Evaluation of nootropic effects of galantamine and sildenafil as a combination in mice

Bhavna Shantilal Bohra & Pravin Popatrao Kale*
Department of Pharmacology, Dr. Bhauuben Nanavati College of Pharmacy, Mumbai-400 056, Maharashtra, India

Received 12 October 2015; revised 05 August 2016

Cognitive decline is one of the age related mental problems and a characteristic symptom of various neurodegenerative disorders. Nootropic effects of combination of sildenafil and galantamine was evaluated in different learning and memory paradigms viz. Elevated plus maze (EPM) and Morris water maze (MWM) against scopolamine induced cognitive impairment. Moreover, the influence on central cholinergic activity via estimating the whole brain acetylcholinesterase enzyme was also assessed. Sildenafil (8 mg/kg, i.p.) and galantamine (3 mg/kg, i.p.) were administered per se to Swiss albino mice for successive 14 days. In addition, combination of sildenafil (4 mg/kg, i.p.) and galantamine (1.5 mg/kg, i.p.) were administered. Scopolamine (1 mg/kg, i.p.) was used to induce amnesia. Inflexion ratio and time spent in target quadrant were determined in EPM and MWM, respectively. Further, whole brain acetylcholinesterase enzyme was estimated through Ellman’s method. Treatment with sildenafil and galantamine combination significantly increased inflexion ratio and time spent in target quadrant in EPM and MWM, respectively. Combination treatment also showed reduction in brain acetylcholinesterase enzyme activity when compared separately against sildenafil and galantamine per se. The present study results suggest the augmentation of benefits of galantamine and sildenafil combination in the treatment of cognitive impairments.

Keywords: Acetylcholinesterase activity, Cognitive impairment, Elevated plus maze (EPM), Inflexion ratio, Learning and memory, Morris water maze (MWM)

Cognitive impairment is a major health problem in normal aged life as well as in some disease conditions. According to WHO, 47.5 million people have dementia, with just over half (58%) living in low and middle-income countries, and there are 7.7 million fresh cases added every year worldwide1. A novel approach for cognition enhancement is the application of allosteric modulators of nAChRs2,3. Allosteric modulators (like galantamine) are drugs which interact with the receptor through binding sites that are distinct from those of acetylcholine and nicotinic agonists and antagonists. Since these modulators are not directly involved in the neurotransmission process, they typically do not induce compensatory processes such as desensitization and downregulation of receptors that are induced by agonists and antagonists. It is hypothesized that these compensatory processes can be avoided with allosteric modulators4.

Galantamine is considered as a first-line therapy for dementia5. It (2.5-5 mg/kg, i.p.) significantly attenuated scopolamine induced deficits in both learning and memory models6. Phosphodiesterase 5 inhibitors already in clinical use for the treatment of erectile dysfunction in men, recent clinical and preclinical animal studies suggest that they also have central cognitive enhancing effects. Current behavioural pharmacological studies with rodents demonstrate that phosphodiesterase 5 inhibitors improve memory consolidation in young animals using elevated plus maze and object recognition tasks7. Sildenafil at dose of 8 mg/kg, i.p., increased nitric oxide (NO) production in brain leading to cerebral vasodilatation which resulted in enhanced acquisition and retention of memory8. Drugs currently used for the treatment of Alzheimer’s disease have weak beneficial effects on cognitive function or offer some relief of behavioural and psychological symptoms of dementia. Early intervention is critical in dementia because a delay in treatment is associated with non-reversible symptom progression. Hence, the discovery of new drugs that act during the early stages of dementia could be considered a ‘medical need’9. Both drugs have different mechanism and site of action in the brain. Galantamine is known to produce allosteric potentiation of ligand effect at nicotinic receptors and also inhibit acetylcholinesterase (AChE)6, and sildenafil increases NO production in brain and thereby elevates acquisition and retention of memory8. With the above understanding, the present
study postulates synergism with galantamine and sildenafil combination in improving cognition.

Materials and Methods

Animals
Male Swiss Albino mice weighing around 25-30 g were purchased from Bharat Serum Ltd, Thane. Animals were housed separately in groups of 6 per cage (Perpex) under laboratory conditions and kept in a temperature (22-24°C) and humidity (50-60 %) controlled central animal house facility with light and dark cycle of 12 h each. The animals were acclimatized for at least five days before behavioral studies. All experiments were carried out during day time between 09:00 and 16:00 h and they had proper access to standard food and water. Study protocols were approved by the Institutional Animal Ethics Committee (Project Approval number: CPCSEA/IAEC/BNCP/P-15/2014).

Drugs
The drugs used were sildenafil (Watson Pharmaceuticals Pvt. Ltd., Ambernath, Thane), galantamine (Beijing Packbuy M&C CO., China). Scopolamine hydrobromide, acetylthiocholine iodide, 5,5'-dithiobis (2-nitrobenzoic acid) were purchased from Sigma-Aldrich chemicals Pvt. Ltd., USA. Drug solutions were prepared in normal saline immediately before use.

Experimental protocol
Thirty healthy male swiss albino mice were selected randomly and divided into five groups of six animals each to evaluate their responses on exteroceptive behaviour models. The groupings of animals were as follows: Group I (control), saline (8 mL/kg, i.p.); Group II (negative control), scopolamine (1 mg/kg, i.p.); Group III, scopolamine (1 mg/kg, i.p.) + sildenafil (8 mg/kg, i.p.); Group IV, scopolamine (1 mg/kg, i.p.) + galantamine (3 mg/kg, i.p.); and Group V, scopolamine (1 mg/kg, i.p.) + sildenafil (4 mg/kg, i.p.) + galantamine (1.5 mg/kg, i.p.)

Both control groups (Gr. I & II) received normal saline treatment for 14 days period. The volume of injection delivered in each animal was 8 mL/kg/day, i.p. for each drug treatment separately. Remaining groups such as III, IV, and V received respective drug treatments except scopolamine for 14 days period. Each drug was given separately in the sequence mentioned above. In addition, scopolamine (1 mg/kg, i.p.) was delivered only on 14th day of treatment schedule in groups II-V after 30 min of regular administration of respective drug dose to induce amnesia. Transfer latency (TL) was observed in elevated plus maze (EPM) trials 45 min after the administration of scopolamine on the 14th day of treatment. TL was also recorded 24 h (15th day) of the first exposure. Escape latency (EL) in Morris water maze (MWM) was observed from 8th day to 13th day of treatment. The mean time spent in the target quadrant (TSTQ) in search of the missing platform was noted as index of retrieval of memory on 14th day of treatment.

Nootropic models

Elevated plus maze (EPM) test
EPM served as the exteroceptive behaviour model to evaluate learning and memory in mice. The procedure, technique, and end point for testing learning and memory was followed as per the parameters described earlier10. The apparatus consisted of two open arms (30×5 cm) and two enclosed arms (30×5×12 cm). The arms extended from a central platform (5×5 cm) and the maze was elevated to a height of 35 cm from the floor. On the first day (i.e., 14th day of treatment), each mouse was placed at the end of an open arm, facing away from the central platform. TL was the time taken by mouse with all its four legs to move into one of the enclosed arms. TL was recorded on the first day. If the animal did not enter into one of the enclosed arms within 90 s, it was gently pushed into one of the two enclosed arms and the TL was assigned as 90 s. Retention of this learned-task was examined 24 h (15th day) after the first day trial11 and recorded video was used to evaluate TL by a single trained person. The “Inflexion Ratio” (IR) was calculated using formula IR = (L1–L0)/L0, where L0 was TL after 24 h (15th day) and L1 was initial TL (14th day) in seconds12.

Morris water maze (MWM)
The procedure, technique, and end point for testing memory were followed as per the parameters described earlier13. MWM for mice consisted of a circular pool (4 ft in diameter i.e., 1.22 m, 40 cm in height) filled to a depth of 30 cm with water maintained at 25°C. The water was made opaque with milk powder. The pool was divided into four quadrants. Boundaries of these quadrants were marked on the edges of the pool with masking tape and labeled: North, South, East and West. A submerged platform (with top surface 15×15 cm and painted in white) was placed inside the target quadrants (SE in present study) of this pool one cm below surface of water. The position of platform was kept unaltered throughout the training session. EL, the time taken by the animal to move from the starting quadrant to find the hidden platform in the target
quadrant, was recorded on the 8th day to 13th days of treatment for each animal. Each animal was subjected to training trials for six consecutive days, the starting position was changed with each exposure and target quadrant (SE in the present study) remained constant throughout the training period. During the training session, mouse was gently placed in the water between quadrants, facing the wall of pool with drop location changing for each trial, and allowed 120 s to locate submerged platform. If the mouse failed to find the platform within 120 s, it was guided gently on to the platform and allowed to remain there for 20 s. On the 14th day, the platform was removed and mouse was placed in any of the three quadrants and allowed to explore the target quadrant for 300 s. The mean TSTQ was noted as index of retrieval of memory. Video camera was fixed on the ceiling to record the behaviour of the mice in the pool. The observer always stood at the same position. Care was taken not to disturb the relative location of water maze with respect to other objects in the laboratory14.

**Biochemical estimation of AChE**

**Collection of brain sample**

Immediately after behavioural testing, animals were sacrificed by cervical dislocation. The whole brain was carefully removed from the skull and kept in ice cold 0.1 M phosphate buffer pH 8. Weight of brain was noted. Samples were homogenized (Kinematic Polytron PT-MR 2500 E) in 5 mL of ice cold 0.1 M phosphate buffer at around 6000 rpm and resulting mixture was centrifuged (Eppendorf 5810 R) at 16356×g for 20 min at 4°C. The supernatant was stored at −80°C until the time of analysis15.

**Estimation of brain AChE activity**

Brain AChE was estimated using the method described earlier16. The esterase activity was measured by providing an artificial substrate, acetylthiocholine (ATC). Thiocholine released because of the cleavage of ATC by AChE was allowed to react with the Ellman’s reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), which was reduced to thionitrobenzoic acid, a yellow coloured anion with absorption maxima at 412 nm. The concentration of thionitrobenzoic acid was detected using a UV spectrophotometer (Shimadzu UV 1800) and taken as a direct estimate of the AChE activity16.

Briefly, 0.4 mL of brain homogenate was added to a cuvette containing 2.6 mL of phosphate buffer (0.1 M, pH 8) and 100 µL of DTNB. The contents of the cuvette were mixed thoroughly by bubbling air and absorbance was measured at 412 nm. When absorbance reaches a stable value, it was recorded as the basal reading. 20 µL of substrate i.e., ATC was added and change in absorbance was recorded for a period of 10 min at intervals of 2 min. Change in absorbance per min was calculated. The enzyme activity was calculated using the following formula:

$$ R = \frac{\Delta A}{1.36 \times 10^4 \times \left(\frac{400}{3120}\right) \times \frac{1}{CO}} = 5.74 \times 10^{-4} \times \frac{\Delta A}{CO} $$

where, $R =$ Rate in moles of substrate hydrolyzed/min/g tissue; $\Delta A =$ Change in absorbance/min; $CO =$ Original concentration of the tissue (mg/mL)15.

**Statistical analysis**

One way ANOVA followed by Tukey’s honest significant difference (HSD) post-hoc test was used for the calculation of statistical significance. The GraphpadInStat for 32 bit Windows version 3.06 was used for statistical assessment. The data was represented as mean ± SEM values and n = 6 per group.

**Results**

**Effect on TL in EPM**

The inflexion ratio (IR) was significantly decreased with group II compared to the control group but significantly increased in groups III-V groups when compared with group II (Fig. 1). On independent

![Fig. 1 — Elevated plus maze (EPM). Inflexion Ratio. Data is presented as mean ± SEM (n=6/group). Significant difference is denoted by * P <0.05 as compared against the control group I; ! P <0.05, !!! P <0.01, !!!! P <0.001 -as compared against Group II (NC); ## P <0.01 as compared against Group III (S); @ P <0.05 as compared against Group IV (G). Gr. I, control; Gr. II (NC), scopolamine treated group; Gr. III (S), sildenafil treated group; Gr. IV (G), galantamine treated group; and Gr. V (SG), sildenafil + galantamine treated group](https://example.com/fig1.png)
comparison, group V showed significant increase in IR against groups III and IV.

**Effect on EL and TSTQ in MWM**

The training session related data i.e. EL of drug treated groups for the period of 8th-13th day of treatment were statistically insignificant (Fig. 2A) when compared against control and NC groups, separately.

Group II showed significant decrease in TSTQ when compared against control group (Gr. I). Comparison of groups III-V showed significant increase in TSTQ than group II (Fig. 2B). There was significant increase in TSTQ with group V when compared against groups III and IV, separately (Fig. 2B).

**Effect on AchE activity**

The AchE activity in whole brain was significantly increased with group II than control group. Groups III-V showed significant decrease in AchE activity when compared against group II. The separate comparison of groups III and IV against group V (combined) showed significant decrease in AchE activity in later group (Fig. 3).

**Discussion**

Combination approach having half of respective monotherapy dose of each drug showed significant memory enhancing benefits in terms of Inflexion ratio (IR), Time spent in target quadrant (TSTQ), and AchE activity in mice than monotherapy. The most widely employed behavioural models such as Elevated plus maze (EPM) and Morris water maze (MWM) were used for screening of cognitive functions in the present study. Effect of scopolamine treatment is in line with available reports. Memory related benefits observed in EPM and MWM with sildenafil and galantamine per se treatment are in line with available reports.
Scopolamine, a muscarinic receptor antagonist, decreases central cholinergic processes and thereby induces memory impairment. Interestingly, co-administration of sildenafil with a central muscarinic blocker such as scopolamine resulted in enhancement of antidepressant activity. In addition, Patil et al. showed cholinomimetic effect of sildenafil and its involvement of cholinergic-NO-cGMP mediation in antinociceptive effect. Therefore, the memory benefits observed in the present study after co-administration of scopolamine with sildenafil suggest the important role of cholinergic-NO-cGMP mediation. The consideration of half of monotherapy dose of sildenafil and galantamine in combination showed synergy in nootropic benefits due to allosteric modulation of nAChRs, inhibition AChE activity and cholinergic-NO-cGMP mediation.

Sildenafil is metabolized principally by cytochrome P450 (CYP) 3A4 and to a lesser extent by CYP2C9. Inducers or inhibitors of these isozymes can affect the clearance of sildenafil. Galantamine is metabolized by hepatic cytochrome P450 enzymes. In vitro studies indicate that cytochrome CYP2D6 is the major cytochrome P450 iso-enzyme involved in the metabolism of galantamine. Owing to different metabolic enzymes pattern, there is low potential of metabolism of galantamine. Owing to different major cytochrome P450 iso-enzyme involved in the metabolism of galantamine, there is low potential of metabolism of galantamine. Owing to different major cytochrome P450 iso-enzyme involved in the metabolism of galantamine, there is low potential of metabolism of galantamine. Owing to different major cytochrome P450 iso-enzyme involved in the metabolism of galantamine, there is low potential of metabolism of galantamine. Owing to different major cytochrome P450 iso-enzyme involved in the metabolism of galantamine, there is low potential of metabolism of galantamine. Owing to different major cytochrome P450 iso-enzyme involved in the metabolism of galantamine, there is low potential of metabolism of galantamine. Owing to different major cytochrome P450 iso-enzyme involved in the metabolism of galantamine, there is low potential of metabolism of galantamine. Owing to different major cytochrome P450 iso-enzyme involved in the metabolism of galantamine, there is low potential of metabolism of galantamine. Owing to different major cytochrome P450 iso-enzyme involved in the metabolism of galantamine, there is low potential of metabolism of galantamine. Owing to different major cytochrome P450 iso-enzyme involved in the metabolism of galantamine, there is low potential of metabolism of galantamine.

Sildenafil is metabolized principally by cytochrome P450 (CYP) 3A4 and to a lesser extent by CYP2C9. Inducers or inhibitors of these isozymes can affect the clearance of sildenafil. Galantamine is metabolized by hepatic cytochrome P450 enzymes. In vitro studies indicate that cytochrome CYP2D6 is the major cytochrome P450 iso-enzyme involved in the metabolism of galantamine. Owing to different metabolic enzymes pattern, there is low potential of possible pharmacokinetic drug interactions between sildenafil and galantamine. There are no preclinical or clinical reports available with the consideration of half of monotherapy dose of sildenafil and galantamine. There are no preclinical or clinical reports available with the consideration of half of monotherapy dose of sildenafil and galantamine. There are no preclinical or clinical reports available with the consideration of half of monotherapy dose of sildenafil and galantamine. There are no preclinical or clinical reports available with the consideration of half of monotherapy dose of sildenafil and galantamine. There are no preclinical or clinical reports available with the consideration of half of monotherapy dose of sildenafil and galantamine.

Acknowledgement
Authors are thankful to Watson Pharmaceuticals Pvt. Ltd., Ambernath, Thane and Beijing Packbuy M & C CO., China for supply of drugs as a gift samples.

References


