RESEARCH ARTICLE

Role of Piperine in Cognitive Behavior and the Level of Nicotinic Receptors (nAChRs) in Mouse Brain

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Abstract

Piperine is an alkaloid found in black pepper and related plants, which has been used in traditional medicine for improving memory and learning. However, its mechanism of action remains unclear. The objective of this study was to investigate the role of piperine in modulating cholinergic function related to memory and learning in normal mice by using the Morris water maze (MWM) test, a behavioral tool commonly used to study spatial learning and memory. The results showed that repeated intraperitoneal injections of piperine at 5, 10, and 20 mg/kg/day for 2 weeks in mice decreased escape latency (11.00 ± 1.05, 10.00 ± 0.43 and 4.00 ± 0.23 vs. 32.00 ± 4.49 s, respectively) and increased retention time (32.00 ± 0.73, 31.00 ± 0.62 and 32.00 ± 1.28 vs. 19.00 ± 0.54 s, respectively) compared with controls. Significant increases in nicotinic acetylcholine receptor levels (nAChRs) in mouse brains of all piperine-treated groups were also observed (28.09 ± 13.73, 19.55 ± 2.60 and 18.20 ± 2.13 vs. 15.97 ± 3.04 fmol/mg, respectively; $p < 0.05$). This indicates that the possible mechanism of action of piperine for improving cognitive function and memory in mice may involve increased brain nAChRs. These findings suggest that piperine might be useful for further development of compounds that improves learning and memory in patients with cognitive impairment.

Keywords: Piperine, Morris water maze test, MWM, nicotinic acetylcholine receptor, nAChR, learning and memory

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บทบาทของพิเพอรีนต่อพฤติกรรมการเรียนรู้และระดับของตัวรับนิโคตินิกในสมอง ของหนูขาวเล็ก

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บทคัดย่อ

พิเพอรีนจัดเป็นสารในกลุ่มอัลคาลอยด์ที่สามารถพบได้ในพริกไทยดำและพืชชนิดอื่นที่ ใกล้เคียง นิยมใช้เพิ่มความจำและการเรียนรู้ แต่กลไกการออกฤทธิ์ยังไม่ชัดเจน การศึกษานี้มี วัตถุประสงค์เพื่อศึกษาผลของพิเพอรีนต่อกระบวนการเรียนรู้และความจำในสัตว์ทดลองโดยใช้ มอร์ริสวอเทอร์เมซ (Morris water maze) ซึ่งเป็นเครื่องมือเชิงพฤติกรรมที่นิยมใช้ในการ ประเมินผลการเรียนรู้และความจำในหนูทดลอง ผลการทดสอบพบว่า การให้พิเพอรีนเข้าทางช่อง ท้องหนูขาวเล็กที่ความเข้มข้น 5, 10 และ 20 มิลลิกรัม/กิโลกรัม/วัน เป็นเวลานาน 2 สัปดาห์ สามารถลด escape latency (11.00±1.05, 10.00±0.43 และ 4.00±0.23 เทียบกับ 32.00±4.49 วินาที ตามลำดับ) และเพิ่ม retention time (32.00±0.73, 31.00±0.62 และ 32.00±1.28 เทียบกับ 19.00±0.54 วินาที ตามลำดับ) ของหนูขาวเล็กได้อย่างมีนัยสำคัญเมื่อเทียบกับกลุ่ม ควบคุม นอกจากนี้ยังสามารถเพิ่มระดับของตัวรับนิโคตินิกอะเซติลโคลีนในสมองของหนูขาวเล็ก (28.09±13.73, 19.55±2.60 และ18.20±2.13 เทียบกับ 15.97±3.04 เฟมโตโมล/มิลลิกรัม ตามลำดับ) อย่างมีนัยสำคัญ (p<0.05) จากการศึกษานี้สรุปได้ว่า ผลของพิเพอรีนในเพิ่มการ เรียนรู้และความจำในหนูขาวเล็กอาจเกี่ยวข้องกับการเพิ่มขึ้นของตัวรับนิโคตินิกอะเซติลโคลีนใน สมอง และพิเพอรีนอาจมีประโยชน์ในการพัฒนาเป็นยาเพิ่มการเรียนรู้และความจำในผู้ป่วยที่มี ความบกพร่องทางการเรียนรู้ต่อไป

คำสำคัญ: พิเพอรีน, มอร์ริสวอเทอร์เมซ, ตัวรับนิโคตินิกอะเซติลโคลีน, การเรียนรู้และความจำ

Introduction

Alzheimer's disease (AD) is a progressive brain disorder and the most common neurodegenerative disorder affecting higher brain functions such as memory and learning skills. There are more than 20 million AD sufferers worldwide.[1] Patients with AD exhibit a progressive deterioration of memory and cognitive function, which is finally leading to severe dementia, and death. The typical neuropathological features of AD are loss of neurons and synapses in the temporal, parietal and frontal lobe, resulting in gross atrophy of these three regions accompanied by cognitive decline.[2] The pathophysiology of AD is associated with abnormal binding of neurotransmitters, receptors and their signal cascades. There are many hypotheses for the etiology of AD including central cholinergic dysfunction, mitochondrial and vascular insufficiency, and aberrant β-amyloid processing.[3]

The central cholinergic system is responsible for neuronal plasticity, arousal and reward. The loss of cholinergic fibers in the brain has long been recognized as an early process in the development of AD. Brainstem and basal forebrain cholinergic neurons project to many areas of the brain[3] and their impairment have been associated with the memory deficits seen in AD patients.[4] The action of acetyl-choline is mediated through brain nicotinic acetylcholine receptors (nAChRs), which involves neuronal development, learning and memory, and reward.[5] Neuronal nAChRs are post-synaptic excitatory neurotransmitter receptors. Most nAChRs are heteromeric, comprising both α and β subunits. About 90% of mammalian CNS nAChRs is α4β2 nAChR subtype[6], but α7, and α3β4 nAChRs found in some areas suggests that they may also be involved in the control of cell excitability at the postsynaptic level.[7] A recent study suggested that the loss of α7 nAChRs in transgenic mice and AD patients, accompanied by mutation of amyloid precursor protein (APP) and tau protein may trigger β-amyloid accumulation.[8] In AD brains, a reduced expression of nAChRs but no change in its mRNA were observed.[9,10] Thus, loss of nAChRs contributes to cognitive dysfunction in AD patient brains and in AD mouse model.[11] Since α4β2 and α3β4 nAChR heteromeric subtypes are associated with neurons that accumulate Aβ[12], the neuroprotection via nAChRs is an interesting target in the treatment of AD cognitive decline.

Most drugs for AD treatment relieve symptoms of patients by inhibiting the acetylcholinesterase enzyme (AChE), thereby causing acetylcholine (ACh) accumulation and promoting restoration of central cholinergic function. Donepezil remains the first-line of treatment in early AD. It is a reversible, noncompetitive and selective acetylcholinesterase inhibitor which readily crosses the blood-brain barrier and shows selectivity compared to other cholinesterase inhibitors i.e., tacrine or physostigmine.[13] Donepezil inhibits brain AChE activity causing an increased ACh in both normal and ACh-deficient animals.[14] In addition to AChE inhibition, donepezil also has neuroprotective effect by enhancing Akt and GSK-3β phosphorylation and reducing phosphorylation of tau and glycogen synthase.[15] Thus, donepezil may have ability to reduce β-amyloid (Aβ) toxicity.

However, donepezil only slows the progression of disease and it is very expensive. Donepezil also causes adverse reactions such as nausea, vomiting, and diarrhea. Medicinal plants are an interesting sources for developing medicines for the treatment of AD and/or improving memory and learning. Piperine is a natural

compound extracted from pepper, which has several effects, e.g., protection against pentylenetetrazole-induced seizure[16,17], potent antidepressant-like properties[18], and anti-inflammatory effect[19,20] and, in particular, improvement of memory and learning in animal models.[21,22] Nevertheless, its mechanism of action involved in cognitive improvement is still unclear. Therefore, this study aimed to investigate the role of piperine in cognitive behavior and level of nAChRs in mouse brain.

Materials and Methods

Materials and chemical

Piperine, donepezil and polyethyleneimine (PEI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Epibatidine, [5,6-bicycloheptyl-^{3}H] (specific activity = 46.2 Ci/mmol) was purchased from Perkin-Elmer (Perkin-Elmer NEN, Shelton, CT, USA). All chemicals were analytical grade.

Animals and drug administration

Male ICR mice (20-22 g) were purchased from National Laboratory Animal Center, Nakhon Pathom, Thailand. They were housed in groups of six per cage in standard cages (7×11×5 inches) under controlled condition (at 24±2°C, 12:12 h light–dark cycle). Food and water were provided *ad libitum*. All mice were allowed at least 1 week to acclimatize to their housing environment before each experiment. The experimental protocols were approved by Naresuan University Animal Care and Use Committee (NUACUC).

Each group (n=6) was given normal saline, donepezil 1 mg/kg body weight, or piperine (5, 10 and 20 mg/kg body weight) intraperitoneally once daily for 14 days.

Behavior testing by Morris water maze (MWM) test

The MWM test is a behavioral tool widely used for assessing spatial learning and memory. Mice were placed in a large circular pool (69 cm diameter and 29 cm height with cues at the sides) filled with water to a depth of 20 cm and maintained at 25°C. The pool was divided into 4 quadrants. A 10 cm diameter escape platform was located in the middle of one quadrant and submerged 1.2-1.3 cm below the surface. Tapioca starch was added into the water to make the platform invisible from swimming mice.

Before MWM testing, mice were trained for 4 days (6 training trials per day with 20 min inter-trial intervals). On each day, each mouse was moved to the procedure room 30 min prior to training. The mouse was placed into the water and allowed to swim for up to 90 sec to locate the platform. "Finding the platform" was defined as staying on it for at least 2 sec. After staying on the platform for 10 sec, the animal was gently moved back to its home cage. The mouse that failed to find the platform within the allotted time was placed on the platform for 10 sec and then returned to its home cage.

MWM testing was performed on day 15 (after 14 days of treatment) and the escape latency (time to find the escape platform) was measured. On day 16, the platform was removed and the retention time (time to search for the non-existent platform) was determined. If the mouse swam near or in the quadrant where the

platform had been located, a retention time was assigned. A decrease in escape latency and increase in retention time would reflect cognitive enhancement.

Whole brain sample collection and preparation

Brain sample collection. Mice were sacrificed by rapid neck dislocation and the brains were quickly removed. The brain samples were stored in -80°C until used for [³H] epibatidine saturation binding assay.

Preparation of whole brain samples. The frozen brains were thawed on ice and homogenized on ice in 10 volumes of cold lysis buffer (50 mm Tris-HCl, pH 7.4 containing with protease and phosphatase inhibitor) using WiseStir®HS-30E homogenizing stirrer (Wisd Laboratory Instruments, Germany). The homogenate was centrifuged at 1,000×g for 10 min at 4°C. The supernatant was re-centrifuged at 40,000×g for 20 min at 4°C and the supernatant was decanted and then replaced with the same lysis buffer. Two or three homogenization-centrifugation cycles were performed in order to washout endogenous ligands. The final pellet was resuspended in the same buffer and homogenized again and stored at -80°C until performing the binding assay.

Measurement of nAChRs using [³H] epibatidine saturation binding assay

Epibatidine is a highly selective nAChR ligand used to assess the brain content of nAChRs. Ninty-six-well plates (a final volume of 125 µL per well) with Whatman GF/C glass-fiber filters were used in the saturation binding assay. Twenty-five microlitres of the [³H] epibatidine (10 nM) solution were added to each well followed by 25 µL of incubation buffer containing 140 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM HEPES and 0.1% bovine serum albumin (pH 7.5; for total binding) or 25 µL of 10 µM nicotine (for non-specific binding). The reaction was started by adding 75 µL of membrane protein (50 µg per well) and then incubated in the dark at room temperature for 90 min. After vacuum filtration, the reaction was stopped by adding cold 0.3% polyethyleneimine (PEI) using a 96-well harvester. Then the samples were washed 3 times with cold buffer. The amount of radioactivity in the samples was measured by a microplate liquid scintillation counter (TopCount NXT™, Perkin Elmer, MA, USA). The binding capacity (B_{max}) and equilibrium dissociation constant (K_D) values of [³H] epibatidine were determined by using GraphPad Prism® Version 5.00 (GraphPad Software, Inc., CA, USA).

Statistical analysis

Data were expressed as means±standard errors (SEM). The differences between sample means were analyzed using independent samples one-way ANOVA with a significance level of 5% ($\alpha = 0.05$).

Results

Piperine enhanced cognitive function in mice

In this study, we examined the effect of piperine on cognitive function in mice treated with either donepezil (1 mg/kg/day) or piperine (5-20 mg/kg/day) using MWM test. The results showed that piperine decreased escape latency in a dose-dependent manner with a significant result in the highest dose group (20

mg/kg/day) (Figure 1) and significantly increased retention time in all dose groups (Figure 2). The donepezil-treated group seemed to require less time to reach platform (Figure 1) and more time to spend at the position that platform had been placed (Figure 2) when compared to the control group without any significant differences. Therefore, our data indicated that piperine at all dosages used in this study has desirable effect on spatial memory and such effect occur more than donepezil.
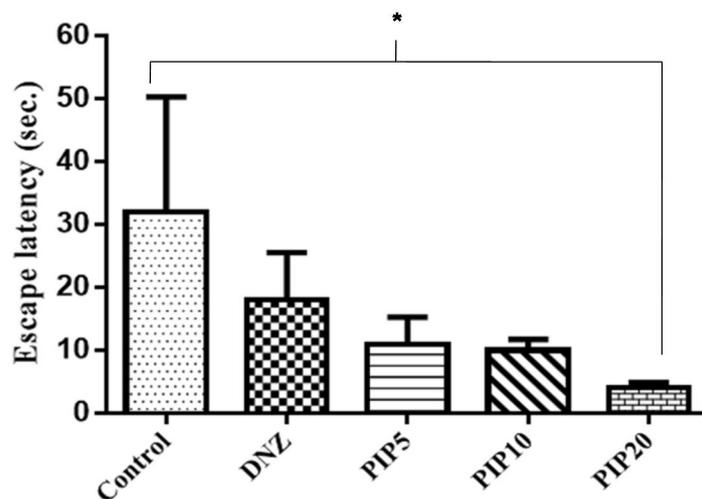
Figure 1.   Effect of piperine on escape latency in mice.
DNZ = donepezil (1 mg/kg/day); PIP5 = piperine (5 mg/kg/day); PIP10 = piperine (10 mg/kg/day); PIP20 = piperine (20 mg/kg/day). Data are expressed as mean ± SEM (N = 6, *$p < 0.05$ vs. control).
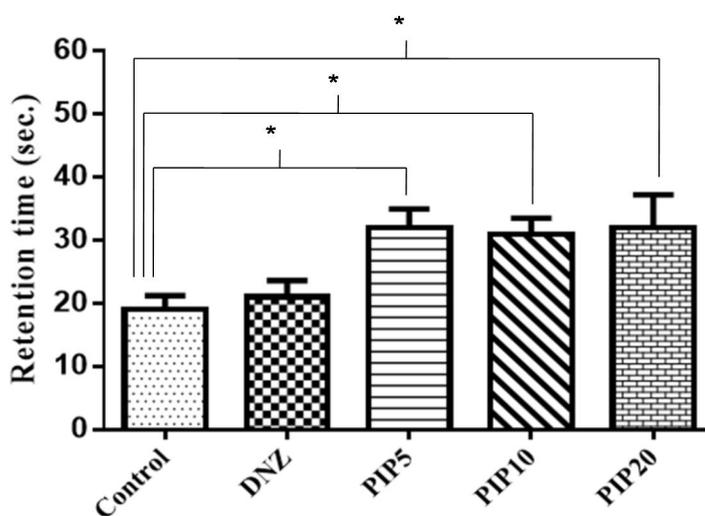
Figure 2.   Effect of piperine on retention time in mice.
DNZ = donepezil (1 mg/kg/day); PIP5 = piperine (5 mg/kg/day); PIP10 = piperine (10 mg/kg/day); PIP20 = piperine (20 mg/kg/day). Data are expressed as mean ± SEM (N = 6, *$p < 0.05$ vs. control).

Piperine increased nAChR level in mouse brain

In this study, we determined the nAChR level in mouse brain after PIP and donepezil administrations using [^{3}H] epibatidine saturation binding assay. The results showed that donepezil-treated group had the highest B_{max} (42.39±17.99 vs. 15.97±3.04 fmol/mg protein) and the highest K_D (3.03±1.69 vs. 0.99±0.33 nM) values when compared with the control group. The B_{max} of [^{3}H]epibatidine in the brains of mice receiving all doses of piperine were significantly greater than that in the brains of control group with the highest B_{max} at the dose of 5 mg/kg piperine. However, the K_D of [^{3}H]epibatidine determined in the brains of donepezil and piperine-treated groups were not significant differences from controls (Table 1).

Table 1. Saturation binding assay of [^{3}H] epibatidine to nAChRs in mouse brain.

Group	[^{3}H] Epibatidine Binding, fmol/mg protein			
	K_D, nM	SEM	B_{max}	SEM
Control	0.99	0.33	15.97	3.04
DNZ	3.03	1.69	42.39*	17.99
PIP5	2.02	1.43	28.09*	13.73
PIP10	0.76	0.19	19.55*	2.60
PIP20	0.67	0.15	18.20*	2.13

Note: DNZ = donepezil (1 mg/kg/day); PIP5 = piperine (5 mg/kg/day); PIP10 = piperine (10 mg/kg/day); PIP20 = piperine (20 mg/kg/day). Data are expressed as mean ± SEM (N = 6, *p < 0.05 vs. control).

Discussion

Piperine is an alkaloid extracted from Thai black pepper (5-10%), long pepper (1-2%) or pepper species.[23] Piperine is used as traditional herbal medicine with evidences in relieving symptoms of various diseases such as seizure[16,21], depression[25,26] or cognitive dysfunction.[27,28] Previous studies demonstrated efficacy of piperine in improving learning and memory in several mouse models.[21,29,30] However, mechanism of piperine involved with learning and memory still uncertain although preventing of lipid peroxidation and decreasing soluble β-amyloid were revealed to be associated with piperine administration.[21]

The Morris water maze (MWM) test is a behavioral neuroscience paradigm for screening the neural mechanisms of spatial learning and memory in rodents and widely used to investigate pharmacological actions on such behaviours[31,32] in rodent model of human disease. This study used MWM for determining the effect of piperine on memory improvement in mice compared to donepezil. Donepezil is a potent AChE inhibitor and the action of this drug inhibited the cholinesterase enzymes leading to enhancing ACh in the synaptic cleft and overall brain cholinergic neurotransmission.[33] Many studies demonstrated that donepezil can enhance learning and memory in animals and humans.[34-37] Donepezil administration was associated with an increase in ACh in mouse brain homogenate by inhibiting cholinesterase

enzyme.[38] The key brain areas associated with spatial memory are hippocampus and prefrontal cortex[39] and donepezil produced increases in ACh in the prefrontal cortex and hippocampus in normal and AD-model rodents.[40, 41] The result from this study demonstrate enhancing effect on spatial memory of donepezil by reducing escape latency time and slight increasing retention time. The result confirms the action of donepezil on increment of ACh in the prefrontal cortex and hippocampus. All doses of piperine in the study produced less escape latency time and more retention time than donepezil and the maximal effect was occurred with the dose 20 mg/kg. There was an evidence to show that piperine exhibited neuroprotective effects in animal hippocampus by suppressing of synchronization of neuronal networks, presynaptic glutamate release, and calcium overloading.[42] It has also been reported improving memory impairment and neurodegeneration in hippocampus of piperine in animal model of cognitive deficit.[21] From the study, it is possible that piperine can enhance spatial memory with a mechanism of action distinct from donepezil leading to differences in spatial memory improvement between piperine and donepezil.

We used [^{3}H] epibatidine saturation binding assay to investigate the alteration of nAChRs in mouse brains after treating with piperine. [^{3}H] Epibatidine has high affinity to nAChRs with a K_D value of ~10 nM.[43-48] Our data showed that piperine and donepezil significantly increased nicotinic receptor binding capacity (B_{max}) with the effect of donepezil being more dominant. The increase in nAChRs in the mouse brain induced by piperine and donepezil were associated with spatial memory improvement in the MWM task. Elevated nicotine binding site density in both the hippocampus and prefrontal cortex was found in donepezil-treated rodents[49] and previous studies reported the increase in nicotine binding sites correlated with improved spatial memory in the MWM.[50,51] There has not been shown evidence of alteration of brain nAChRs after piperine administration and the present study is the first examination of this relationship in mouse. The results showed an increase of nAChRs by piperine which is associated with spatial memory of the animal. However, the less extent in increase nAChRs of piperine leading to more performance improvement than donepezil imply that other mechanisms may also involve with spatial memory in addition to increase nAChRs. As has been reported previously, piperine also has neuroprotective effects[21,42] with possible underlying mechanisms related to the decrease lipid peroxidation and acetylcholinesterase enzyme.[21] Therefore, such results may promote piperine to exhibit memory improvement better than donepezil. The present study suggests that piperine clearly improves learning and memory in the mouse model and piperine could be a valuable source for developing more efficacious drugs aimed at improving learning and memory in the future.

Conclusion

Piperine improved spatial memory with the more pronounced than donepezil in mice using MWM test. The possible mechanism in the memory improvement might be related to increased levels of nAChRs in the mouse brains.

Acknowledgements

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