Evaluation of memory-enhancing effect of flunarizine on active avoidance in experimental model of Alzheimer's disease through calcium homeostasis

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Abstract

Background: Alzheimer's disease is a chronic, neurodegenerative disease which involves complications in cognitive functioning leading to dementia. Flunarizine (FLN) is a selective, calcium channel blocker that is widely used in the treatment of migraine. Moreover, it was investigated that it can improve cognitive functioning by regulating calcium homeostasis. **Objective:** The present study aims to evaluate the memory-enhancing effect of FLN in scopolamine-induced dementia model. **Materials and Methods:** The present study was carried out on male Swiss-albino mice, where Alzheimer's type of dementia was induced by the administration of scopolamine. In the current investigation, the mice were divided into six groups and then test animals received FLN at the doses of 20 mg, 40 mg, and 80 mg after dosing with scopolamine at the dose of 3 mg/kg, intraperitoneally for 7 days. Active avoidance was assessed by the use of elevated plus maze, T-maze, and Morris Water Maze test. The biochemical markers assessed were acetylcholinesterase, catalase, and lipoxygenase activity. **Results:** FLN at the doses of 20 mg, 40 mg, and 80 mg showed significantly increased impedance in learning and memory with all the tests. **Conclusion:** The current study demonstrates significant memory-enhancing effect of FLN.

Key words: Alzheimer's disease, calcium channel blockers, calcium homeostasis, flunarizine

INTRODUCTION

Alzheimer's disease (AD) or simply Alzheimer's can be defined as a chronic, progressive, and neurodegenerative disease which involves degeneration of the cortical regions, specifically the frontal, and temporal regions. AD is represented by the presence of amyloid plaques and bizarre helical protein filaments known as neurofibrillary tangles in neurons.[1] It is a neurological disorder that causes complications with spatial memory, thinking, behavior, spatial learning, and orientation. It normally starts slowly but gets worse over time.[2,3] AD is not a normal part of aging. It leads to dementia, which involves various symptoms like problems with cognitive functions such as behavior, thinking, language, mood swings, disorientation, loss of motivation, and unable to manage self-care. AD makes up 60–70% of cases of dementia which is normally seen in people over 65 years of age.[1,3,4] Currently, there is no efficient treatment for AD, but novel therapies are able to delay its progress and promote the patient's ability to function.

Pathophysiology

The pathophysiology of AD is complicated as it involves various cellular, molecular, and physiological pathologies. Plaques and tangles are the two proteins, which are the main cause of AD. Amyloid plaques, which are formed by the clustering of β-amyloid fragments, produce a toxic effect on

nerve cells causing disruption in cell-to-cell communication. Tau proteins which play a major role in nerve cell internal support and transport system, change shape and align themselves into structures called neurofibrillary tangles which are toxic to cells.[5]

The formation of Aβ peptides by "amyloid-cascade hypothesis" causes the accumulation of Aβ plaques, which triggers the degeneration of neurons in sporadic and familial AD. Many genes either directly or indirectly enhance the risk for AD, as they all involved in the alteration of some stage of the formation or stability of Aβ. By applying the same logic, it can be said that calcium dyshomeostasis plays a central role in the pathophysiology of AD, as every gene involved in the progression of AD also alters some stage of calcium signaling. Therefore, calcium dyshomeostasis is involved in the degeneration of neurons in Alzheimer's.[6]

Role of Calcium Homeostasis in AD

Calcium channels play a major role in the alteration of resting potential leading to stimulation or inhibition of cell activity.[7] Calcium homeostasis, i.e., a dynamic equilibrium in the levels of calcium in cells is regulated by calcium channels, and any disturbance in this equilibrium can become the cause of many disorders. One such disorder is cognitive dysfunction, which includes problems with learning and memory, as cognitive function. Calcium-reliant processes resulting in gene expression and synaptic plasticity are associated with learning and memory.[8-11] Many investigators have proved that significant imbalance in calcium homeostasis can influence the accumulation of Aβ and the hyperphosphorylation of Tau, which are the primary cause of AD. Hence, calcium dyshomeostasis is a proximal cause of neurodegeneration in AD.

Role of Calcium Channel Blockers (CCB's) in the Treatment of AD

Calcium dyshomeostasis is mainly characterized by the increased intracellular calcium ion concentration and CCB's block excess calcium influx into the cells, thus preventing cell damage.[12,13] CCB's, along with their vasodilating action, show a significant effect on the betterment of cognitive abilities. Many scientists studied the therapeutic effect of CCB's on cognitive functioning and dementia in hypertensive patients. Many recent investigations showed that these drugs improved cognitive functioning and also delayed the onset and incidence of dementia, specifically of Alzheimer's type. Hence, CCB's have a good scope in the treatment of cognitive dysfunction and dementia in AD.[14]

Flunarizine (FLN)

FLN is a highly lipophilic, Class IV, selective, and CCB which also has the ability to block sodium channels, has shown its use in the treatment of a number of neurological and cerebrovascular disorders.[15,16] It is a diphenylmethylpiperazine derivative and is associated with antihistamine hydroxyzine.[17] FLN prevents cell damage in calcium overload by specifically blocking the excess influx into the cells. As it has the ability to cross blood-brain barrier efficiently and can cause a blockade of calcium influx during neurotransmission,[12] it can be used to treat disorders related to cognitive functions such as thinking, behavior, spatial memory, and spatial learning.[13]

Active Avoidance

Active avoidance is an operative procedure, where a specific reaction enables the animal to avoid suffering. It is a variant of a place avoidance task[18] which is used for the evaluation of spatial learning and memory in rats[19,20] and mice.[21,22] The apparatus consists of a dry and smooth, metallic circular field consisting of an unmarked to-be-avoided area, entering which the animal is suffered by a mild foot shock. The animal has to remember this area and should have continual locomotion[19] and navigation[23] to escape from shock.

MATERIALS AND METHODS

Materials

Experimental animals

The studies were performed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, on the male Swiss albino mice weighing 20–25 g, procured from Gentox Bioservices, Hyderabad. The study was carried out after getting approval from the Institutional Animal Ethical Committee. A week before the initiation of neurobehavioral studies, animals were acclimatized under standard laboratory conditions (12 h light/dark cycle, air-conditioned room at 25°C ± 2°C temperature) and fed with pellet diet and water *ad libitum*.

Preparation of doses

Different doses of FLN (20 mg, 40 mg, and 80 mg) were selected based on the acute toxicity studies data. The drug is administered orally by dissolving it in distilled water. The standard drug (Donepezil 3 mg/kg) was administered orally by dissolving it in distilled water. Dementia was induced by administering scopolamine hydrobromide (3 mg/kg) intraperitoneally (i.p) by dissolving it in water for injection. Every day fresh drug solutions were prepared before dosing.

Experimental design and treatment schedule

In the present study, the male Swiss albino mice were categorized into six groups with a minimum of six animals in each group and submitted to various interoceptive and exteroceptive models.
- Group-I: Normal control animals received distilled water in the dose of 10 ml/kg orally for 7 successive days.

- Group-II: Negative control animals received scopolamine hydrobromide in the dose of 3 mg/kg, i.p for 7 successive days
- Group-III: Positive control animals received donepezil in the dose of 3 mg/kg orally for 7 successive days and after 60 min of last dose administration, dementia was induced by injecting scopolamine hydrobromide (3 mg/kg, i.p)
- Group-IV, V and VI: The animals were pretreated orally with FLN in the dose of 20 mg (group IV), 40 mg (group V), and 80 mg (group VI) for 7 consecutive days, and after 60 min of the last dose administration, dementia was induced by injecting scopolamine hydrobromide (3 mg/kg, i.p) and retention memory was evaluated after 30 min of dementia induction using various exteroceptive models.

Methods

Neurobehavioral studies

Elevated plus maze

Elevated plus maze is a widely used test to evaluate the anxiety in experimental animals such as rats and mice. The apparatus consists of a four-armed maze with two arms crossing each other in the middle and the remaining two arms enclosed by walls and is elevated off the floor. The animals are placed at the junction of the four arms of the maze, facing an open arm. The entries or duration in each arm by each animal are observed and recorded for 5 min. The number of entries into each arm and the time spent by the animal in the open arms are recorded. The distance traveled, the number of entries into each arm and the time spent in each arm are calculated. Furthermore, the ratio of time spent in the open arms to the time spent in the closed arms is calculated. An increase in open arm activity represents anxiolytic behavior.[24]

T-maze

T-maze is a simple maze used to test anxiety and cognitive functioning in animals. It is used to evaluate learning, spatial memory, and spatial orientation. It consists of five segments, a starting, and a goal box. The rats are trained to run through the maze within 10 s without entering the sidearm by keeping food as the reward. The time taken to reach the goal box is recorded. Before the administration of the drug, the animals are trained to reach the goal box as soon as possible by moving to the correct segment in the t-maze. After the animals get trained, the time taken to reach the goal box by the drug-treated animals is compared with the time taken by the same animals before treating with the drug and control animals.[25]

Morris water maze

Morris water maze is an excellent test to evaluate spatial learning and memory. The apparatus is kept in a room with visual cues, and it consists of a circular pool of 180 cm diameter and 80 cm height, filled to a depth of 25 cm with colored water at room temperature. A movable platform is placed beneath the surface of the water. Animals are placed in the water and the time taken by them to find the hidden platform is recorded. The experiment is repeated for 8–10 days, and the animals learn the visual cues to find the platform. The decrease in time to reach the platform indicates learning.[25]

Biochemical markers

After sacrificing the animals, the brains of all the animals were collected. According to brain weight brains are triturated in the prepared solution, centrifuged, the supernatant collected was used for performing the following studies.

Evaluation of acetylcholinesterase enzyme (AChE) inhibitory activity

Inhibition of AChE activity is evaluated by Ellman's method.

Reagents used

0.1 M phosphate buffer, Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB) (39.6 g of DTNB with 15 mg sodium bicarbonate is dissolved in 10 ml of 0.1 M phosphate buffer [pH 7.0]), acetylthiocholine iodide (ATCI) (21.67 mg of acetylthiocholine iodide is dissolved in 1ml of distilled water).

Principle

The enzyme hydrolyzes the substrate ATCI to thiocholine and acetic acid. Thiocholine is allowed to react with DTNB, and this reaction resulted in the development of a yellow color. The color intensity of the product is measured at 405 nm, and it is proportional to the enzyme activity.

Procedure

On the 9th-day animals after neurobehavioral studies, the brains were decapitated. The whole brain was taken out in normal saline later suspended in phosphate buffer pH:7.4. The brain was homogenized in tissue homogenizer and then 0.4 ml of the homogenate is mixed with 10 ml of DTNB. The absorbance is recorded in ultraviolet (UV) spectrometer. After a few minutes, the sample is mixed with the acetyl thiocholine and readings were taken, change in the absorbance per minute was noted.

Calculations

The percentage inhibition for each test solution was then calculated using the following equation:

$$\text{Inhibition}\,(\%) = \frac{\text{Abs}_{control} - \text{Abs}_{test}}{\text{Abs}_{control}} \times 100$$

Where Abs_{test} is the absorbance of the test drug and $\text{Abs}_{control}$ is the absorbance of the blank.[26]

Catalase (CAT) activity

CAT is a heme protein located in the microperoxisomes.

Principle

It reduces the hydrogen peroxide produced by dismutation reaction and prevents generation of hydroxyl radicals thereby protecting the cellular constituents from oxidative damage in the peroxisome. The enzyme catalyzes the decomposition of hydrogen peroxide to water and oxygen and thus protecting the cell from oxidative damage by hydrogen peroxide.

Reagents used

50 mM phosphate buffer (pH-7.0), 12.5 mM hydrogen peroxide used in phosphate buffer.

Procedure

0.4 ml of homogenate was diluted 20 times with phosphate buffer. A mixture of 2 ml of diluted homogenate and 1 ml of hydrogen peroxide was taken as a test sample while a mixture of 4 ml of dilute homogenate and 1 ml of phosphate buffer was taken as blank. Absorbance was measured for both blank and test at 240 nm for 2 min with 60-s interval using a UV-visible spectrophotometer.[27]

Lipid peroxidase (LPO) activity
Reagents used

0.1 M Tris-HCl buffer (pH-7.4), 10% w/v trichloroacetic acid (TCA), and 0.67% w/v thiobarbituric acid (TBA) were used.

Principle

Oxidative stress is associated with the peroxidation of cellular lipids, which is determined by measurement of TBA reacting substance (TBARS). The concentration of LPO products may reflect the degree of oxidative stress. The increased levels of TBARS result in an increase of oxygen free radicals which attacks polyunsaturated unsaturated fatty acids in cell membranes and cause LPO. The malondialdehyde content, a measure of lipid peroxidation was assayed in the form of TBARS.

Procedure

0.5 ml of PMS was taken. To it add Tris-HCl buffer and incubate at 37πc for 2 h. Then add 1 ml of ice-cold TCA and centrifuged at 1000 rpm for 10 min. From above, a mixture of 1 ml supernatant and 1 ml TBA was taken in boiling tubes which were kept in a water bath for 10 min. Tubes were removed, cooled to room temperature and 1ml of distilled water is added. Absorbance was measured at 532 nm using UV-visible spectrophotometer. Blank is prepared without tissue homogenate.[28]

Calculations

3* absorbance of sample/50.156*(mg of tissue taken) = μm/mg tissue.

RESULTS

Neurobehavioral Studies

T maze

Animals showed increased impedance in learning and memory on the 7th day when compared to that of the 8th day

because of treatment with scopolamine. Donepezil, when given as standard showed an enhanced process of learning and memory through its movement against scopolamine. FLN (20 mg, 40 mg, and 80 mg) showed varied rate modification on the 8th day when compared to that of the seventh against scopolamine. The concentrations of FLN administered showed improved memory, learning, and behavior against scopolamine [Figure 1].

Elevated plus maze

Animals on the 7th day were found to have exchange idleness in higher esteems than on the 8th day, which proved disabled memory and learning on scopolamine treatment. Donepezil, when given as standard showed an enhanced process of learning and memory through its activity against scopolamine. FLN (20 mg, 40 mg, and 80 mg) showed varied rate modification on the 8th day compared to that of the 7th day against scopolamine. The concentrations of FLN administered showed improved memory and learning behavior of the animals against scopolamine. The intraperitoneal administration of FLN promoted the opportunity of entering into the arms demonstrating reduced exchange idleness of animals in contrast to scopolamine [Figure 2].

Water Morris

Animals showed diminished escape latency period on the 7th day when compared to that of the 8th day through its movement. The varied drug concentration showed its action by reducing time on the 8th day when compared to that of the 7th day distinguishing submerged concealed stage [Figure 3].
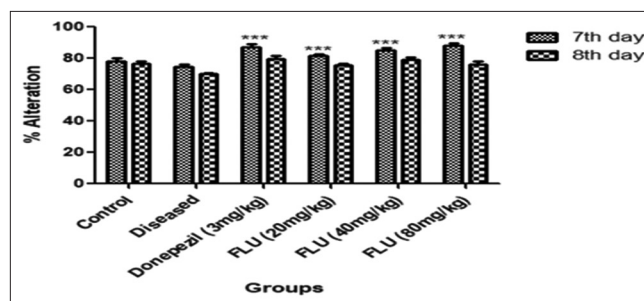

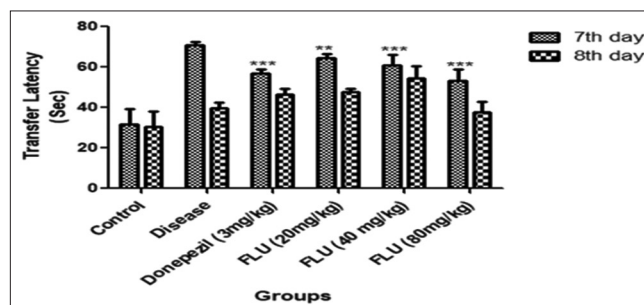**Figure 1:** Effect of treatment on behavior alterations in T-maze

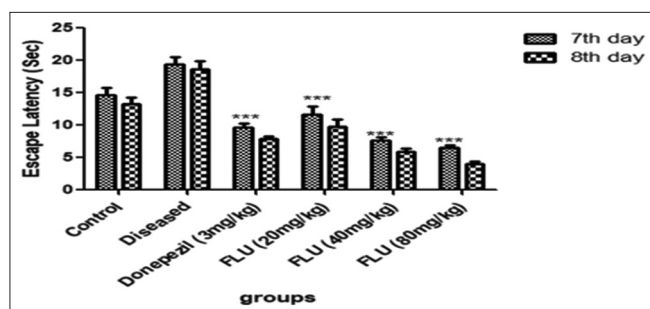**Figure 2:** Effect of treatment on transfer latency in the elevated plus maze

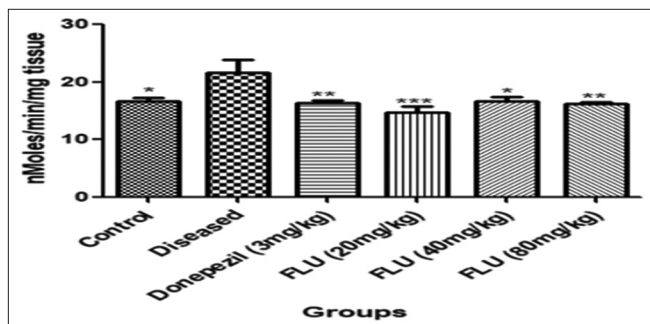**Figure 3:** Effect of treatment on escape latency in water Morris

Figure 4: Effect of treament on acetylcholinesterase activity

Biochemical Markers

Acetylcholinesterase activity

Varied doses of FLN showed a significant reduction in the levels of AChE when compared to the control group [Figure 4].

LPO

The animals showed increased antioxidant activity when administered with standard varying doses of FLN when compared to that of control and disease group [Figure 5].

CAT

A significant increase in the activity of CAT enzyme was observed in the test group when compared to control and disease group [Figure 6].

DISCUSSION

The study resulted in increased active avoidance behavior of FLN treated group when compared to that of the control group. The statistical significance was observed through the FLN treated mice than that of normal groups. The results of the administration of various doses of FLN showed improved behavior of learning and memory at various considered doses (20 mg, 40 mg, and 80 mg). An increase in dose indicated an increase in behavioral changes through active avoidance. The mechanism of action by which FLN showed memory-enhancing effect is through the regulation of calcium
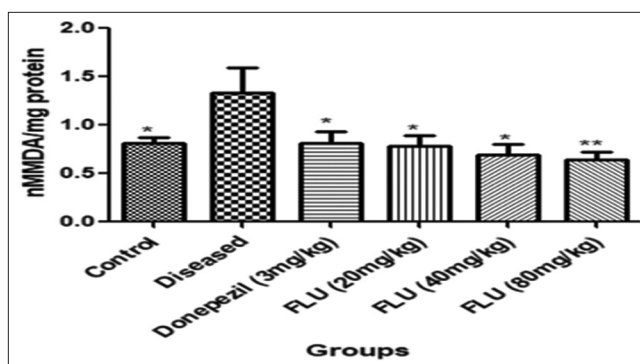

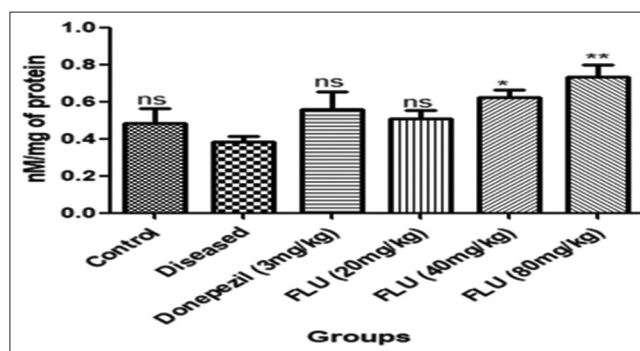**Figure 5:** Effect of treatment on a lipid peroxidase

Figure 6: Effect of treatment on catalase activity

homeostasis. The calcium levels have been regulated by a protein during normal age, whereas an increase in age and disease conditions such as AD and dementia may result in dysregulated calcium levels which in turn impair memory and learning behavior. FLN, a potent CCB proved its efficacy in maintaining calcium homeostasis, thus demonstrated improved neurobehavioral of animals. It proved its efficacy of enhancing memory retrieval by blocking inactivated and resting calcium channels. The results of T-maze showed an increased impedance of memory and learning in animals treated with FLN against scopolamine. The idleness esteems were found to be high on the 7th day compared to that of the 8th day when measured against the scopolamine treatment through its behavior in elevated plus maze. There is an improved escape latency period through the administration of FLN against scopolamine was observed FLN treated animals in Morris water maze test. FLN showed significant antioxidant activity by reducing the levels of LPO and increasing the levels of CAT enzyme. FLN also showed significant inhibition of acetylcholinesterase activity compared to the control group.

CONCLUSION

The earlier studies revealed the role of FLN in migraine and cognitive dysfunction. The present study evaluated the behavioral changes of animals treated with various doses of FLN when compared against scopolamine treated groups, the alterations in behavior observed through active avoidance

phenomenon and calcium homeostasis mechanism. FLN, though commonly used the drug in the treatment of migraine, may have its efficacy in the treatment of behavioral changes and retrieval of memory through its calcium homeostasis mechanism in diseases like AD. However, further research is required in evaluating the exact mechanism of FLN in AD.

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