Original Article

α-Pinene Influence on Pulpal Pain-Induced Learning and Memory Impairment in Rats Via Modulation of the GABAA Receptor

Abstract

Background: This study investigated the effect of central administration of α-pinene and the interaction of α-pinene with GABAA receptor on pulpal nociception-induced changes in learning and memory performances in rats. **Materials and Methods:** Sixty-six adult male Wistar rats were used. Pulpal nociception was induced by intradental application of capsaicin (100 μg/rat). α-pinene (0.1, 0.2, and 0.4 μg/rat) was injected centrally 10 min before the administration of capsaicin. In addition, α-pinene (0.4 μg/rat) was co-injected with bicuculline (0.5 μg/rat). Spatial and passive avoidance learning and memory were assessed using Morris water maze (MWM) and shuttle box tasks, respectively. **Results:** Experimental results of the MWM test showed that capsaicin increases escape latency and distance traveled to the hidden platform ($P < 0.01$). The effect was prohibited by α-pinene at the dose of 0.4 μg/rat. Moreover, capsaicin-treated animals spent less time in the target zone than capsaicin + α-pinene (0.4 μg/rat)-treated rats ($P < 0.05$). In the shuttle box test, α-pinene (0.2 μg and 0.4 μg) prevented an increased number of acquisition trials and time spent in the dark chamber induced by capsaicin, whereas it increased step-through latency ($P < 0.01$). However, the effects of α-pinene (0.4 μg/rat) in both tests were prohibited by bicuculline (0.5 μg/rat). **Conclusion:** The data showed that central administration of α-pinene might reduce pulpalgia-induced learning and memory impairment, at least partially, via modulation of $GABA_A$ receptors.

Keywords: *Alpha-pinene, bicuculline, dental pulp, learning, memory, pain*

Forouzan Rafie, Razieh Kooshki[1], Mehdi Abbasnejad[2], Iran Rahbar[2], Maryam Raoof[3], Amir Hossein Nekouei[4]

Oral and Dental Diseases Research Center, Neuroscience Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, [2]Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, [4]Endodontology Research Center, Kerman University of Medical Sciences, Kerman, [1]Department of Biology, Faculty of Sciences, Lorestan University, Khorramabad, Iran, [3]Department of Orofacial Pain and Dysfunction, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

Address for correspondence:
Dr. Razieh Koshki, Department of Biology, Faculty of Sciences, Lorestan University, Khorramabad, Iran.
E-mail: kooshki.r@lu.ac.ir
Mr. Amir Hossein Nekouei, Endodontology Research Center, Kerman University of Medical Sciences, Kerman, Iran.
E-mail: nekouei.amir@gmail.com

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Introduction

Orofacial pain with a high degree of prevalence can arise from different regions that are innervated predominantly by various branches of the trigeminal nerve. Indeed, it is considered a major clinical problem and results in deleterious social and economic conditions.[1,2] Besides sensory features, trigeminal problems are often accompanied by substantial adverse neurobehavioral outcomes.[3,4] Dental pulp pain is the most common type of orofacial pain with considerable neurobehavioral dysfunctions including learning and memory deficits.[5,6]

Because of the difficulties and limitations of clinical trials, animal models are valuable tools for studying different aspects of pulpal nociception and pain-related neurobehavioral dysfunctions.[7,8] Capsaicin is considered well-suited for pulpal nociceptive assessments in rodents.[9,10] Capsaicin activates transient receptor potential cation channel subfamily V member 1 (TRPV1) on primary polymodal C and A-delta nociceptive fibers, resulting in increased calcium influx. The release of pro-nociceptive mediators in primary sensory nerves is then mediated.[11] It has been indicated that *in vitro* treatment with capsaicin prevents the stimulated release of calcitonin gene-related peptide from nerve terminals in the human dental pulp. The capsaicin effect has been inhibited with capsazepine, a TRPV1 antagonist.[9]

It has already been reported that capsaicin-evoked trigeminal nerves can disrupt memory acquisition, consolidation, and retention in rats.[6] Moreover, intradental administration of capsaicin stimulates the induction of pro-inflammatory and pro-apoptotic molecules in the hippocampus of rats. Whereas, capsaicin-induced cognitive impairments have been shown to be reversed by analgesic drugs.[6,12]

α-pinene, as the main component of essential oils from aromatic coniferous trees,[13] presents various therapeutic effects

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including anti-inflammatory, anticancer, and antibacterial activities.[14-16] Recent studies have focused on the capacity of α-pinene in modulating neurobehavioral responses. It has been reported that chronic oral administration of α-pinene suppresses 6-hydroxydopamine-induced passive avoidance learning and memory impairment in rats.[17] In addition, central administration of α-pinene attenuates capsaicin-evoked dental pulp nociception and inflammation.[18]

α-pinene also has an interface with neurotransmitter systems in the brain. It enhances the duration of nonrapid eye movement sleep, predominantly via the activation of $GABA_A$ receptors.[19] Moreover, α-pinene has anti-acetylcholinesterase activity, which may result in an increased acetylcholine transmission, a critical neurotransmitter for facilitating learning and memory.[20,21]

The effects of α-pinene on nociceptive signals and cognitive functions have been previously documented. However, the ability of α-pinene to modulate pain-related changes in learning and memory performances has not yet been understood. The present study was designed to investigate the effect of central administration of α-pinene on pain-induced changes in learning and memory performances using a rat model of capsaicin-evoked pulpal nociception. α-pinene interaction with $GABA_A$ receptors, in such conditions, was also evaluated.

Materials and Methods

Animals

This experimental study was carried out on adult male Wistar rats (230–250 g). The animals were maintained on a 12-h light: 12-h dark cycle, at a temperature of 22°C ± 2°C. Food and water were *ad libitum*.[5,6] Two-week before initiating behavioral experiments, the subject rats (in their home cage) were moved to the test room and habituated with lab environment at least 30 min/day. All ethical principles were considered in this study. The experimental procedures were permitted by the Ethical Committee of Kerman University of Medical Sciences, Kerman, Iran (IR.KMU.REC.1396.2535), which is based on standard ethical guidelines for care and use of laboratory animals.

Drugs

The drugs including α-pinene, bicuculline methiodide, and capsaicin were purchased from Sigma-Aldrich (Merk, Burlington, MA, United States). α-pinene was diluted in a normal saline solution. Bicuculline methiodide was dissolved in dimethyl sulfoxide and further diluted into the artificial cerebrospinal fluid. Capsaicin was dissolved in distilled water: ethanol: tween 80 (8:1:1) solution.[4]

Surgical

The rats were deeply anesthetized by intraperitoneal injection of ketamine (100 mg/kg) combined with xylazine (10 mg/kg).[22] The animals were fixed on a stereotaxic apparatus and stainless steel guide cannula (23G), which were bilaterally implanted in the lateral ventricle according to coordinates given by Paxinos and Watson atlas (0.9 mm posterior to the bregma, 1.8 mm lateral from the midline, and 3.8 mm depth to the cortical surface).[23] Before microinjection of the drugs, each rat was allowed at least 1 week to recover from surgery.

Microinjection

The drugs were injected into the lateral ventricles using a 30G stainless steel cannula connected by a polyethylene tube to a 1-μL Hamilton syringe. To reach the lateral ventricles, the injection needle was extended 1 mm beyond the tip of the guide cannula. All injections were delivered over a minute at a volume of 1 μL/side. The injection needle was left in place for 30 s to allow drug diffusion.

Experimental design

Experimental groups (n = 6 per group) were defined as follows: control group (no injection), sham group which was intracerebroventricularly (i. c. v.) injected by α-pinene vehicle (normal saline) and then received intradental capsaicin solvent; pain group that received intradental capsaicin solution (100 μg); α-pinene groups treated by α-pinene (0.1, 0.2, and 0.4 μg/rat/i. c. v.) 10 min prior to capsaicin application; bicuculline (0.5 μg/rat/i. c. v.) and bicuculline plus α-pinene-treated group that was administrated with bicuculline (0.5 μg/rat/i. c. v.) and then α-pinene (0.4 μg/rat/i. c. v.) before capsaicin application.[18]

Nociceptive induction

Dental pulp pain was induced by intradental application of capsaicin, as previously reported. Briefly, after short-duration anesthesia with a low concentration of carbon dioxide, a small cavity (2 mm^3) was prepared in the gingival third of the distal aspect of the left mandibular incisor's crowns using a small fissure bur in a high-speed handpiece with a water coolant. With the help of magnification (×2.5), pulp exposure was prevented. A small cotton pellet moistened with capsaicin solution (100 μg) was left in the cavity under a light-cured glass-ionomer (Fuji II, GC, Japan) restoration.[10]

Assessing spatial learning and memory

Spatial learning and memory were assessed by the Morris water maze (MWM) pool. It consisted of a dark circular pool (136 cm in diameter and 60 cm in height) filled with water (22°C ± 1°C) to a depth of 25 cm. The extra maze cues were placed in consistent locations on the walls which were visible to the rats. The pool was divided into four quadrants, defined by the four cardinal directions. A circular platform was located 2 cm below the water surface in the middle of one of the quadrants. At the beginning of the experiment, each rat was lightly placed in the water facing the wall of the pool from one

of the directions. The location of each rat was tracked by a digital TV system and analyzed using the EthoVision video tracking system (Noldus Information Technology, the Netherlands). One day before the beginning of training, the rats were adapted to the pool by allowing them to swim for 60 s without the platform.

The test included acquisition and probe trials. The acquisition test was performed on three consecutive blocks with four trials per block with a 5-min interval between the trials. The rats were allowed to swim within 60 s to find the hidden platform at each trial. Once the platform was found, the animal would have to stay on the platform for 30 s. If unsuccessful within 60 s, it was gently guided to the platform for 30 s. The escape latency traveled distance and velocity for each rat was evaluated. In the probe test, 24 h after the acquisition test, the hidden platform was removed from the pool, and the rats were placed in the quadrant opposite to the target quadrant and allowed to swim freely for 60 s. The time spent in the target quadrant was recorded and analyzed as a measure of spatial memory retention.

Assessing passive avoidance learning and memory

The shuttle box consists of two separate light and dark chambers. A plexiglass gate connects the chambers. The learning test included habituation and acquisition trials. In the habituation trial, for 10 min, the rats were individually located in the light chamber and permitted to freely enter the dark chamber. In the acquisition phase, each rat was placed in the light chamber and after 5 s, the guillotine gate was opened. Once the rats entered the dark chamber, the guillotine door was closed, and a constant electric shock (0.5 mA, 50 Hz, for 4 s) was applied via the grid floor. After 1 min, the rats were sent to their home cage and 5 min later, the same course was repeated. If the animals did not enter into the darkroom during 300 s, the acquisition of passive avoidance learning was considered successful. The number of acquisition sessions was recorded. After 24 h, in the retention test, the rats were placed in the light chamber and allowed to cross into the dark chamber. The maximum cutoff time for step-through latency (STL) and time spent in the dark chamber (TDC) was 300 s.

Assessing locomotor activity behaviors

The effect of central drug administration on the motor coordination of rats was assessed by an open field (OF) test. The OF test was performed in a square wooden box (70 cm × 70 cm) that was bordered by 30-cm-high walls. The rats were placed in the field and permitted to explore the maze space for 5 min. The locomotor activity of rats was measured by the total distance and velocity.

Statistical analysis

All data were presented as means ± standard error of the mean. The acquisition test data related to three training blocks of the MWM test were analyzed using repeated-measures analysis of variance (ANOVA). Statistically significant differences among groups in probe and shuttle box tests were assessed by one-way ANOVA followed by *post hoc* Tukey's test. $P < 0.05$ was regarded as statistically significant.

Results

Morris water maze

During the acquisition blocks, escape latency to find the hidden platform was significantly different among the groups (F [2, 230] = 19.003, $P = 0.001$). As shown in Figure 1a, in blocks 2 and 3, capsaicin-treated rats showed a reduced ability to find the hidden platform compared to that of control animals ($P < 0.01$). The capsaicin effect was prohibited by α-pinene at the dose of 0.4 μg but not 0.1 μg/rat and 0.2 μg/rat. Besides, microinjection of α-pinene (0.4 μg/rat) significantly decreased the traveled distance to find the hidden platform during the second ($P < 0.01$) and third ($P < 0.001$) blocks of acquisition in comparison to the capsaicin and capsaicin plus α-pinene (0.1 μg/rat and 0.2 μg/rat)-treated rats [Figure 1b].

Central pretreatment with bicuculline at the dose of 0.5 μg exhibited no significant effect on escape latency and distance traveled to reach the hidden platform in rats treated with capsaicin ($P < 0.01$) [Figure 2a]. However, bicuculline blocked improvements in the escape latency

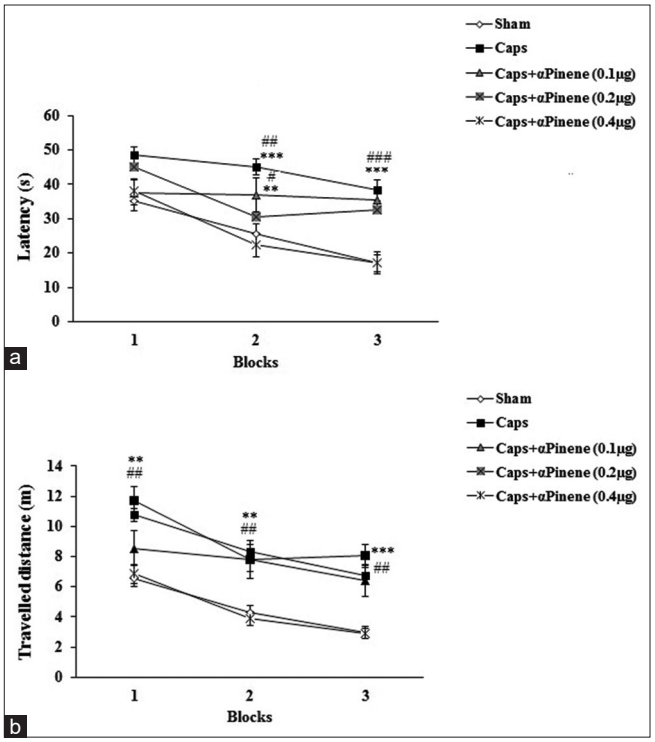

Figure 1: Effects of central administration of α-pinene (0.1, 0.2, and 0.4 μg) on escape latency (a) and distance traveled (b) to reach the hidden platform in Morris water maze test. Data were presented as mean ± standard error of mean. ***$P < 0.001$, **$P < 0.01$ versus sham, ###$P < 0.001$, ##$P < 0.01$, #$P < 0.05$ Caps+ α-pinene (0.4 μg)-treated group. Caps: capsaicin

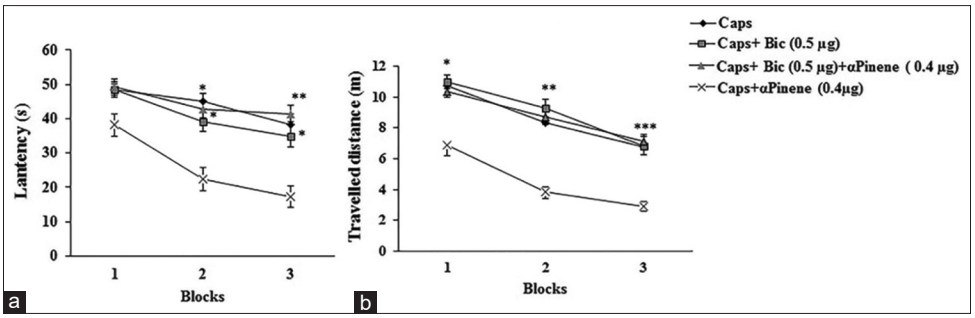

Figure 2: Effects of co-administration of bicuculline (0.5 µg) and α-pinene (0.4 µg) on escape latency (a) and distance traveled (b) to reach the hidden platform in Morris water maze test. Data were presented as mean ± standard error of mean. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ versus Caps+ α-pinene (0.4 µg) group. Caps: capsaicin, BIC: bicuculline

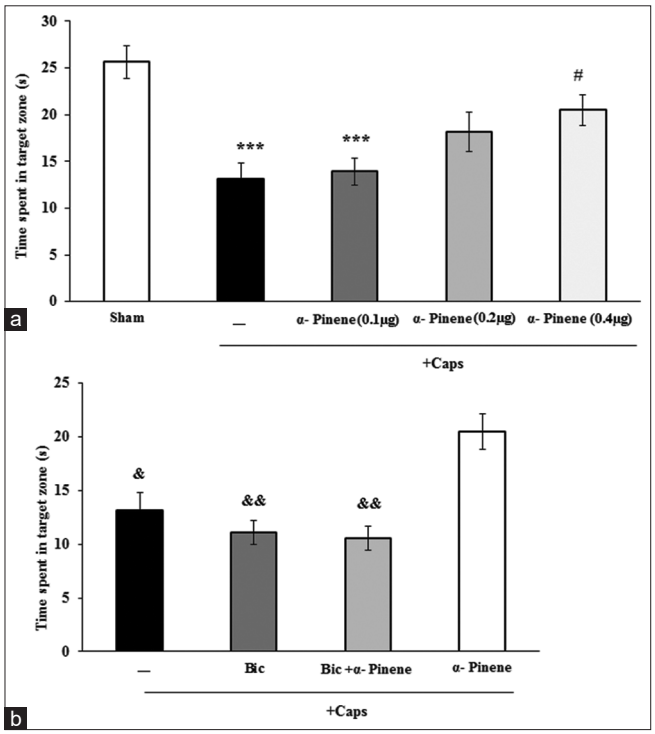

Figure 3: Effects of central administration of α-pinene (0.1, 0.2, and 0.4 µg) (a) and bicuculline (0.5 µM) plus α-pinene (0.4 µg) (b) on swimming speed in the Morris water maze test. Data were presented as mean ± standard error of mean. Caps: Capsaicin, BIC: Bicuculline

and distance traveled to reach the platform during acquisition trials produced by α-pinene (0.4 µg/rat) in capsaicin-treated rats ($P < 0.01$) [Figure 2a and b]. The groups showed no significant differences in the swimming speed [Figure 3a and b].

The probe test results showed that capsaicin-treated animals spent significantly less time in the target zone than sham ($P < 0.001$) and capsaicin + α-pinene (0.4 µg/rat) ($P < 0.05$) groups [Figure 4a]. In addition, as shown in Figure 4b, animals receiving bicuculline (0.5 µg/rat) before α-pinene (0.4 µg/rat), showed a significantly decreased time spent in the target zone as compared to those in the α-pinene (0.4 µg/rat) plus capsaicin-treated group ($P < 0.05$).

Passive avoidance

The number of trials in the acquisition step significantly increased in rats interdentally treated by capsaicin compared with that of the sham group ($P < 0.05$) [Figure 5a]. In the retention trial, capsaicin-treated rats showed decreased STL and increased TDC as compared to those of the sham group (both $P < 0.001$) [Figure 5b and c]. As shown in Figure 4a, α-pinene (0.2 µg/rat and 0.4 µg/rat) could prohibit the increased number of acquisition trials induced by capsaicin ($P < 0.01$). In the retention trial, capsaicin-induced decreased STL ($P < 0.01$) and increased TDC ($P < 0.05$) were attenuated by α-pinene (0.4 µg/rat) [Figure 5b and c].

As displayed in Figure 5a, the number of acquisition trials, STL, and TDC did not alter following central blockage of $GABA_A$ receptors by bicuculline at the dose of 0.5 µg. However, bicuculline (0.5 µg/rat) prevented α-pinene (0.4 µg/rat), decreased the number of acquisition trials and TDC, and increased STL in rats treated by capsaicin [Figure 6].

Assessment of locomotor activity

Central administration of α-pinene, at all doses, bicuculline (0.5 µg/rat), and bicuculline (0.5 µg/rat) + α-pinene (0.4 µg/rat) could not change the velocity (F [6, 41] = 0.563, $P = 0.757$) and the total traveled distance (F [6, 41] = 2.071, $P = 0.082$) in capsaicin-treated rats exposed to the OF test [Figure 7a and b].

Discussion

The current study determined a dose-related preventive effect of the central administration of α-pinene on memory consolidation and retention impairment induced by intradental application of capsaicin. This was shown by decreased escape latency time and traveled distance to find the platform in the MWM test, increased STL, and decreased TDC in the shuttle box test. However, the effects of α-pinene at the dose of 0.4 µg, which was the most effective dose, were prevented by the blockage of $GABA_A$ receptor by a noneffective dose of bicuculline (0.5 µg).

There is some evidence to support that α-pinene has neuronal proficiency. Especially, α-pinene showed

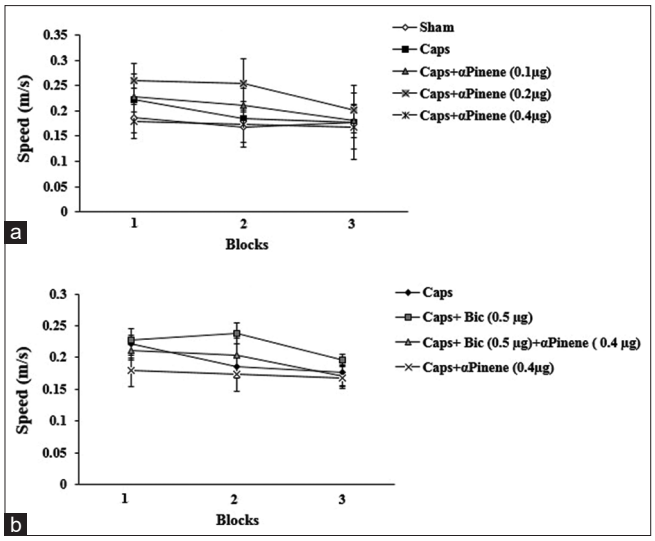

Figure 4: Effects of central administration of α-pinene (0.1, 0.2, and 0.4 μg) (a) and bicuculline (0.5 μM) plus α-pinene (0.4 μg) (b) on time spent in target zone in probe trial. Data were presented as mean ± standard error of mean. ******P* **< 0.001 versus sham group, #*P* **< 0.05 versus Caps, &&***P* **< 0.01, &***P* **< 0.05 versus Caps+ α-pinene (0.4 μg). Caps: capsaicin, BIC: bicuculline**

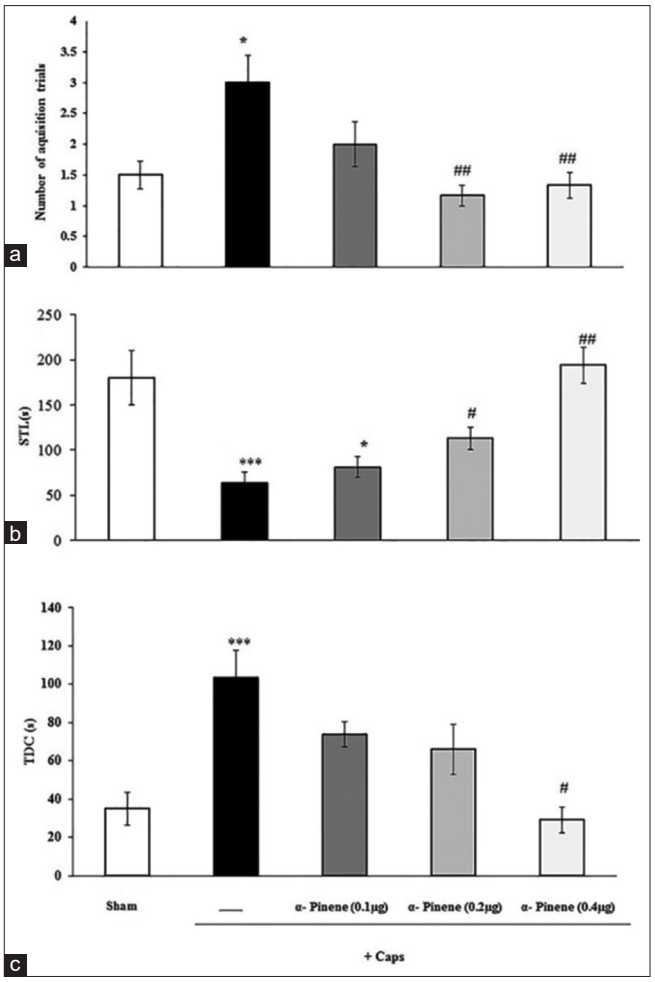

Figure 5: Effects of central administration of α-pinene on passive avoidance acquisition (a) and retention performances (b and c) of capsaicin-treated rats. Data were presented as mean ± standard error of mean. ******P* **< 0.001, ****P* **< 0.05 versus sham group, ##*P* **< 0.01, #***P* **< 0.05 versus caps group. STL: Step-through latency, TDC: Time spent in the dark chamber, Caps: capsaicin**

antioxidant, anti-anxiety, and anticancer activities.[14,15,24] Inconsistent with the current study results, Goudarzi and Rafieirad, using the 6-hydroxydopamine model of Parkinson's disease, showed that chronic daily administration of oral α-pinene for 2 weeks increases passive avoidance performance in rats.[17] In addition, it has been reported that systemic administration of α-pinene significantly improves scopolamine-induced cognitive dysfunction in mice. The effect was associated with enhanced levels of hippocampal antioxidant enzymes and choline acetyltransferase mRNA expression in the cortex of mice.[21] To the best of our knowledge, no study has demonstrated *α*-pinene association with learning and memory processing in humans.

High levels of oxidative markers during chemical-induced inflammation, disrupt neurobehavioral performances including learning and memory.[25] Antioxidant phytochemicals suppress cognitive deficiency and pain stimulus sensitivity in some neuroinflammatory situations.[26,27] Accumulating evidence indicates that *α*-pinene is a potent antioxidant. α-pinene can also improve memory performance in Parkinsonian rats by decreasing hippocampal and striatal levels of malondialdehyde as an oxidative stress marker.[17] Moreover, α-pinene at low concentrations can increase total antioxidant capacity in cultured primary neurons.[28] Similarly, intraperitoneal administration of α-pinene increased mRNA expression of choline acetyltransferase in the cortex and protein levels of antioxidant enzymes.[21] Therefore, it is likely that α-pinene antioxidant property, at least in part, is responsible for reduced memory deficits due to pulpal nociception.

Interestingly, it has been reported that natural antioxidants exert neuroprotective functions via the modulation of brain neurotransmitter systems including $GABA_A$ receptor.[26,29] According to Figure 5, the effects of α-pinene are prevented by the $GABA_A$ receptor antagonist, suggesting the interaction of α-pinene with the GABAergic system. It has been previously reported that α-pinene has a specific binding site on aromatic residues of $GABA_A$-benzodiazepine receptors α1-and -γ2 subunits. Oral administration of α-pinene enhances the duration of nonrapid eye movement sleep, predominantly via the activation of $GABA_A$ receptors.[19] A recent study indicated that capsaicin-induced pulpal nociception and COX-2 induction are reduced by the central administration of α-pinene. In addition, blocking the $GABA_A$ receptors suppressed the mentioned effect.[18]

Tonic activities of $GABA_A$ receptors are essential for optimal regulation of various aspects of learning and memory.[30,31] It has been indicated that central blocking of $GABA_A$ receptors can improve learning and memory. This may be mediated via increased induction of

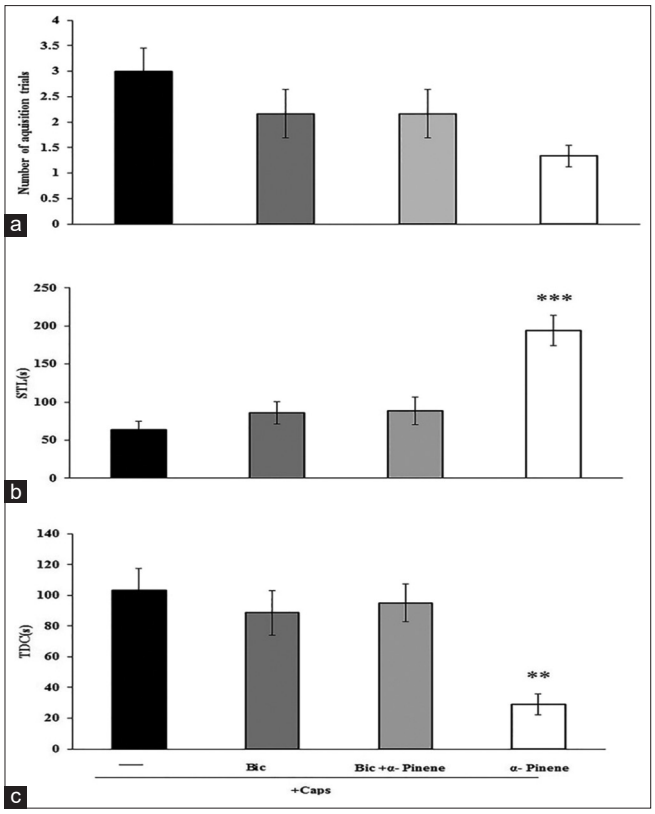

Figure 6: Effects of co-administration of bicuculline (0.5 μg) and α-pinene (0.4 μg) on passive avoidance acquisition (a) and retention performances (b and c) of capsaicin-treated rats. Data were presented as mean ± standard error of mean. ***$P < 0.001$, **$P < 0.01$ versus other groups. STL: Step-through latency, TDC: Time spent in the dark chamber, Caps: Capsaicin, BIC: Bicuculline

synaptic mediators and activation of excitatory inputs to neuronal networks involved in learning and memory.[32,33] Interestingly, α-pinene inhalation for one hour could increase the expression of brain-derived neurotrophic factor (BDNF) mRNA, the most important molecule involved in memory storage and retrieval, in the olfactory bulb and hippocampus of mice.[34] Chemical inflammation induced by formalin and complete Freund's adjuvant as well as capsaicin has been shown to disrupt rat's spatial learning and memory performance via hippocampal downregulation of BDNF.[35] Thus, it is possible to assume that α-pinene interaction with $GABA_A$ receptors could modulate pulpal pain-induced learning and memory impairment.

In this study, neither α-pinene nor bicuculline exhibited a measurable change in the locomotor activity in capsaicin-treated rats. However, it has been already reported that systemic α-pinene (0.2 and 0.4 mg/kg) can decrease the locomotor activity of intact rats in the OF test.[24] Some studies revealed heightened locomotor-activating effects of bicuculline following direct brain administration. However, this extremely dose-related effect was also influenced by the injection site and the number of injection trials.[36-38] Further experiments are still required to better understand

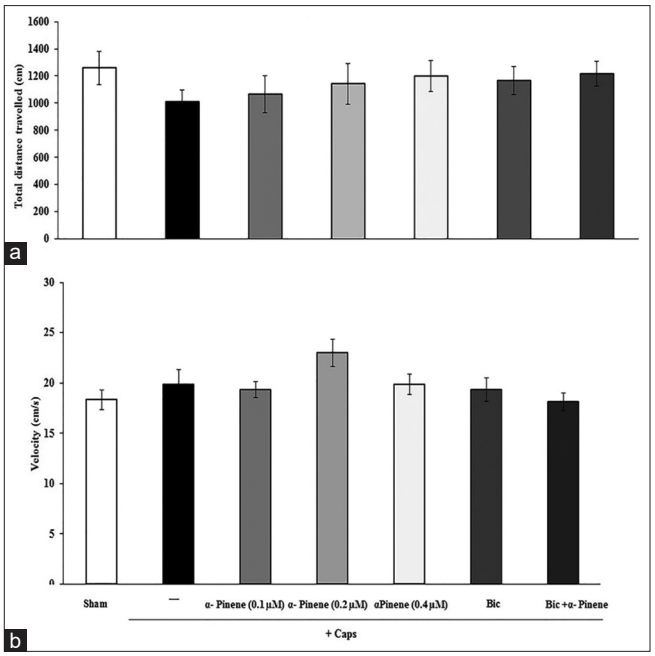

Figure 7: Comparison of the total distance traveled (a) and velocity (b) between the experimental groups in open field test. Data were presented as mean ± standard error of mean. Caps: Capsaicin, BIC: Bicuculline

the effects of α-pinene and bicuculline on locomotor behaviors.

Conclusion

The data show that central administration of α-pinene suppresses capsaicin-induced learning and memory impairment in rats. This effect is at least partially mediated through $GABA_A$ receptors.

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Conflicts of interest

There are no conflicts of interest.

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