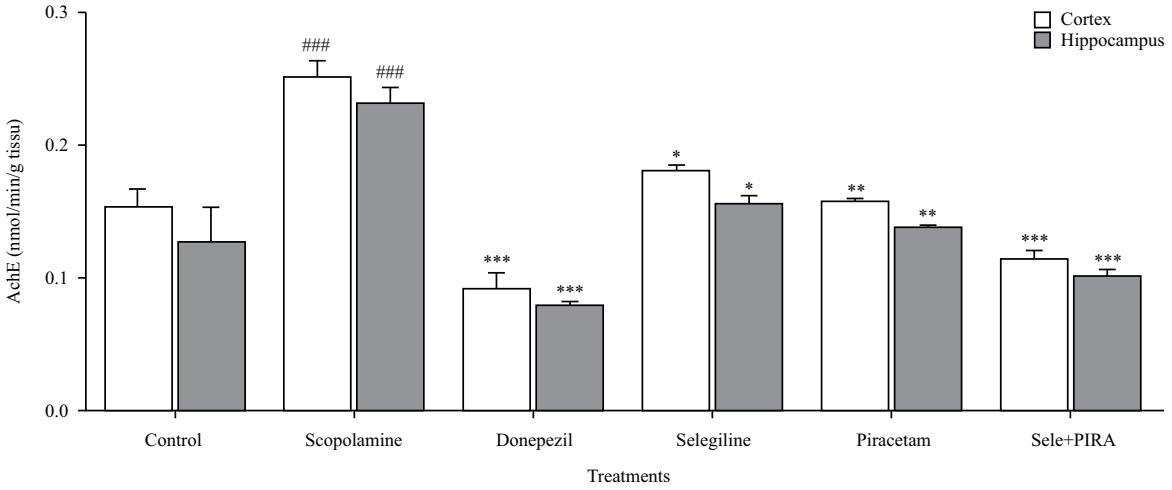

Fig. 3: Effect of combined administration of selegiline with piracetam on AChE level in scopolamine-induced memory impaired rat brain

Data are expressed as mean AChE level (μmol min^{-1} g^{-1} tissue) ±S.E.M. (n = 4). Significant difference (###p<0.001) scopolamine vs control group, **p<0.01 piracetam, *p<0.05 selegiline, ***p<0.001 selegiline+piracetam, ***p<0.001 donepezil vs scopolamine group

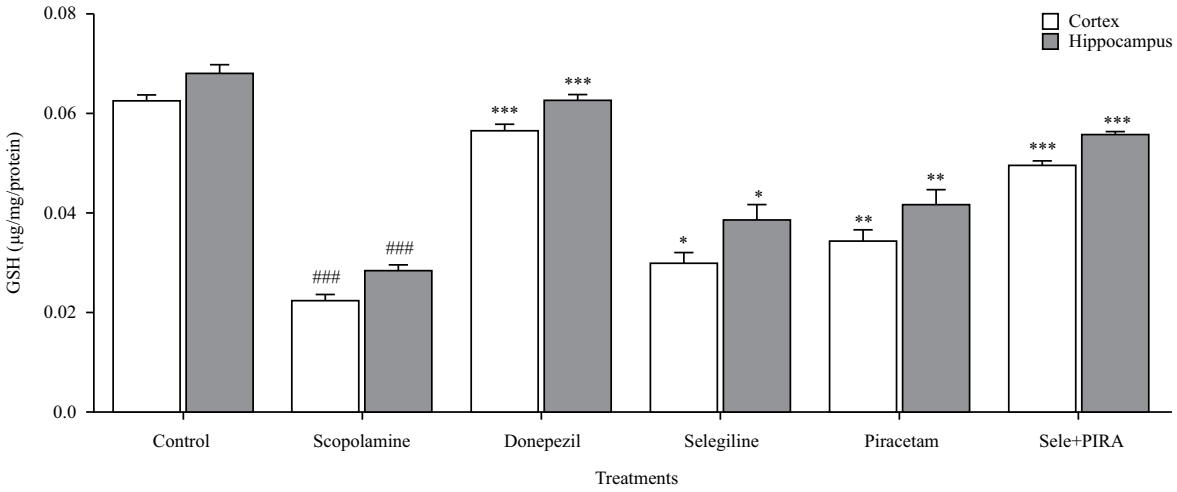

Fig. 4: Effect of combined administration of selegiline with piracetam on GSH level in scopolamine-induced memory impaired rat brain

Data are expressed as mean GSH level (μg mg^{-1} protein) ±S.E.M. (n = 4). Significant difference ###p<0.001 scopolamine vs control group, **p<0.01 piracetam, *p<0.05 selegiline, ***p<0.001 selegiline+piracetam ***p<0.001 donepezil vs scopolamine group

levels were measured in the brain after scopolamine administration. The levels of MDA increased in scopolamine-induced rats (###p<0.001) than the controlled group rats. Both the test drugs selegiline (*p<0.05) and piracetam (**p<0.01) versus the scopolamine groups, showed a significant decrease in MDA levels. The combined effects of both the test drugs selegiline with piracetam (***p<0.001) showed a marked decrease in MDA levels as compared to scopolamine treated groups, which was equivalent to standard drug donepezil (***p<0.001).

Effect of combined administration of selegiline with piracetam on AChE level in scopolamine-induced memory impaired rat brain: As shown in Fig. 3, AChE levels (μmol/min/g tissue) in the brain were measured on the 5th day after scopolamine administration on different brain parts which were cortex and hippocampus. The level of AChE is increased notably in scopolamine treated (###p<0.001) rats when compared with controlled group rats. Both the test drugs selegiline (*p<0.05) and piracetam (**p<0.01) showed a significant decrease in the AChE level as compared

scopolamine group. The combined administration of selegiline with piracetam (***p<0.001) showed a decrease in AChE as compared to a scopolamine treated rat which is comparative to the standard drug donepezil (***p<0.001).

Effect of combined administration of selegiline with piracetam on GSH level in scopolamine-induced memory impaired rat brain: As shown in Fig. 4, GSH was estimated on the 5th session after scopolamine administration on different brain parts which were cortex and hippocampus. A decrease in the levels of GSH was observed in the scopolamine-induced group ($^{###}$p<0.001) as compared to the control group. Both the test drugs selegiline (*p<0.05) and piracetam (**p<0.01) showed a marked increase in the GSH level as compared to the scopolamine group. The combined administration of selegiline with piracetam (***p<0.001) showed a significant increase in GSH level as compared to the scopolamine treated group which is equivalent to the standard drug donepezil (***p<0.001).

Effect of combined administration of selegiline with piracetam on STZ-induced memory impairment in the MWM test: As shown in Fig. 5, the combined effect of selegiline with piracetam on spatial memory impairment was evaluated from 21 days after the first's dose of STZ administration using Morris water maze. As compared to session 1, the control group showed a significant decrease in escape latency time in the 3rd, 4th and 5th sessions. In comparison to the 2nd, 3rd and 4th session, there was no marked decrease in escape latency time among all sessions. In aCSF treated group in there was a significant decrease in escape latency time in the 3rd, 4th and 5th session as compared to the 1st session. In comparison to the 2nd, 3rd and 4th session, there was no marked decrease in escape latency time among all sessions.

In the STZ group throughout the training sessions, i.e. 1st-5th session there was no notable decrease, in comparison to all sessions. Following the oral administration of selegiline, a significant decrease in escape latency time for the 4th and 5th sessions was recorded in comparison to the 1st session. When compared to the 2nd session, there was a significant decrease in the 5th session only and there was no significant decrease in escape latency time in the 3rd, 4th session as in comparison to all sessions. Intraperitoneal administration of piracetam showed a marked decrease in escape latency time during the 3rd, 4th and 5th sessions when compared to the 1st session.

In comparison to the 2nd session, there was a significant decrease in the 5th session only and there was no decrease in escape latency time in the 3rd and 4th session as in comparison among all sessions. Whereas, the combined

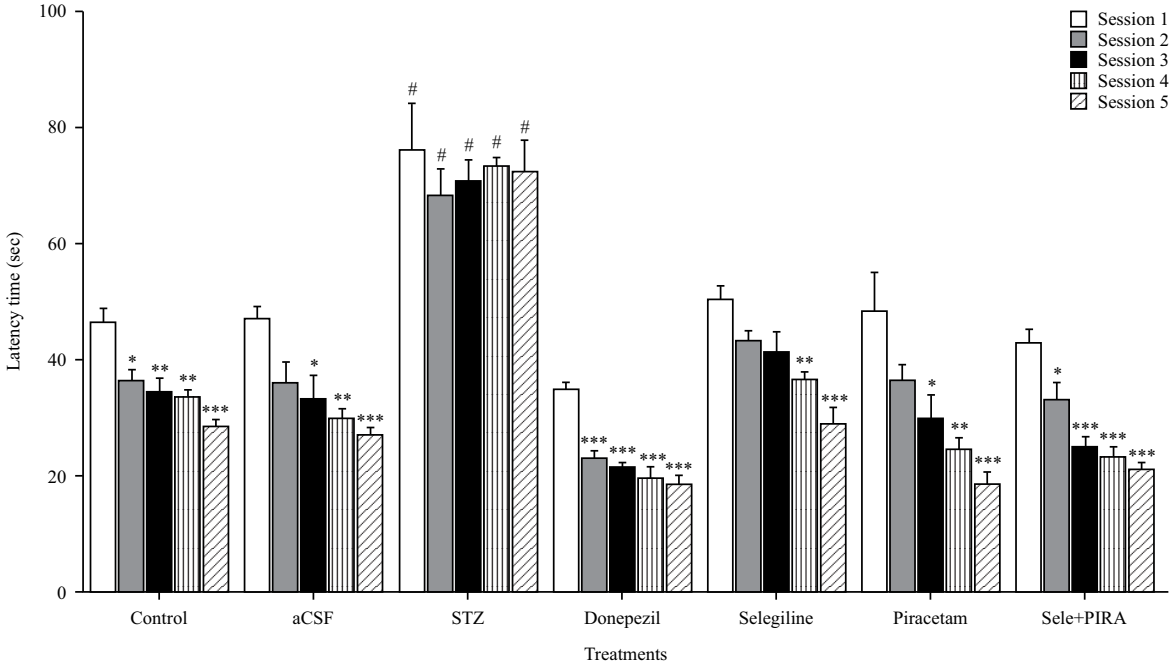

Fig. 5: Effect of combined administration of selegiline with piracetam on STZ-induced memory impairment in the MWM test

Data are expressed as mean escape latency time (s) ±S.E.M. (n = 4), *Significant difference (*p<0.05, **p<0.01 and ***p<0.001) in compare to the session 1, whereas, $^{#}$p denotes no significant decrease in escape latency time

administration of selegiline with piracetam showed a decrease in escape latency time 2nd, 3rd, 4th and 5th session as compared to the 1st session. On comparing the 2nd session with the 4th and 5th sessions, there was a significant decrease in the 4th and 5th sessions only. There was no significant decrease in escape latency in the 3rd and 4th sessions in comparison among all sessions. Donepezil, used as standard, showed a significant decrease in escape latency time 2nd, 3rd, 4th and 5th sessions in comparison to 1st session. In comparison to the 2nd session, there was a significant decrease in the 4th and 5th sessions only. In comparison to the 3rd session, there was a significant decrease in the 5th session only. In comparison to the 4th session, there was no significant difference among all sessions.

Effect of combined administration of selegiline with piracetam on MDA level in STZ-induced memory impaired mice brain: As shown in Fig. 6, The MDA levels (nmol mg^{-1} protein) in the brain were measured on the 25th day after the first dose of STZ. The level of MDA increased significantly in *i.c.* STZ treated mice ($^{###}p<0.001$) as compared to control and aCSF treated. On the other hand, both the test drugs showed a significant decrease in MDA level as compared to STZ treated group selegiline ($^{*}p<0.05$) and piracetam ($^{**}p<0.01$). The combined administration of selegiline with piracetam ($^{***}p<0.001$) yielded a marked decrease in MDA when compared to that in STZ treated mice, which are equivalent to the standard drug donepezil ($^{***}p<0.001$).

Effect of combined administration of selegiline with piracetam on AChE level in STZ-induced memory impaired mice brain: As shown in Fig. 7, on the 25th day, after the first dose of STZ, the AChE levels (µmol min^{-1} g^{-1} tissue) in the brain were measured. The levels of AChE were found to have increased in i.c. STZ ($^{###}p<0.001$) treated mice as compared to that in controlled groups and also in aCSF treated groups. Both the test drugs, selegiline ($^{*}p<0.05$) and piracetam ($^{**}p<0.01$) marked a decrease in AChE levels as compared to STZ treated groups. The combined effects of selegiline with piracetam ($^{***}p<0.001$) yielded a significant decrease in AChE as compared to STZ treated mice which were similar to the standard drug donepezil ($^{***}p<0.001$).

Effect of combined administration of selegiline with piracetam on GSH level in STZ-induced memory impaired mice Brain: As shown in Fig. 8, GSH estimation was performed on the 25th day, after the first dose of STZ was administered. There was a marked decrease in the levels of GSH in the STZ treated groups ($^{###}p<0.001$) as compared to the controlled group and aCSF treated groups. On the other hand, both the test drugs showed a significant rise in GSH level as compared to STZ treated group selegiline ($^{*}p<0.05$) and piracetam ($^{**}p<0.01$). The combined administration of selegiline with piracetam ($^{***}p<0.001$) yielded a significant rise in GSH level as compared to STZ treated mice which were similar to the standard drug donepezil ($^{***}p<0.001$).

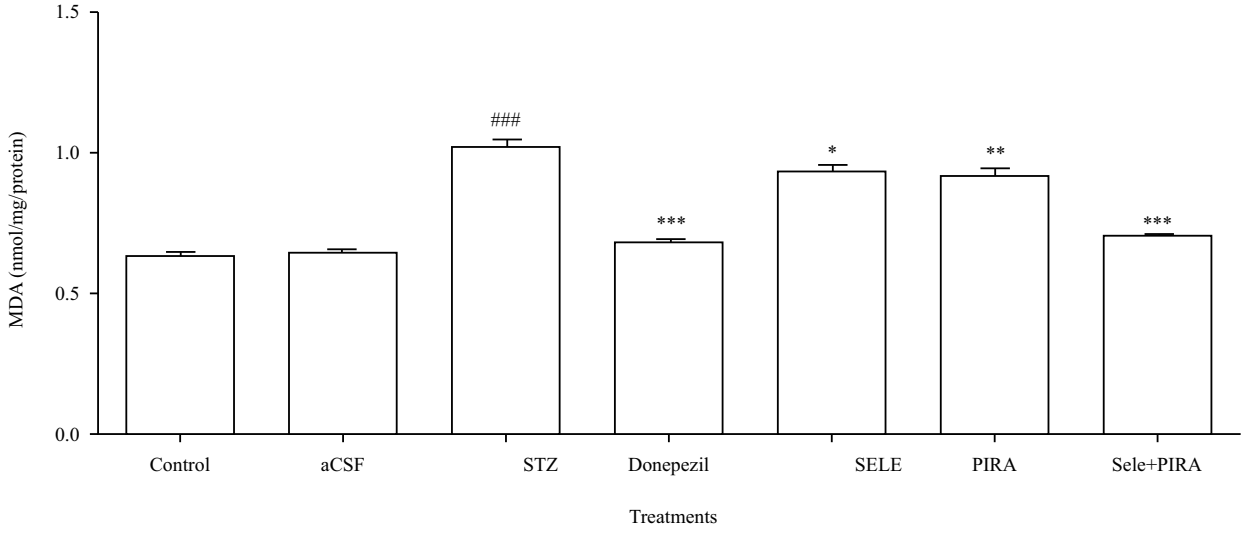

Fig. 6: Effect of combined administration of selegiline with piracetam on AChE level in STZ-induced memory impaired mice brain

Data are expressed as mean AChE level (µmol/min/g tissue) ±S.E.M. (n = 4). Significant difference (###p<0.001) STZ vs control and aCSF group; **p<0.01 piracetam; *p<0.05 selegiline; ***p<0.001 selegiline+piracetam; ***p<0.001 donepezil vs STZ group

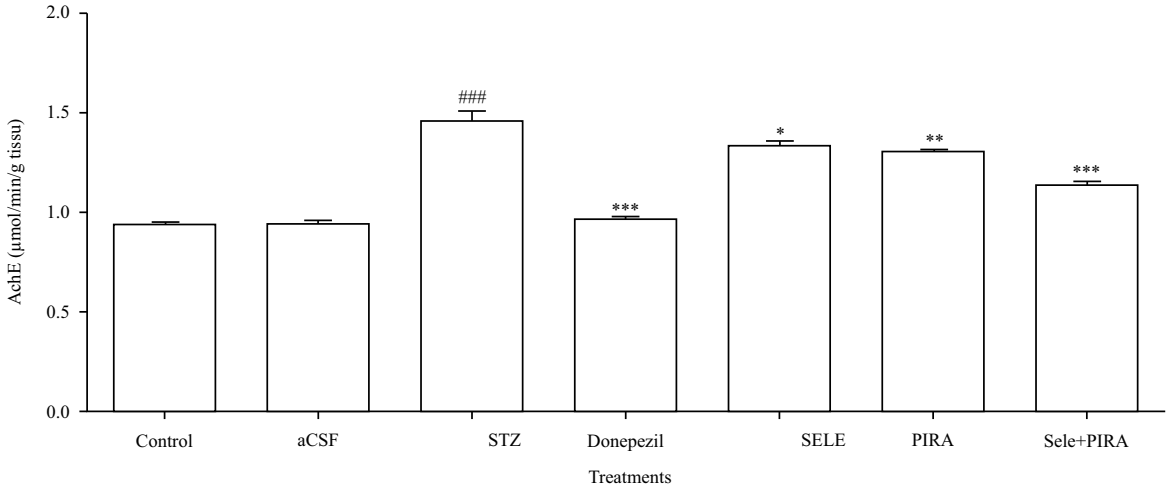

Fig. 7: Effect of combined administration of selegiline with piracetam on AChE level in STZ-induced memory impaired mice brain.
Data are expressed as mean AChE level (µmol/min/g tissue) ±S.E.M. (n = 4). Significant difference (###p<0.001) STZ vs control and aCSF group; **p<0.01 piracetam; *p<0.05 selegiline; ***p<0.001 selegiline+piracetam; ***p<0.001 donepezil vs STZ group

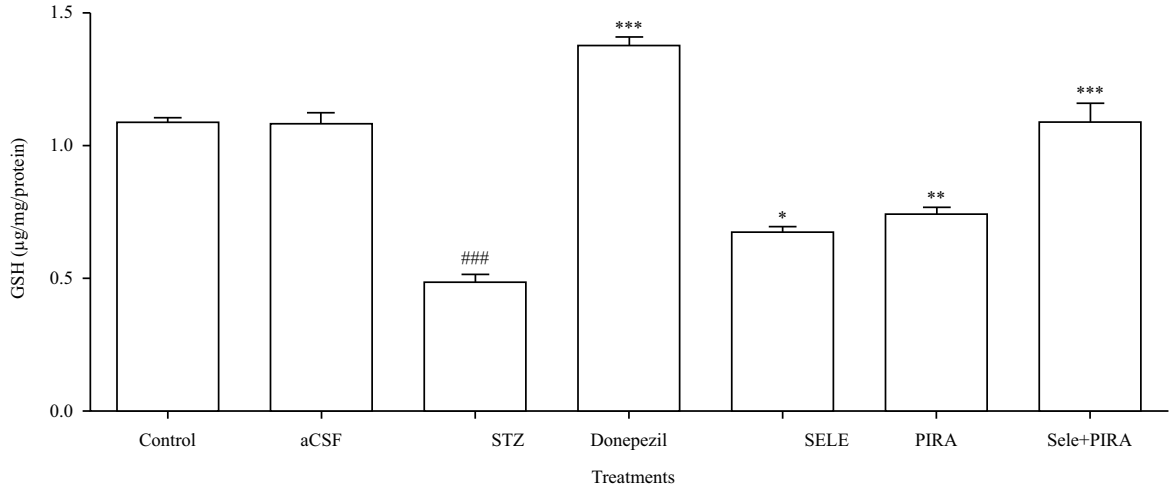

Fig. 8: Effect of combined administration of selegiline with piracetam on GSH level in STZ-induced memory impaired mice brain
Data are expressed as mean GSH level (µg mg-1 protein) ±S.E.M. (n = 4). Significant difference ###p<0.001 STZ vs control and aCSF group. **p<0.01 piracetam; *p<0.05 selegiline; ***p<0.001 selegiline+piracetam ***p<0.001 donepezil vs STZ group

RT-PCR and western blot analysis: Expressions of Bcl-2, Bak and Bax mRNA are demonstrated in Fig. 9 for excluding the variations due amount and nature of RNA, the results recorded were adjusted according to the expression of GAPDH. Hippocampal tissue in the STZ group was indicated by considerably amplified the levels Bak and Bax and significantly declined the levels of Bcl-2. Their levels were appreciably inverted in donepezil, selegiline, piracetam and selegiline with piracetam groups. Selegiline with piracetam group showed the best results which were almost comparable to healthy control group Fig. 9a. In comparison to the piracetam group, selegiline with piracetam demonstrated a more significant effect in the up-regulation of Bcl-2 protein and down-regulation of p53 protein. In the mitochondrial pathway cell apoptosis chiefly involves the Bcl-2 gene. Bcl-2 and Bax (both belong to the Bcl family) control the secretion of proapoptotic factors from mitochondria. These findings indicated that selegiline with piracetam in combination could exert its memory enhancement by interacting with these proteins. This analysis was carried out for recording the memory enhancer activity of selegiline with piracetam. In the hippocampus region, NR1, NR2B and GAP-43 levels in STZ treated mice were significantly lower than in the control group. In contrast, the selegiline with piracetam group had significantly higher NR1,

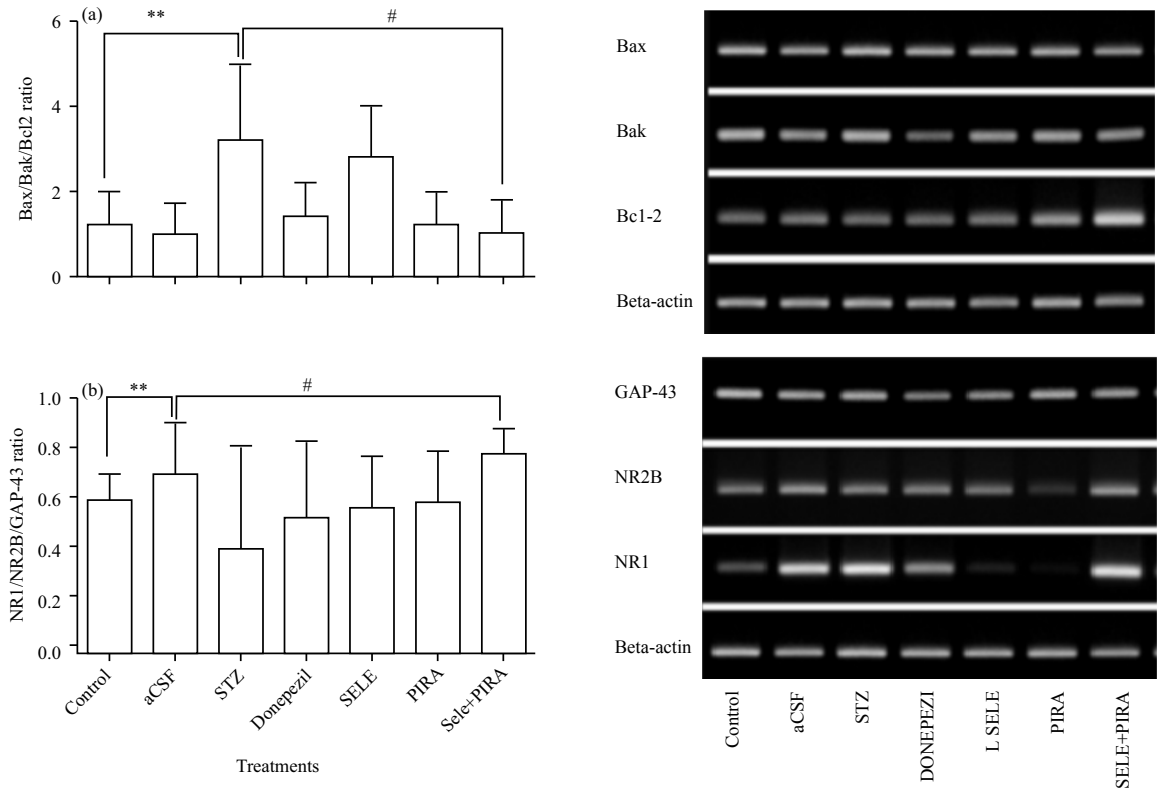

Fig. 9: Protein expression of Bcl-2, Bak and Bax in the hippocampus of the experimental mice in RT-PCR analysis (a) and (b) expression of NR1, NR2B and GAP-43 with western blot analysis

Statistical analysis was carried out by 1-way ANOVA and after that Dunnett's multiple comparison assay was done. *Denotes significance when compared to the control group

NR2B and GAP-43 protein levels in the hippocampus than did the STZ group. As shown in Fig. 9b, protein expressions of NR1 and NR2B were augmented by almost two times and five times in the hippocampus of mice of the STZ group as compared to the mice of the control group, whereas their expressions were appreciably reduced in selegiline, piracetam and selegiline with piracetam groups but best rest was shown in selegiline with piracetam mice. NR1 expression was amplified by almost six-folds in STZ treated mice while selegiline with piracetam group treated mice partially prevented this effect. NR1, NR2B and GAP-43 proteins lead to a reduction of brain cell damage. Selegiline with piracetam group showed a significant effect by shielding against STZ-induced brain damage.

DISCUSSION

The present study evaluates the neuroprotective effect of co-administration of selegiline (0.49 mg mg kg^{-1}) with piracetam (100 mg mg kg^{-1}) in scopolamine and STZ-induced memory impairment in animals. Screening methods such as the Morris water maze was performed to screen the effect of the drugs. Furthermore, using rats and mice brain homogenate MDA, GSH, AChE level, RT-PCR and western blot analysis were performed.

Reports suggested that amnesia can be induced by a single i.p. administration of scopolamine or i.c. injection of STZ[8,9]. To check the combined effect of selegiline with piracetam animals were pretreated for 7 days and the last day amnesia was induced by scopolamine in scopolamine model and for 21 days and on days 1 and 3 amnesia was induced by STZ in STZ model.

The finding of the current studies showed that selegiline with piracetam prevented STZ and scopolamine-induced memory impairment in all paradigms of behavioral studies in MWM test in animals, these results were correlated with the previous reports of Abbott and Means[20].

In the Morris water maze, mean time spent by the animals in the target quadrant was significantly improved as compared to individual therapy, which was correlated with

the previous reports of Jawaid *et al.*[21-23]. These results suggest an improvement in the retrieval of memory in rats and mice as compared to individual treatment.

One of the most promising therapies to treat a cognitive deficit in AD is to increase the cholinergic activity and inhibition of AChE enzyme[24]. In this study, selegiline with piracetam significantly reduced the level of AChE which, in turn, increased the availability of acetylcholine for improving memory, increased GSH level and marked reduction in MDA level which were correlated with the previous reports of Zaki *et al.*[25]. This study indicated that the combined treatment of selegiline with piracetam had a greater effect on the improvement of spatial learning and memory impairment in Alzheimer's disease model as compared to individual treatment[26]. The main action of the piracetam is mainly unidentified but piracetam was frequently thought to adapt cholinergic receptors in the hippocampus to amplify the special effects of acetylcholine, which is well-known for enhancing memory as it is profoundly implicated with the programming of new memories[27].

In neurodegenerative disease, brain is especially vulnerable to oxidative damage, due to the imbalance between the generation of oxygen free radicals and antioxidant defense system. In this study, MDA level was elevated while a decrease in GSH level was observed in scopolamine and STZ-treated group which indicated oxidative damage in the brain. On the other side, pretreatment with selegiline, piracetam and their combination significantly reversed the endogenous antioxidant enzymes and decreased oxidative damage. These results were consistent with the previous reports of Singh *et al.*[28]. Standard donepezil significantly increased the level of GSH and reduced the MDA level in the brain of mice suggesting its antioxidant action as earlier reported by Alikatte *et al.*[29].

In the present study, Bax and Bak mRNA (proapoptotic) were down-regulated, while Bcl-2 mRNA and protein which are antiapoptotic were up-regulated in the selegiline with piracetam group opposite to the STZ group. Also, p53 regulates the Bcl-2 family proteins its expression in the given experiment was appreciably reduced in selegiline with piracetam group opposite effect was recorded in the STZ groups. Reported findings suggest that the interaction of the death receptor and its ligand e.g., the interaction of NR1/NR2B are important for initiating apoptosis (extrinsic pathway). Prior studies have shown that suppressing NR1, NR2B and GAP-43 proteins lead to a reduction of brain cell damage.

Hence, these findings suggest that the pretreatment with combined administration of selegiline with piracetam may act synergistically to enhance memory and learning in a scopolamine and STZ-induced amnesia model by reversing the oxidative stress, decreasing the AChE level and suppressing NR1, NR2B and GAP-43 proteins in animal brain which may be the probable mode of actions for its beneficial effect as compared to the individual treatment. Thus, multi-drug therapy would be interesting to get the best response in the treatment of AD.

CONCLUSION

Oxidative stress, neuroinflammation and biochemical changes in the various brain regions induced by scopolamine and STZ can produce memory impairment linked to a significant increase in levels of AChE and MDA and decreased GSH. This is the first study to determine the effect of selegiline, piracetam and selegiline with piracetam as a neuroprotective agent. From the present study, it can be concluded that the administration of scopolamine and STZ cause a decline in memory function which is significantly ameliorated by the treatment of selegiline with piracetam. The combined effect of selegiline with piracetam was found to be therapeutically more effective than the individual administration. Thus, this can be set as a new therapeutic approach for the treatment of memory impairment. The drug can thus be utilized in the form of pharmaceutical after safety assessment for the treatment of dementia.

SIGNIFICANCE STATEMENT

This study exposed the neuroprotective effect of co-administration of selegiline with piracetam on scopolamine and streptozotocin-induced learning and memory impairment in animals. It showed a significant neuroprotective effect by decreasing the activity of AChE enzyme in various brain regions, increased acetylcholine level that helped to increase learning and memory. This study will help the researchers to know the synergistic effect of two drugs for the treatment of learning and memory that many researchers were not able to explore. Thus, a novel approach that co-administration of both the drug is effective and safe to treat a patient suffers from Alzheimer's disease may be arrived at.

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