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RESEARCH ARTICLE

NEUROPROTECTIVE EFFECT OF NICOTINE AND SIMVASTATIN ON CEREBRAL ISCHEMIA INDUCED COGNITIVE IMPAIRMENT

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ABSTRACT

Cerebral ischemia is a condition in which there is insufficient blood flow to the brain to meet metabolic demand. This vascular obstruction and the subsequent decrease in the cerebral blood flow results in immediate drop in the neurological activity that might end in death of brain tissue or cerebral infarction / ischemic stroke. In the present study, the effects of Nicotine and Simvastatin and their combinational effect on cerebral ischemia-induced cognitive impairment in rats were investigated. The animals were pre-treated with Nicotine and Simvastatin for a period of 10 days. Cerebral ischemia was induced by Bilateral Common Carotid Artery Occlusion (BCCAO) of rats for 30 minutes followed by reperfusion. The treatment was continued for another week after surgery. To evaluate the learning and memory parameters, Rectangular maze test, Morris Water maze test, locomotor activity tests were conducted. Catalase activity and DPPH assay were also assessed. Donepezil was used as standard drug. This study demonstrates the neuroprotective effect of Nicotine and Simvastatin on improving the cognitive function and by increasing the free radical scavaging activity. The memory enhancing capacity of the drugs was very significant when compared to disease control ($p<0.001$).

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INTRODUCTION

Cerebral ischemia results from decreased or interrupted blood supply leading to reduced availability of glucose and oxygen in the area of affected vascular bed which in turn leads to cellular energy crisis (Muge *et al*., 2008). Shortage of energy interrupts the activity of cellular ionic pumps and disturbs the ionic gradient homeostasis which results in increased release of neurotransmitters particularly glutamate from the presynaptic terminals, within 1–2 min after the onset of ischemia (Jeon *et al*., 2004). A massive release of glutamic acid, an excitatory neurotransmitter activates the glutamate receptors, leading to membrane depolarization and accumulation of free cytosolic calcium by cellular influx at the postsynaptic site (Oliver *et al*., 1990). The accumulation of calcium plays a key role in the propagation of the irreversible neuronal damage by activation of series of neurotoxic events such as lipid peroxidation, free radical generation, activation of proteolytic enzymes and pathological gene activation. All the above changes lead to the formation of zone of infarction in the brain area where blood supply is interrupted (Michael and Patrick, 2002).

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Following reperfusion there will be increased production of superoxide, Nitric Oxide (NO) and peroxynitrate free radicals. Formation of these radicals in the vicinity of blood vessels plays an important role in reperfusion-induced injury (Sevgin *et al*., 2007). These radicals activate matrix metalloproteases (MMPs), which degrade collagen and laminins in the basal lamina, which disrupts the integrity of the vascular wall leading to increase in permeability of blood brain barrier (BBB). Oxidative stress, a condition of cellular pro-oxidant-antioxidant disturbance in favour of the pro-oxidant state, also induces the production of reactive oxygen species ROS, leading to serious functional impairments (Shereen *et al*., 2013). Oxidative and nitrative stress are also known to trigger recruitment and migration of neutrophils and other leukocytes to the cerebral vasculature, which release enzymes which further increase basal lamina degradation and vascular permeability (Coyle and Puttfarcken, 1993). These events can lead to parenchymal haemorrhage, vasogenic brain oedema and neutrophil infiltration into the brain (Crack and Taylor, 2005). Simvastatin is a hypolipidemic drug used with exercise, diet, and weight-loss to control elevated cholesterol, or hypercholesterolemia. It is a member of the statin class of pharmaceuticals. Simvastatin is a synthetic derivative of a fermentation product of Aspergillus terreus. The drug is marketed generically following the patent expiration, and

under the trade name Zocor (9). Simvastatin administration has been shown to have pronounced neuroprotective effects including anti-inflammatory effects due to NMDA receptor modulation (Liu *et al*., 2012). Nicotine is a potent parasympathomimetic alkaloid found in the nightshade family of plants (Solanaceae) and a stimulant drug. It is a nicotinic acetylcholine receptor (nAChR) agonist (Iuphar, 2014). Nicotine also activates the sympathetic nervous system (Yoshida *et al*., 1994) acting via splanchnic nerves to the adrenal medulla, stimulating the release of epinephrine. Acetylcholine released by preganglionic sympathetic fibers of these nerves acts on nicotinic acetylcholine receptors (King *et al*., 2009). The main purpose of the present study was to investigate the synergistic effect of simvastatin and nicotine in cerebral ischemia induced cognitive impairment and oxidative stress model.

MATERIALS AND METHODS

Animals

Male Wister albino rats weighing 200-250 g were used in the present study. They had free access to food and water and were maintained under standard laboratory conditions with alternating light and dark cycles of 12 h each. They were acclimatized to laboratory conditions for 2 days before behavioural studies. All the readings were taken during the same time of the day, that is, between 10 am and 2 pm. The experiments were planned after the approval of Institution Animal Ethical Committee (IAEC), Vaagdevi College of Pharmacy, Warangal (A.P) and India.

Chemicals

Hydrogen peroxide, paraformaldehyde was purchased from Finar Reagents. DPPH (1,1-diphenyl-2-picrylhydrazyl) were purchased from local dealers of Sigma-Aldrich Company. Thiopentone was purchased from Neon Laboratories.

Experimental design

Animals (30) were weighed and kept in cages accordingly and randomly divided into 5 groups (n=6). Drug extract were given orally daily for 10 days. On day 10, 60 min after last dose, all rats except normal control groups were subjected to 30 min bilateral common carotid artery occlusion (BCCAO) with aneurysm clips and all rats were sacrificed after 7 days of induction, their brains were removed and subjected to biochemical analysis and histopathological evaluation. On day 1, the training sessions for all the animals were given. Drug extract of three different concentrations were administered to their respective groups, after 1hr the retention time (RT) was calculated.

Table 1: Experimental Design

Sno.	Groups	Treatment
1	Normal	Vehicle saline (saline)
2	Standard	BCCAO + Donepazil
3	Disease control	BCCAO for 30 min
4	Test dose 1	BCCAO + Test dose 1 (100mg/kg)
5	Test dose 2	BCCAO + Test dose 2 (200mg/kg)
6	Test dose 3	BCCAO + Test dose 3 (400mg/kg)

This is followed on consecutive 3,5,7,9 of cerebral ischemia. The doses of drugs were taken according to the body weights of the animals given in Table 1.

Induction of Cerebral ischemia

Rats were anaesthetized with Thiopentone sodium at a dose of 50mg/kg, i.p (Jintanporn *et al*., 2011). A midline incision was made in the region between neck and sternum and trachea was exposed. Both the left and right common carotid arteries were located lateral to sternocleiomastoid, freed from the surrounding tissues and vagus nerve was separated. Cerebral ischemia was induced by clamping both the arteries with the help of aneurysm clips. After 30 min of cerebral ischemia, the clips were removed from both the arteries to allow the reflow of the blood through carotid arteries, the incision was sutured back in layers with the surgical suture. The sutured area was cleaned with spirit and was sprayed with antiseptic powder. After completion of surgical procedure, the body temperature was maintained at 37°C. All the surgical instruments used in the surgical procedure were sterilized prior to use (Kalyani Pitta *et al*., 2014).

Behavioural tests

All the animals were trained for 7 days before drug administration.

Rectangular Maze Test

Assessment of learning and memory can be effectively done by this method. The maze consists of completely enclosed rectangular box with an entry and reward chamber appended at opposite ends. The box is partitioned with wooden slats into blind passages leaving just twisting corridor leading from the entry to the reward chamber. Animals were trained prior to the experiment by familiarizing with the rectangular maze for a period of 10 min for 2 hrs. Transfer latency (time taken to reach the reward chamber) was recorded. For each animal, four readings were taken and the average is taken as learning score (transfer latency) for that animal. Lower scores of assessment indicate efficient learning while higher scores indicate 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 (Goverdhan Puchchakayala *et al*., 2012; Vogel *et al*., 2002).

Morris Water Maze Test

Morris water maze was used to assess learning and memory in experimental mice. There are several advantages of Morris water maze over other models of learning and memory including absence of motivational stimuli such as food and water deprivation, electrical stimulations, and buzzer sounds (Vogel *et al*., 2002; Morris, 1984). Briefly, it consists of a circular water tank, filled with opaque water, and one centimeter submerged platform. First, animals were trained to locate the platform. During acquisition, trial escape latency time (ELT), time measure to locate the hidden platform, was noted as an index of acquisition. Each animal was subjected to the four acquisition trials per day for 4 consecutive days. The time spent by the animal, searching for the missing platform in target quadrant Q2 with respect to other quadrant (Q1, Q3, and Q4) on 5th day, was noted as an index of retrieval. For studying the effect of drug on acquisition, the drug solution was administered before acquisition trial (Saraf *et al*., 2011).

Locomotor Activity

Locomotor activity is influenced by most of the CNS drugs in both 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 photocell is cut off by the animal, then a count is recorded. Animals are placed individually in the activity cage for 10min and the activity was monitored. The test is done before 30 min and after the drug administration. The photo cell count is noted and decreases or increase in locomotor activity is calculated (Vogel *et al*., 2002).

Histopathological Studies

After 8-day treatment, the brains of different groups were perfusion-fixed with 4% paraformaldehyde in 0.1M 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 40 magnification (Yu *et al*., 1997)

Dissection and Homogenization

On day 9, after behavioral assessments, animals were scarified by cervical dislocation. The brains were removed. Each brain was separately put on ice and rinsed with ice-cold isotonic saline. A (10% w/v) homogenate was prepared in 0.1M phosphate buffer (pH 7.4). The homogenate was centrifuged at 3000 rpm for 15 minutes and aliquots of supernatant were separated and used for biochemical estimation (Yu *et al*., 1997).

Biochemial tests

Catalase Activity

Catalase activity was assessed by the method of Luck (Luck *et al*., 1971), wherein the breakdown of hydrogen peroxide is measured. In this 3mL of H_2O_2 phosphate buffer was added to 0.05mL of the supernatant of the tissue homogenate. The absorbance was recorded at 240nm using Perkin Elmer Lambda 20 spectrophotometer. The results were expressed as micromoles of H2O2decomposed perminute per mg protein (Kalyani Pitta *et al*., 2014; Goverdhan Puchchakayala *et al*., 2012).

DPPH (2, 2-Diphenyl-1-picrylhydrazyl) Assay

In this, measurement is made from the bleaching of purple-coloured methanol solution of DPPH. To the 1000 μL of diverse conc. of the sample, 4mL of 0.004% methanolic solution of DPPH was added. After 30 min incubation, absorbance was read at 517 nm. Inhibition of free radical by DPPH in % was calculated in the following way:

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

A_{blank}: absorbance of control reaction. A_{sample}: absorbance of test sample. Values of inhibition were calculated (Kalyani Pitta *et al*., 2014; Goverdhan Puchchakayala *et al*., 2012).

Statistical Analysis

The statistical analysis of data was done by the one way analysis of variance (ANOVA) followed by the Dunnett's test. The probability level less than 0.05 were considered as significant. Results were expressed as mean± SD.

RESULTS

Behavioural tests

Rectangular maze test

The activity of the test drug was evaluated by using rectangular maze. The transfer latency measured for all groups of animals before and after induction of ischemia compared against disease control group which was given in fig.1. Results are compared with the disease control using Dunnet's test and found to be significant ($p<0.05$).

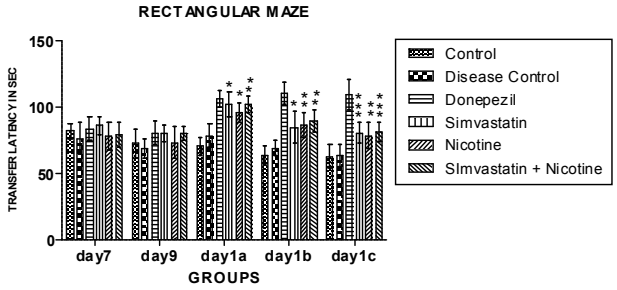


Fig 1. Effect Simvastatin and Nicotine on latency time compared to the disease control group (Mean ± SD, n = 6). Values are expressed as Mean±SD of latency time in seconds. * p<0.05, ** p<0.01, *** p<0.001 as compared with corresponding values of disease control group. Days 1a, 1b and 1c are the days after inducing ischemia

Locomotor activity test

The activity of the test drug was evaluated by using locomotor activity. The transfer latency measured for all groups of animals before and after induction of ischemia compared against disease control group which was given in fig.2. There was an increase in locomotor activity to the test groups compared with the disease control using Dunnet's test and the reslts were found to be significant ($p<0.05$).

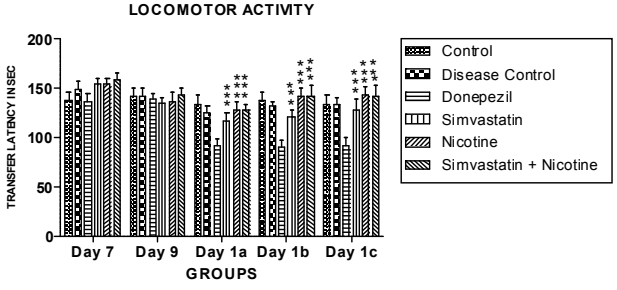


Fig 2. Effect Simvastatin and Nicotine latency time compared to the disease control group (Mean ± SD, n = 6). Values are expressed as Mean±SD of latency time in seconds. * p<0.05, ** p<0.01, *** p<0.001 as compared with corresponding values of disease control group. Days 1a, 1b and 1c are the days after inducing ischemia

Morris water maze test

Learning abilities of animals were evaluated by this morris water maze test. The animal groups are compared before and after induction of ischemia against the disease control group using Dunnet's test which was given in fig. 3 and the results were found to be significant ($p<0.0$

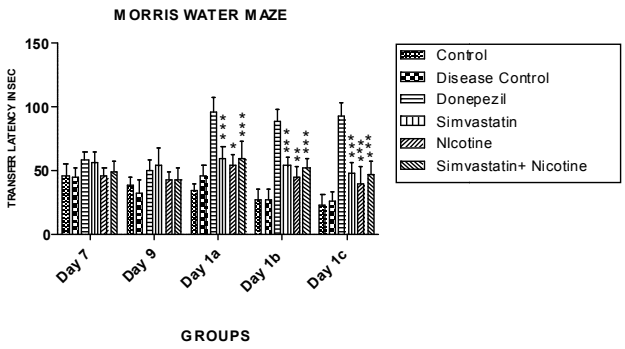


Fig 3. Effect Simvastatin and Nicotine on latency time compared to the disease control group (Mean ± SD, n = 6). Values are expressed as Mean±SD of latency time in seconds. * p<0.05, ** p<0.01, *** p<0.001 as compared with corresponding values of disease control group. Days 1a, 1b and 1c are the days after inducing ischemia

Biochemical tests

Catalase activity test

Percentage of inhibition of hydrogen peroxide was increased in test groups compared to disease control which was given in fig. 4. Significant difference was found in drug treated groups.

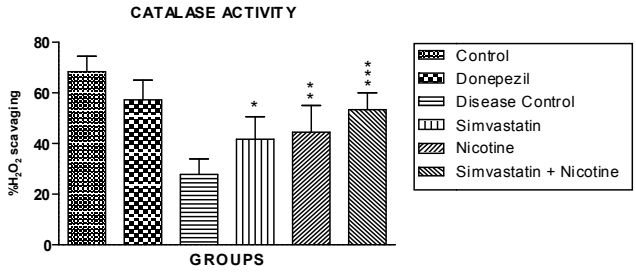


Fig 4. Effect Simvastatin and Nicotine on % H2O2 scavaging activity compared to the disease control group (Mean ± SD, n = 6). Values are expressed as Mean±SD. * p<0.05, ** p<0.01, *** p<0.001 as compared with corresponding values of disease control group

DPPH method

Percentage of inhibition of DPPH was decreased in disease control groups compared to control which was given in fig. 5. Significant difference was found in drug treated groups compared to disease control group.

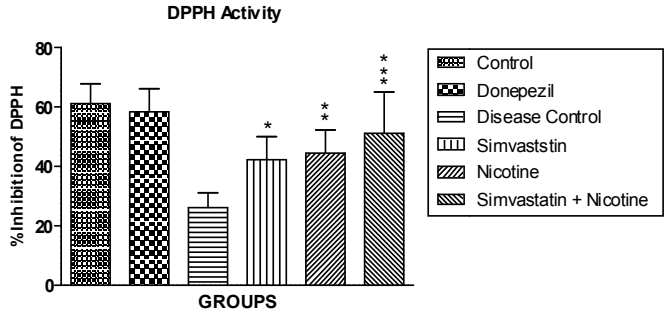
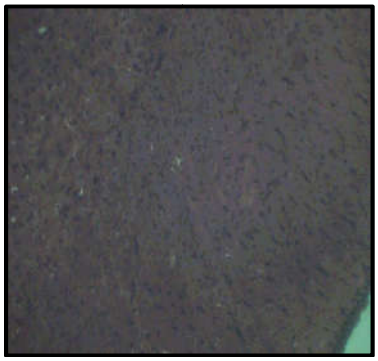


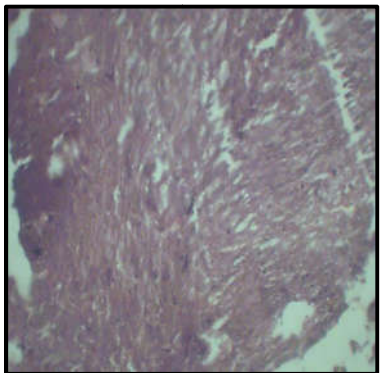
Fig. 5: Effect Simvastatin and Nicotine on % DPPH inhibition activity compared to the disease control group (Mean ± SD, n = 6). Values are expressed as Mean±SD. * p<0.05, ** p<0.01, *** p<0.001 as compared with corresponding values of disease control group.

Histopathologial studies

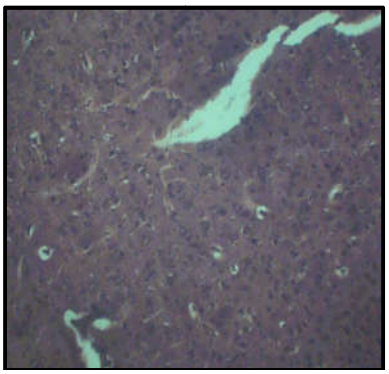
From fig. 6 it is clear that drug treated groups shown less neuronal damage, which is indicated by the gaps in slides, compared to the disease control group. Control group showed optimum sized and normal cells.



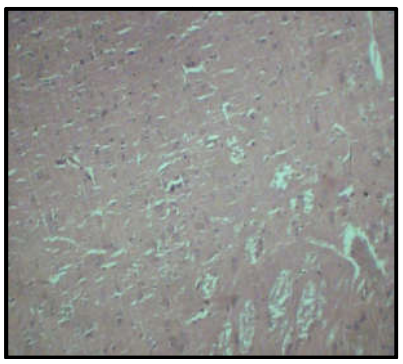
a. Control



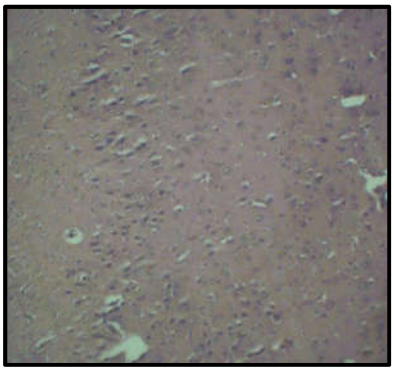
b. Disease Control



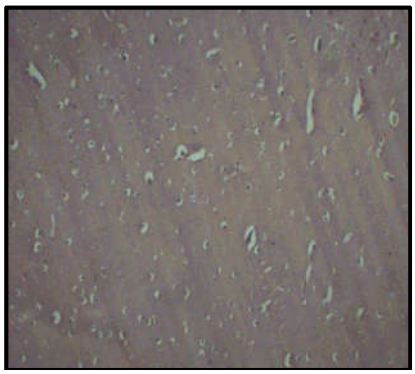
c. Donepezil



d. Simvastatin



e. Nicotine



f. Simvastatin + Nicotine

Figure 6. Histopathological studies these Figures a, b, c, d, and e represents the histological sections of the brain tissue showing neurological lesions

DISCUSSION

The present investigation showed the neuroprotective potential of Pyritinol and Fluvastatin against ischemia induced oxidative stress as well as histopathological alterations. Reducing oxidative stress is a consideration as potential therapeutic approaches for ischemic stroke. Oxidative stress occurs when the production of free radicals overpowers the endogenous scavenging capacity of cellular antioxidant defences. There is considerable evidence that reactive oxygen and nitrogen molecules are important mediators of tissue injury in acute ischemic stroke (Brouns and De Dey, 2009). Studies have demonstrated that nicotine administration to AD patients enhances their attention and information processing (Jones *et al*., 1992; Linert *et al*., 1999); in addition, it has been found that nicotine treatment improves the cognitive function of both young and aged rats (Arendash *et al*., 1995; Socci *et al*., 1995). Simvastatin administration has been shown to have pronounced neuroprotective effects including anti-inflammatory effects due to NMDA receptor modulation (Liu *et al*., 2012). In the present study, the synergistic effect of Simvastatin and Nicotine were studied. Their antioxidant activity was measured by catalase activity and DPPH activity test. The disease control group showed increased H_2O_2 and DPPH levels compared to control group and simvastatin + nicotine treated animals showed decreased levels when compared to their individual effects. From the behavioral test, that is, rectangular maze test and Morris water maze test, locomotor activity it is clearly seen that there was a general decrease in the transfer latency in all treated groups compared to disease control group. In comparison with Donepezil, the drug treated groups had almost equal performance which indicates synergistic effect of Simvastatin and Nicotine against memory loss.

Conclusion

In conclusion, the results suggests that Simvastatin, Nicotine and co-administration of these test drugs had potential therapeutic effects on improving cognitive impairment and attenuated the oxidative stress induced by cerebral ischemia. Mostly their combination showed more improvement than individual effect.

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