Research Article

Menthol causes reduction of Scopolamine induced Glutamatergic neurotoxicity and cognitive deficits

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Abstract

Background: Enhancement in the life-span of human beings in developed and developing countries has resulted in proportionate increase in the number of patients suffering from senile age-associated dementia. Memory impairment during aging is believed to be a consequence of decline in neuronal function and increase in neurodegeneration. Impaired neurotransmission at cholinergic synapses may contribute to the devastating loss of cognitive abilities in Alzheirmer's disease. It is suggested that a selective decrease in acetylcholine level may account for deterioration in learning and memory process, considering the important role for cognitive functions. Nootropic agents like piracetam and anticholinesterase inhibitors are commonly used for improving memory, mood and behaviours. **Objective:** Menthol, a natural alcohol and found in mint leaves and many essential oils. The present study was undertaken to investigate the impact of menthol on cognitive functions employing long term exteroceptive and interoceptive behavioral model in young and aged male mice. **Materials and methods:** to evaluate learning and memory, Menthol (100, 200, 400 mg/kg, s.c.) and Piracetam (200mg/kg, i.p.) were administered for 10 days passive avoidance and 11 days for morris water maze test.Brain amino acid level was measured after 10 days of passive avoidance. Whole brain acetylcholinesterase level and antioxidant level estimations were done after 11 days of morris water maze test. **Results and conclusion:** Lower dose of menthol produced significant improvement in spatial learning and memory by improving whole brain acetylcholine level, antioxidant level and brain amino acid in scopolamine and aged group of mice.

Keywords: Menthol, nootropic agents, alzheimer's disease, cognitive disorders, memory, glutamate

Introduction

Neuronal function and increase in neurodegeneration is the result of consequence of memory impairment during aging, may be due to accumulation of oxidative damage and impairment of antioxidant defense system (Haddadi et al., 2014). At old age and neurodegenerative disorders, cognitive impairment is a prominent feature) (Sharma et al., 2008). Alzheimer's disease (AD) is a neurodegenerative disorder expressed by progressive loss of memory and cognitive impairment. AD prevalence is approximately 1% between 65 and 69 years and is higher than 50% in individuals above 95 years (Vasto et al., 2008). As said with increasing age, the demage of the cerebral neurons is one of the commonest and vital causes for Alzheimer's like dementia (Agarwal et al., 2002). Neuronal loss leads to memory loss or impairment that result in progressive disability and eventual incapacitation (Negishi and Kawahara, 2008; Hung et al., 2008).

Attention and concentration, learning and memory, problem-solving ability, spatial abilities, mental flexibility, psychomotor efficiency and manual dexterity, these are the multidimensional concept of cognitive impairment (Bhadania et al., 2012). Cognitive impairment disturbs complex brain function like memory, consciousness, alertness, and learning which are controlled by neurotransmitter and neuromodulatory systems (Bliss and Collingridge, 1993). These type of neuronal damage associated with two major protein: amyloid plaques and neurofibrillary tangles (Goedert, 1996; Chong et al., 2005;

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Parihar and Hemnani, 2004).

Cholinergic transmission is linked with normal cognitive function and in the elderly the activity of choline-acetyltransferase, a marker enzyme for acetylcholine (Roger and Cate, 2008). Acetylcholine is the most important neurotransmitter at synaptic level, involved in the regulation of cognitive functions. There is extensive evidence linking the central cholinergic system to memory (Peng et al., 1997; Olney, 1990; Bhattacharya et al., 1993). Loss of cholinergic neurons and decrease in cholineacetyltransferase activity was reported to be a characteristic feature of senile dementia (Agnolli et al., 1983; Joshi and Parle, 2007).

Mental disorders have traditionally been treated with various essential oils derived from plants. The medicinal use of essential oils that originated in ancient Egypt has continued until the present (Balchin, 1997; Buckle, 1999; Perry and Perry, 2006). Menthol, a natural alcohol found in mint leaves and many essential oils (e.g. peppermint oil) (Bakkali et al., 2008).

A Ca^{2+} imaging study on DRG neurons demonstrated that menthol could directly release Ca^{2+} from intracellular Ca^{2+} stores. Menthol can act directly on pre-synaptic Ca^{2+} stores of sensory neurons to release Ca^{2+}, resulting in a facilitation of glutamate release and a modulation of neuronal transmission at sensory synapses (Tsuzuki et al., 2004). Menthol was reported to be effective in treating prostate cancer in vitro possibly due to TRPM8 activation and its resulting increase in Ca^{2+}. It indicates that TRPM8-independent mechanisms are involved in menthol-induced increase in Ca^{2+} (Kim et al., 2005; Bhadania et al., 2012). It is unclear that the consequence of excitotoxic changes produced by glutamatergic overactivity or result from a decrease in glutaminergic function (Advokat and Pellegrin, 1992).

AD exhibits extensive oxidative stress throughout the body (Wang et al., 2014). Glutathion (GSH) has a variety of neuroprotective functions in the brain. Accordingly, brain GSH depletion has been implicated in neurologic disorders. GSH is produced from three amino acids, glutamate, cysteine and glycine (Aoyama and Nakaki, 2015).

Materials and methods

Animals

Animal Species: Male Swiss albino mice. Young (3-4 months old) mice weighing around 25 g and aged (22-24 months old) mice weighing around 40 g were used. The animals had free access to food and water, and they were housed in a natural (12h each) light-dark cycle. The animals were acclimatized for at least 7 days to the laboratory conditions before behavioral experiments. Experiments were carried out between 0900 h and 1800 h. The experimental protocol was approved by the IAEC and the guidelines of CPCSEA, Govt. of INDIA.

Research design

The mice were divided into 32 different groups comprising of 6 animals each for investigations using various interoceptive as well as exteroceptive memory models and bio-chemical estimations. Piracetam, an established nootropic agent, was used as standard drug. Menthol (100, 200, 400 mg/kg, s.c.) and Piracetam (200mg/kg, i.p.) were administered for 10 days passive avoidance and 11 days for morris water maze test. Animal received training session using passive avoidance for 9 days and morris water maze for 10 successive days daily. Retention (memory) of the learned task was recorded after 24 hours. Amnesia was induced in separate groups of young mice by scopolamine (0.4 mg/kg, i.p.) on last day of learning session of memory test model. Phenytoin (200 mg/kg, i.p.) was given to separate group of young and aged mice. Brain amino acid level was measured after 10 days of passive avoidance. Whole brain acetylcholinesterase level and anti oxidant level estimations were done after 11 days of morris water maze test.

Memory models

Morris water maze test

To assess place learning and memory performance of mice, a cylindrical test apparatus was used. The water maze was slightly modified from the morris water task. The experimental apparatus consisted of a circular water tank (diameter 100 cm; height 55 cm) containing water was maintained at 24°C to a depth of 45 cm. A slightly submerged silvered platform to which the mice could escape was hidden from view by making the water opaque with a white bio-safe material i.e. milk was used. The position of the platform was fixed during 90 sec test period. A platform was positioned inside the tank with it stop submerged 2 cm below the water surface in the target quadrant of the maze. After the training session of 10 days, the test was conducted on the day of injection of scopolamine10^{th} day. In each training trial, the transfer latency, the time (in second) required to escape onto the hidden platform was recorded. On 11^{th} day, the time spend in target quadrant was measured (Um et al., 2006; Morris, 1984).

Passive Shock Avoidance Paradigm

Passive-avoidance behavior based on negative reinforcement was recorded to examine long-term memory. The apparatus consisted of a box (27 x 27 x 27 cm3) having three walls of wood and one wall of Plexiglas, featuring a grid floor (3 mm stainless steel rods set 8 mm apart), with a wooden platform (10 x 7 x 1.7 cm3) in the center of the grid

floor. The box was illuminated with a 15 W bulb during the experimental period. Electric shock (20 V AC) was delivered to the grid floor. Training was carried out in two similar sessions. Each mouse was gently placed on the wooden platform set in the center of the grid floor. When the mouse stepped down and placed all its paws on the grid floor, shocks were delivered for 15 s and the step-down latency (SDL) was recorded. SDL was defined as the time taken by the mouse to step down from wooden platform to grid floor with its entire paw on the grid floor. Mice showing SDL in the range (2–15 s) during the first test were used for the second session and the retention test. The second session was carried out 90 min after the first test. When mice stepped down before 60 s, electric shocks were delivered for 15 s. During the second test, animals were removed from shock free zone if they did not step down for a period of 60 s. Retention was tested after 24 h in a similar manner, except that the electric shocks were not applied to the grid floor. Each mouse was again placed on the platform, and the SDL was recorded, with an upper cutoff time of 300 s (Parle et al., 2005; Dhingra et al., 2004).

Evaluation of biochemical parameters

Collection of brain samples

The animals were sacrificed by cervical dislocation. After sacrifice, the brain was rapidly removed and placed on an ice-cold plate. Whole brain was homogenized with perchloric acid. The resultant cloudy supernatant liquid was used for biochemical parameters.

Brain glutathione

The brain glutathione was determined by the method of Moron et al. The assay was based on the formation of a relatively stable yellow product when sulphydryl groups react with 5,5-dithiobis 2-nitro benzoic acid (DTNB). Briefly, proteins were precipitated using 10% trichloro acetic acid, centrifuged and 0.5 ml of the supernatant was mixed with 0.2 M phosphate buffer (pH 8.0) and 0.006 mM 5,5-dithiobis 2-nitro benzoic acid. This mixture was incubated for 10 min and the absorbance was measured at 412 nm against appropriate blank. The glutathione content was calculated by using the standard plot under same experimental conditions. Standard curve of absorbance against glutathione concentration (0.005–0.042 µmol) was plotted which showed linear correlation coefficient (r2=0.999) (Moron, 1979).

Brain lipid peroxidation

As a marker for lipid peroxidation, the level of thiobarbituric acid reactive substances in the brain homogenate was measured by the method of Braughler et al. Briefly, the homogenate was incubated with 15% trichloro acetic acid, 0.375% thiobarbituric acid and 5 N hydrochloric acid at 95°C for 15 min, the mixture was cooled, centrifuged and the absorbance of the supernatant was measured at 532 nm against appropriate blank. The amount of lipid peroxidation was determined using the formula $\varepsilon=1.56\times105\ M^{-1}\ cm^{-1}$ and expressed as malondialdehyde n mol per gram of tissue (Braughler et al., 1987).

Brain acetyl cholinesterase

Brain acetyl cholinesterase activity was measured by the method of Ellman et al with a slight modification. 0.5 ml of the cloudy supernatant liquid was pipetted out into 25 ml volumetric flask and dilution was made with a freshly prepared DTNB (5.5-dithiobis-2-nitrobenzoic acid) solution (10mg DTNB in 100ml of Sorenson phosphate buffer, pH 8.0). From the volumetric flask, two 4ml portions were pipette out into two test tubes. Into one of the test tubes, 2 drops of eserine solution was added. 1 ml of substrate solution (75 mg of acetylcholine iodide per 50 ml of distilled water) was pipette out into both the tubes and incubated for 10 min at 30°C. The solution in the tube containing eserine was used for zeroing the colorimeter. The resulting yellow color is due to reduction of DTNB by certain substances in the brain homogenate and due to non-enzymatic hydrolysis of substrate. After having calibrated the instrument, change in absorbance per min of the sample was read at 420 nm. Acetyl cholinesterase (AChE) activity was determined on 11 days after phenytoin (12mg/kg, p.o.) injected to acetyl cholinesterase group (Joshi and Parle, 2007; Ellman, 1961).

Brain amino acid

In this experiment, mice were decapitated after 11 days of behavioral experiments and their brain regions were removed and homogenized. Glutamate was assayed using a fully automated high pressure liquid chromatography system with electro chemical detection and standard biochemical methods. HPLC analysis was performed using a Luna C18, 25 cm, 5 µm reverse-phase columns. Compounds were eluted isocratically with mobile phase delivered at flow rate of 0.75 ml/min using a Shimadzu Class VP LC 10AD pumps. An Antec electrochemical detector with a flow-through cell linked to a Shimadzu class VP Integrator SCL-10 Avp was used. A high-density glass carbon working electrode (Antec) was operated at +0.85 V. A Rheodyne injection valve with a 20 µl sample loopwas used to manually inject the samples. Preparation of the mobile phase and the derivatizing agents were based on the methods described by Rowley et al. The mobile phase consisted of 0.1 M monosodium phosphate and 0.5 mM EDTA with 25% methanol (v/v) in water adjusted to pH 4.5 with 1 M phosphoric acid. Then, it was filtered through 0.45 µm filters and degassed for 15 min. stock solutions (0.01M) of amino acid standards were prepared in triple distilled water and kept at 4 °C for five days. To prevent adhesion to

the glass, standardswere prepared in polyethylene vials. Working solutions were prepared daily by dilution of the stock solution. To obtain agents for derivatization, OPA (22 mg, Fluka) was dissolved in 0.5 ml of absolute ethanol and 0.9 ml of sodium tetraborate buffer (0.1M) adjusted to pH 10.4 with 5 M sodium hydroxide. The reaction of derivatization agent (20 µl) was carried out by reacting with 1 ml of amino acid standard for five min in a polyethylene vial before injection onto the column (Maciejak et al., 2004; Szyndler et al., 2006).

Statistical analysis

Repeated Measures ANOVA followed by Tukey's multiple comparison tests was applied for the statistical analysis of the data in order to account for the inter-subject variability and to facilitate the comparisons of within group as well as inter-group differences. Two ways ANOVA was used for statistical analysis of young and aged group. $P < 0.05$ was considered as statistically significant.

Results

Effect of menthol on time (in second) spent in target quadrant (TSTQ) using morris water maze test

The results of spatial memory with Morris water maze test are shown in figure 1. Old age and scopolamine severely impaired spatial cognition in the water maze task. Pretreatment of menthol significantly reversed memory defect in scopolamine and aging induced memory impairment in mice. Scopolamine injected on 10th day of water maze memory test model. Scopolamine were significant (P<0.001) decrease in time spent in target quadrant as compared to control group of young mice. Pretreatment with menthol showed (P<0.001) increase in time spent in target quadrant as compared to scopolamine treated group, indicating reversed the amnesia induced by scopolamine. Menthol (100 mg/kg, s.c.) showed significant improved in the time as compared to memory defected young and aged mice, indicating reversed the amnesia induced by natural ageing process. Higher dose of menthol means 200 mg/kg, s.c. and 400 mg/kg, s.c. shows decrease in time spent in target quadrant compared to 100 mg/kg, s.c. Menthol 100 mg/kg, s.c. shows significant shows the improvement in long term memory. Pretreatment with piracetam showed significant (P<0.001) improved long term memory as compared to scopolamine and control group of aged mice.

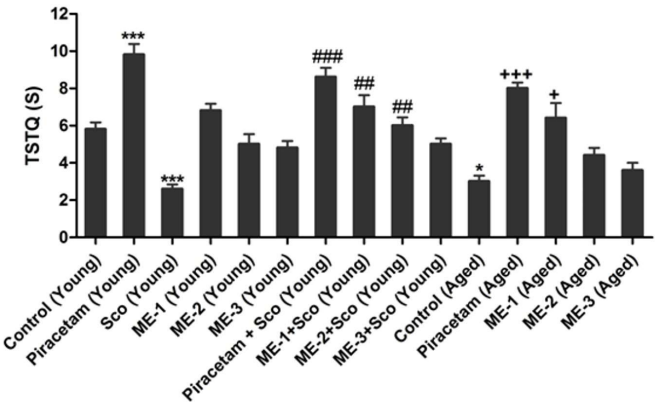

Figure 1. Effect of Menthol 100 mg/kg (ME-1), 200 mg/kg (ME-2), and 400 mg/kg (ME-3) on time spent in target quadrant (TSTQ) of young and aged mice using water maze. Values are mean ± SD (n=6, F=19.28). *** P<0.001 as compared to control young mice, * P<0.05 as compared to control young mice, ### P<0.001 as compared to scopolamine treated mice, ## P<0.01 as compared to scopolamine treated mice, +++ P<0.001 as compared to control aged mice, + P<0.05 as compared to control aged mice.

Effect of menthol on step down latency using passive avoidance

The examination of long term memory was tested by passive avoidance depicted in Figure 2. Scopolamine and control group of aged mice significant (P<0.001) decrease in step down latency as compared to control group of young mice. Among all three dose of menthol, menthol (100mg/kg, s.c.) shows significant improved in young, aged and scopolamine induced memory defect group of mice. Other dose means menthol (200 mg/kg, s.c.) and Menthol (400 mg/kg, s.c.) not shows improvement as compared to lower dose 100 mg/kg, s.c. it is as same as the result indicating in water maze long term memory model. Piracetam significant (P<0.01) improve step down latency as compared to young, aged and scopolamine induced group of young mice.

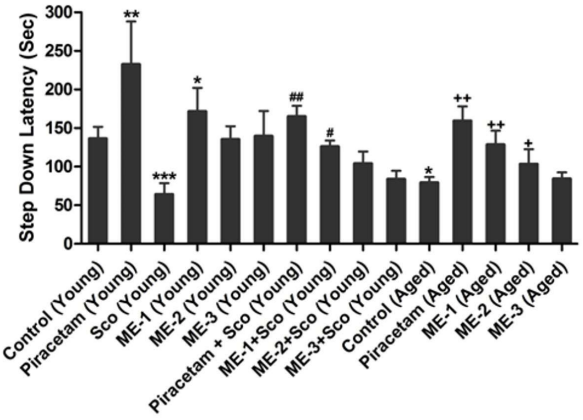

Figure 2. Effect of Menthol 100 mg/kg (ME-1), 200 mg/kg (ME-2), and 400 mg/kg (ME-3) on step down latency in sec of young and aged mice using passive avoidance. Values are mean ± SD (n=6, F=18.69). *** P<0.001 as compared to control young mice, ** P<0.01 as compared to control young mice, * P<0.05 as compared to control young mice, ## P<0.01 as compared to scopolamine treated mice, # P<0.05 as compared to scopolamine treated mice, ++ P<0.01 as compared to control aged mice, + P<0.05 as compared to control aged mice.

Effect of menthol on brain acetyl cholinesterase

The result of whole brain acetyl cholinesterase level

depicted in figure 3. Pre-treatment with menthol 100 mg/kg, s.c. shows significant (P<0.01) maintaining acetylcholine level as compared to control group of young and aged mice. Higher dose of Menthol (400 mg/kg, s.c.) not showing improvement as lower dose. Phenytoin (200 mg/kg, i.p.) was used as negative standard control. Phenytoin significantly increase acetyl cholinesterase level as compare to control group of young mice (P<0.01).

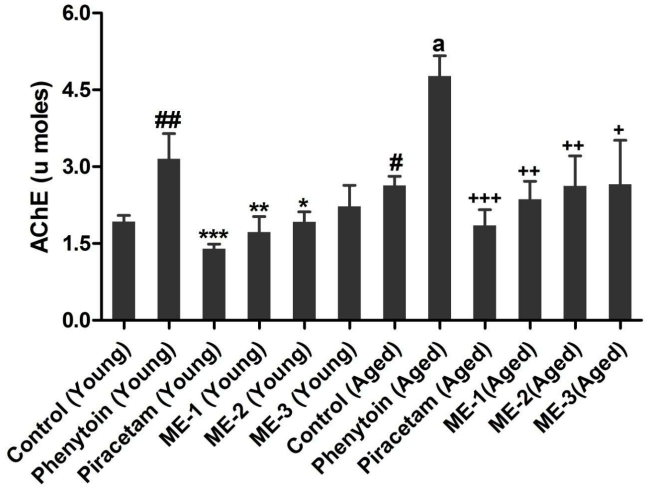

Figure 3. Effect of Menthol 100 mg/kg (ME-1), 200 mg/kg (ME-2), and 400 mg/kg (ME-3) on acetyl cholinesterase (AChE) activity of young and aged mice. Phenytoin was used as a negative standard control. Values are mean ± SD (n=6, F=4.54). ## P<0.01 as compared to control young mice, # P<0.05 as compared to control young mice, *** P<0.001 as compared to phenytoin treated young mice, ** P<0.01 as compared to phenytoin treated young mice, * P<0.05 as compared to phenytoin treated young mice, a P< 0.01 as compared to control aged mice, +++ P<0.001 as compared to phenytoin treated aged mice, ++ P<0.01 as compared to phenytoin treated aged mice, + P<0.05 as compared to phenytoin treated aged mice.

Brain glutathione

Scopolamine administered to young mice on 10th day, (P<0.01) reduced glutathione content, indicating increase in brain oxidant activity. Pre-treatment with menthol showed significant (P<0.01) increase in glutathione content, reversed the oxidant activity as compared to scopolamine treated group of young mice. Piracetam significant (P<0.001) reversed the oxidation produced by scopolamine. Ageing induced amnesia significant (P<0.05) decrease in glutathione content as compared to control group of young mice. Pre-treatment with menthol (100 mg/kg, s.c.) significant improved in glutathione content as compared to control group of young mice (P<0.05) and scopolamine induced memory defect in mice (P<0.01). Menthol (200 mg/kg, s.c.) significant increase in glutathione content as compared to scopolamine group. Higher dose of menthol (400 mg/kg, s.c.) is maintain the glutathione level in young mice. All dose of menthol was maintaining the glutathione content as compared to control group of aged mice (Figure 4).

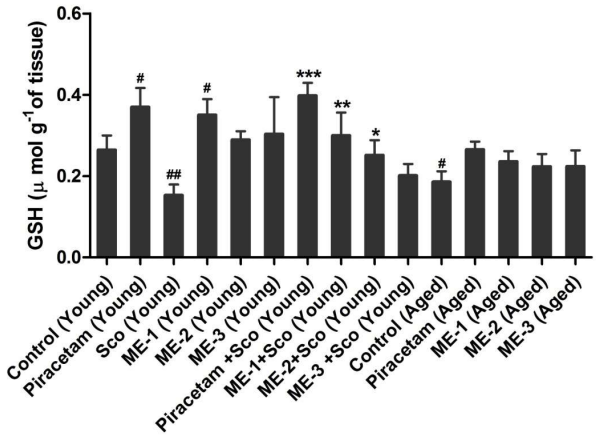

Figure 4. Effect of Menthol 100mg/kg (ME-1), 200mg/kg (ME-2), 400mg/kg (ME-3) on contents of glutathione (GSH) of young and aged mice. Values are mean ± SD (n=6, F=13.99). ## P<0.01 compared to control (Young), # P<0.05 compared to control (Young), ***P<0.001 as compared to scopolamine treated mice, ** P<0.01 as compared to scopolamine treated mice, *P<0.05 as compared to scopolamine treated mice.

Brain lipid peroxidation

Scopolamine and aging of mice showed significant (P<0.05) increase in malondialdehyde content indicating oxidant activity in mice brain. Pre-treatment with piracetam significant (P<0.001) reverse the oxidant activity induced by natural aging and scopolamine. Lower dose of menthol (100 mg/kg, s.c.) significant (P<0.01) reduced in oxidation process induced by scopolamine and natural aging process. Piracetam and menthol were showed significantly reduced, natural ageing induced oxidant activity (Figure 5).

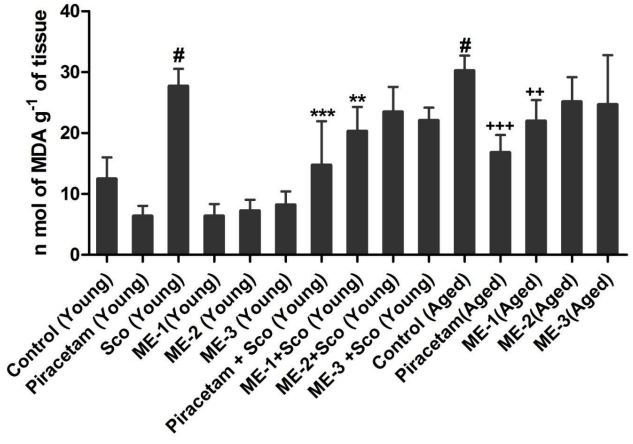

Figure 5. Effect of Menthol 100mg/kg (ME-1), 200mg/kg (ME-2) and 400mg/kg (ME-3) on lipid peroxidation for contents of brain malondialdehyde (MDA) of young and aged mice. Values are mean ± SD (n=6, F=21.30). # P<0.001 compared to control (Young), *** P<0.001 as compared to scopolamine (Sco) treated mice, ** P<0.01 as compared to scopolamine (Sco), +++ P<0.001 compared to control (Aged), ++ P<0.01 compared to control (Aged).

Effect of menthol on brain glutamate level

Table 1. Effect of Menthol on Scopolamine (Sco) and aging induced memory impairment of glutamate level of mice brain.

Groups	Glutamate		
	Cortex	**Hippocampus**	**Hypothalamus**
Control (Y)	1643±155.7	305±26.4	1524±89.2
Sco (Y)	618±55.8[a]	67±16.2[a]	638±46.9[a]
Pira+Sco (Y)	1546±71.1[***]	283±43.1[***]	1594±53.1[***]
ME-1+Sco (Y)	1237±122.4[**]	215±11.6 [**]	1292±120.3[**]
ME-2+Sco (Y)	1014±99.6[**]	196±38.1[**]	1033±147.8[**]
ME-3+Sco (Y)	677±43.8	98±16.9	842±67.5
Control (A)	841±48.2[a]	92±9.1[a]	946±24.8[a]
Pira (A)	1622±133.8[###]	256±23.4[###]	1281±15.7[##]
ME-1 (A)	1333±142.1[##]	176±38.5[##]	1001±36.2[#]
ME-2 (A)	901±98.7	125±24.5[#]	924±29.6
ME-3 (A)	762±63.9	82±16.4	836±49.1

Menthol at 100mg/kg (ME-1), 200mg/kg (ME-2), 400mg/kg (ME-3). Values are mean ± SD, nM per gram of wet brain tissue, [a]P<0.001 compared to control young, ***P<0.001 compared to Scopolamine treated young group, **P<0.01 compared to Scopolamine treated young group, ### P<0.001 compared to control aged, ## P<0.01 compared to control aged, # P<0.05 compared to control aged.

Scopolamine and natural aging induced memory defect group significant (P<0.001) reduced concentration of glutamate in cortex, hippocampus and hypothalamus. It is indicating memory defect in brain parts. Piracetam was used as standard drug was significant (P<0.001) improve in brain glutamate level as compared to scopolamine and control group of aged mice. All result showing that the lower dose of menthol (100 mg/kg, s.c.) significant (P<0.01) increase in glutamate level as compared to scopolamine and control group of aged mice. All dose of menthol significantly improve glutamate level in hippocampus (Table 1).

Discussion

Cholinergic transmission at muscarinic acetylcholine receptor has been implicated in higher brain functions such as learning and memory, and loss of synapses may contribute to the symptoms of Alzheimer disease (Levey, 1996; Biegon et al., 1986; Perry et al., 1978; Perry, 1994). Functional or physiologic damage of a neuron induces impairment of cognitive brain functions (Kim et al., 2005).

Nootropic drugs belong to the category of psychotropic agents with selective facilitator effect on intellectual performance, learning and memory. A number of drugs including piracetam have now been introduced in therapy to ameliorate cognitive deficits (Giurgea, 1973). Piracetam has been used to treat various dementias for several years as it enhances or facilitates various learning and other cognitive functions. It increases the intracellular ATP concentration in the nerve cell which may have an inhibitory effect over the ATP gated potassium channels. It also exhibited similar anticholinesterase inhibition activity, thus our report falls in line with the already published scientific reports (Rehni and Rehni, 2004; Bhadania et al., 2012; Joshi and Parle, 2007).

Phenytoin is one of the cheapest and widely used anticonvulsants. But, as with many other antiepileptic drugs, it is known to adversely affect learning and memory (Smith, 1991; Aldenkamp, 1994; Sudha et al., 1995). Administration of Phenytoin has been shown to significantly impair learning and memory.

In this present study, menthol administered for 10 successive days to assess its dose dependent nootropic activity in marris water maze memory test model. The results show that the subcutaneous administration of menthol caused an increase in the spatial learning (acquisition) and memory (retrieval) as compared to scopolamine group using the Morris water maze task.

Morris water maze is a classical model for testing the anxiety-like behaviour in mice and rats. It was introduced by Morris and colleagues as a spatial localization or navigation task. The task has been used extensively to study the neurobiological mechanisms that underlie spatial learning and memory, age-associated changes in spatial navigation and the ability of nootropic agents to influence specific cognitive processes (Achliya et al., 2004).

Study showed menthol significantly reversed scopolamine and aging induced memory defect in mice. The potentiation of working memory was more profound with menthol (100 mg/kg, s.c.) than menthol (400 mg/kg,s.c.), aging and scopolamine group.

As we have discuss, the protection of neuron or

maintaining acetylcholine levels may be used one method for improving cognitive functions (Gibbs et al., 2004; Riekkinen et al., 1998). It has been suggested that cholinergic neurons are closely related to cognitive functions like learning and memory (Zhang et al., 2002). Menthol (100 mg/kg, s.c.) improve spatial learning and memory compared to higher dose of menthol (400mg/kg, s.c.) by measuring acetyl cholinesterase level in mouse brain. Phenytoin was given to separate group showed significant increase in acetyl cholinesterase level.

Increase in oxidative stress would also contribute to progressive impairment of the antioxidant reserves of the brain. Glutathione is an important antioxidant that limits oxidative damage caused by reactive oxygen species. Several studies have suggested that these increases in oxidative stress vulnerability and the resulting neuronal loss can be reduced through dietary supplementation of plant extracts that prevent brain atrophy as well as learning and memory impairments (Parihar and Hemnani, 2003). The extent of excitotoxic neuronal damage was measured by assaying lipid peroxidation and glutathione. Long term menthol treatment shows opposite effect as our earlier research on short term therapy (Bhadania et al., 2012). Results show that lower dose of menthol (100 mg/kg, s.c.) profoundly neuro protective level of glutathione and melondialdehyde level as compared to scopolamine group and higher dose of menthol. Lower dose for long time therapy increase the neuro protective effect. It indicates that menthol improves long term memory by acetyl cholinesterase and antioxidant activity of mouse brain.

Excitatory amino acids are very well known to modulate memory processes. Particularly important role in this respect is ascribed to the glutamate, N-methyl –D-aspartate (NMDA) receptor and glycine. The NMDA- receptor requires low concentration of glycine as a co-agonist, in addition to glutamate. The entry of excessive amounts of Ca^{2+} produced by NMDA-receptor, activation can result in neuronal cell death or glutamate induced excitotoxocity (Rang et al., 2007). Glutamate is the major excitatory neurotransmitter in the brain and over stimulation of the glutamate receptors and NMDA may cause neuronal death in neurodegenerative diseases. Mitochondrial have critical cellular functions that influence neuronal excitability, such as regulation of Ca^{2+} homeostasis and ATP production to maintain $Na^{+}K^{+}$ATPase in the central nervous system. The mitochondria are also the primary site of reactive oxygen species production and oxidative stress can induce cellular damage (Santos et al., 2014).

Behavior based on negative reinforcement was recorded to examine long-term memory by passive avoidance memory model. Passive avoidance apparatus is a punishment-based exteroceptive model used to test long-term memory (Milind and Nirmal, 2004). The results are same as water maze test. Long term therapy of higher dose of menthol (400 mg/kg, s.c.) decrease the step down latency. To know the effect of different doses the brain amino acid measurement was done. Glutamate level of menthol (100mg/kg, s.c.) in cortex, hippocampus and hypothalamus shows significant improve as compared to scopolamine and aging induced amnesia. Higher dose of menthol 200mg/kg, s.c. and 400 mg/kg, s.c. were not showing proper improvement in glutamate level of brain parts. It indicating that lower dose of menthol (100 mg/kg, s.c.) increases the glutamatergic excitatory neuronal effect. This study sought to determine the neuroprotective effect of lower dose of menthol against higher dose of glutamate toxicity in different brain region. This excitotoxicity may be due to using specific inhibitors of glutamate channels. Menthol decrease intracellular melondialdehyde, most likely by mechanisms involving NMDA and intracellular Ca^{2+} and prevents mitochondrial dysfunction and impairments in $Na^{+}K^{+}$ATPase and glutamine synthesis activity after glutamate activation (Santos et al., 2014).

The results falls within line that menthol (400 mg/kg, s.c.) shows less neuroprotective compare to menthol (100 mg/kg, s.c.). Menthol (100 mg/kg, s.c.) shows highly anticholinesterase activity, indicating improving acetylcholine level in mouse brain. It indicating slow dose for long time give better result. Menthol increases glutamate and acetylcholine level, as a great sign of neuron protective agent.

Conflict of Interest: None

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