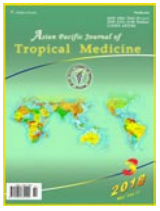

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Nacre extract prevents scopolamine-induced memory deficits in rodents

Tatsuya Fuji, Tatsurou Inoue, Yasushi Hasegawa✉

College of Environmental Technology, Muroran Institute of Technology, 27-1 Mizumoto, Muroran 050-8585, Japan

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ABSTRACT

Objective: To investigate whether the extract from the nacreous layer of pearl oysters (nacre extract) improves impairments in memory caused by scopolamine administration in rodents. **Methods:** Nacre extract was prepared from the inner shell layer of pearl oyster. Effects of nacre extract on scopolamine-induced memory impairment were estimated using novel object recognition test, Y-maze test, and Barnes maze test. Effect of nacre extract on mRNA expressions which are genes associated with memory in the hippocampus was investigated using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. **Results:** Administration of nacre extract led to the protection against scopolamine-induced impairments in object recognition, short-term memory, and spatial memory. Treatment with nacre extract reversed the mRNA expression of brain-derived neurotrophic factor (BDNF) and Homer protein homolog 1 (*Homer-1a*) in the hippocampus, which decreased with the treatment of scopolamine. **Conclusions:** These results suggest that nacre extract has attenuating effects on memory impairments induced by scopolamine through the increase in mRNA expression of BDNF and *Homer-1a*.

1. Introduction

Pearl oyster shells consist of two layers of the prismatic layer and the nacreous layer. Pearls, composed of the nacreous layer, are produced in physiological environments by molluscs such as pearl oyster. Pearl powder is a traditional Chinese medicine and has been used for treating brain dysfunction such as epilepsy, myopia, palpitations, and convulsions[1-3]. Pearl powder has also been used as a cosmetic, anti-aging substance. Extract from the nacreous layer (nacre extract) prevents UVB-induced apoptosis and inflammation in the keratinocyte cell line, HaCaT and promotes dermal wound healing[4-6]. In addition, some beneficial biological activities associated with nacre extract have been reported. The N16 protein in nacre produces mineralization of osteoblastic cell line MC3T3-E1[7-9]. *In vivo* studies further revealed that pieces of nacre implanted in human and animal bone tissues can stimulate bone remodeling[10,11].

Pearl and nacre consist of $CaCO_3$ (> 90%), conchiolin proteins (about 5%-6%), and a small quantity of trace metal elements. A number of nacre matrix proteins have been isolated and identified to investigate the regulation of the formation of nacre, aragonite[12,13]. However, information concerning the substances responsible for the biological activities still remains unknown.

Alzheimer's disease is characterized by a progressive deficit in memory and cognition associated with neurodegeneration[14-17]. Particularly the progressive disruption of cholinergic function contributes to the decline in memory and cognitive function[14]. Scopolamine is a cholinergic blocker that induces learning and memory impairment by interacting with the cholinergic neuronal system; many studies have reported that scopolamine induces memory deficits resembling those observed in patients with Alzheimer's disease[17]. Therefore, scopolamine administration has

First author: Tatsuya Fuji, College of Environmental Technology, Muroran Institute of Technology, 27-1 Mizumoto, Muroran 050-8585, Japan.
E-mail: 17041059@mmm.muroran-it.ac.jp
✉Corresponding author: Yasushi Hasegawa, College of Environmental Technology, Muroran Institute of Technology, 27-1 Mizumoto, Muroran 050-8585, Japan.
Tel: +81-143-46-5745
Fax: +81-143-46-5701
E-mail: hasegawa@mmm.muroran-it.ac.jp

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been widely used as a model of Alzheimer's disease[18-20].

In this study, we investigated whether nacre extract rescues scopolamine-induced memory impairments.

2. Materials and methods

2.1. Materials

Pearl oyster shells, *Pinctada fucata*, were purchased from Uwajima Bay, Ehime, Japan.

2.2. Preparation of nacre extract

Nacre was obtained from the inner region of pearl oyster shells, crushed and ground to a powdered state. The nacre powder (about 50 g) was decalcified in 5 L of 5% acetic acid for 1-2 week. The solution was dialyzed against deionized water, and then lyophilized, resuspended in deionized water, and the supernatant after centrifugation was employed as nacre extract.

2.3. Electrophoresis

Samples (20 μL) were mixed with 20 μL of sodium dodecyl sulfate (SDS) sample solution (2% SDS, 20 mM Tris, 10% glycerol, 1 mM 2-mercaptoethanol, and bromophenol blue), boiled and loaded onto a SDS polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to a previously established method[21]. Gels were stained with silver-staining or periodic acid-schiff staining.

2.4. Animals

Four-week-old male Wistar rats and four-week-old male ICR mice were purchased from CLEA Japan (Tokyo, Japan). They were housed five or six to a cage and maintained at 22 ℃ with free access to water and food. The rats and mice were acclimatized for at least seven days and used in each experiment. Animal experiments were conducted following the guideline of the Muroran Institute of Technology. The experiments were approved by the Committee on the Ethics, Care, and Use of Animal Experiments of Muroran Institute of Technology.

2.5. Novel object recognition test

The novel object recognition test was conducted in an open field box with a diameter of 32 cm and height of 38 cm. Both, mice and rats were subjected to the test on separate occasions. On the test day, the animal was placed in the test box and two objects were introduced (about 30 cm apart from each other). The objects used in this study were about 9 cm × 5 cm × 9 cm plastic blocks with different shapes and colors. Both, mice and rats were allowed to explore the objects during 5-min period (training session). After 24 h, one of the original objects was replaced by a novel object, and animals were returned to the open field box. A novel object exploring time was recorded during 5-min period (test session). The animal was considered to be exploring the object when it was sniffing, biting, or facing the object. A relative exploration time (time exploring the novel object × 100/total exploration time) was measured and compared. Time exploring any one of the two objects was measured in the training session.

Wistar rats were divided into four groups with five rats each. Phosphate buffer saline (PBS) alone (control), scopolamine alone, scopolamine plus nacre extract (10 mg/kg), and scopolamine plus nacre extract (100 mg/kg) was injected intraperitoneally for each group, respectively. ICR mice were also divided into three groups with five mice each and PBS alone (control), scopolamine alone, and scopolamine plus nacre extract (50 mg/kg) was injected intraperitoneally for each group, respectively. In oral administration of nacre extract, PBS alone (control), scopolamine alone, scopolamine plus nacre extract (50 mg/kg), and scopolamine plus nacre extract (100 mg/kg) was administered for each four group (five mice in each group), respectively. The dosages of nacre extract were selected based on results from a preliminary experiment. Experimental schedule was shown in Figure 1A. One hour before the training session, the rats or mice were administered intraperitoneally or orally with PBS or nacre extract. Thirty minutes before the training session, scopolamine (1 mg/kg) was injected intraperitoneally and 24 h later test sessions were performed.

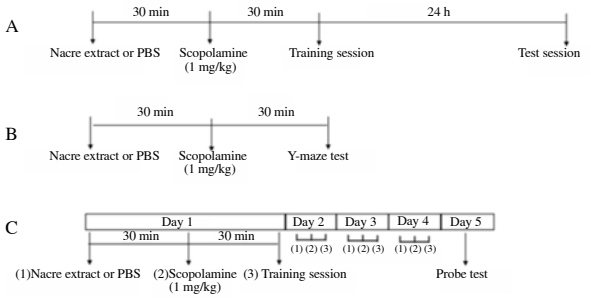

Figure 1. Experimental schedules of (A) novel objection recoginition test, (B) Y-maze test, and (C) Barnes maze test.

2.6. Y-maze test

Y-maze test was performed as described previously[22]. The apparatus with three identical arms (35 cm in length, 10 cm in

width, and 25 cm in height) was set up under lighting conditions. Tests were started by placing animals in the center of the maze. The sequences of arm entries were recorded during 10 min-period. Three successive entries into each of three different arms were defined as an alternation (*e.g.*, ABC, BCA, or CAB, but not BCC). The percent alternation was expressed as the total number of alternations divided by the total number of arm entries minus two. Wistar rats were divided into four groups (five rats in each group). PBS alone (control), scopolamine alone, scopolamine plus nacre extract (50 mg/kg), and scopolamine plus nacre extract (100 mg/kg) was injected intraperitoneally for each group, respectively. ICR mice were divided into three groups (five mice in each group) for intraperitoneal administration and PBS alone (control), scopolamine alone, and scopolamine plus nacre extract (50 mg/kg) was injected intraperitoneally for each group, respectively. In oral administration of nacre extract, ICR mice were divided into four groups (five mice in each group) and PBS alone (control), scopolamine alone, scopolamine plus nacre extract (50 mg/kg), and scopolamine plus nacre extract (100 mg/kg) was administered for each four group, respectively. Experimental schedule was shown in Figure 1B. One hour before the test, the rats or mice were given PBS or nacre extract intraperitoneally or orally. Thirty minutes before the test, scopolamine (1 mg/kg) was injected intraperitoneally and 30 min later the tests were performed (Figure 1B).

2.7. Barnes maze test

The maze platform consists of a circular space (1.15 m in diameter) with 12 holes lining the perimeter. One of the holes was a darkened escape hole that rats can escape the maze. The location of the escape hole remained the same during the training session. Training session was started by placing rats in the center of platform and rats searched for the darkened escape hole for 2 min. If the rat did not find the darkened escape hole within 2 min, rats were gently guided to the escape hole. The time when the rats reached the escape hole (escape latency time) was measured in each training session. Each rat was tested once a day for four days. After four training sessions, a probe test at day 5 was performed (Figure 1C). After the darkened escape hole was removed, a 5-min probe trial was performed. The time taken to search for the area surrounding the darkened escape hole (inside 20 cm from the escape hole) was evaluated. Wistar rats were divided into three groups with five rats each. PBS alone (control), scopolamine alone, and scopolamine plus nacre extract (100 mg/kg) was injected intraperitoneally for each group, respectively. Experimental schedule was shown in Figure 1C. One hour before each training session, rats were given PBS or nacre extract intraperitoneally. Thirty minutes before the session, scopolamine (1 mg/kg) was injected intraperitoneally and 30 min later the training session were performed (Figure 1C).

2.8. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from the hippocampus of each animal at the end of each experiment using an RNAiso Plus kit (Takara, Shiga, Japan). First strand cDNA was synthesized from 10-20 μg of total RNA using oligo (dT) primer and PCR was performed using specific sense and anti-sense primers of β-*actin*, brain-derived neurotrophic factor (*BDNF*), Homer protein homolog 1 (*Homer-1a*), activity-regulated cytoskeleton associated protein (*Arc*), and early growth response protein 1 (*Egr1*). The sequencing primers were as follows: sense, 5′- TTGTTACAGGAAGTCCCTTGCC -3′, and anti-sense, 5′- ATGCTATCACCTCCCCTGTGTG -3′ for β-*actin*; sense, 5′-AGAGCTGTTGGATGAGGACCAG-3′, and anti-sense, 5′-CAAAGGCACTTGACTACTGAGCA-3′ for *BDNF*; sense, 5′-ACCCGATGTGACACAGAACT-3′, and anti-sense, 5′- GTTGCTTCCACTGCTTCACA-3′ for *Homer-1a*; sense, 5′- ATACCGTTAGCCCCTATGCC -3′, and anti-sense, 5′-GTGGTTCTGGATCTGGGACA-3′ for *Arc*; sense, 5′- TTCCTACTCCTCTCCTGGCT-3′, and anti-sense, 5′-AAGGTCGCTGTCATGTCTGA-3′ for *Egr-1*. The band intensities were determined using ImageJ software (NIH). Normalization of the mRNA expression levels was performed with respect to the level of β-*actin* mRNA. The number of cycles were determined on the basis of the relationship between the number of cycles and the amount of amplified PCR product.

2.9. Statistical analysis

All the data were expressed as mean ± SD. Significant difference was determined by Student's *t*-test or one-way analysis of variance and Tukey's multiple-comparison test.

3. Results

3.1. Composition of nacre extract

The nacre extract contained mainly acid- and water-soluble proteins and saccharides. SDS-PAGE analysis showed that the nacre extract contained mainly proteins with a molecular weight of about 10 kDa, 29 kDa, and 66 kDa (Figure 2). Periodic acid-schiff staining showed strong staining for components with a molecular weight of over 66 kDa, suggesting that the nacre extract contains polysaccharide or glycoproteins.

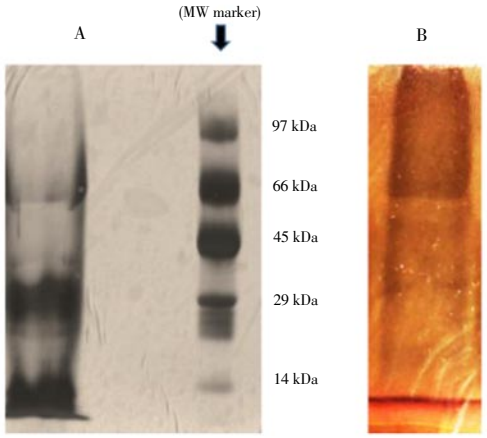

Figure 2. SDS-PAGE analysis of nacre extract.
Gel was (A) silver-stained or (B) PAS-stained.

3.2. Improvement of memory and cognitive impairments by nacre extract in rats

The effect of nacre extract against scopolamine-induced cognitive dysfunction in novel object recognition was assessed. The relative exploration time was about 50% in control, scopolamine-injected, and scopolamine- and nacre extract-injected rats in the training sessions (Figure 3A). After 24 h, the control rats spent more time exploring the new object than the familiar object, and relative exploration time (72%) was significantly increased (Figure 3B). Scopolamine-injected rats did not increase the relative exploration time of a new object compared to those in the control rats, suggesting that scopolamine induced cognitive dysfunction in the object recognition test. However, rats injected with scopolamine and nacre extract (100 mg/kg) retained the relative exploration time. This indicated that nacre extract could improve scopolamine-induced cognitive impairment observed in the novel object recognition test.

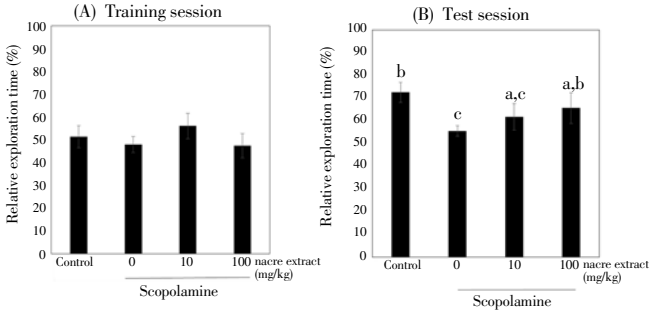

Figure 3. Effects of nacre extract on scopolamine-induced cognitive impairment of rats in novel object recognition test.
(A) Relative exploration time in training session. (B) Relative exploration time in test session. Data were expressed as mean ± SD. Different letters between groups indicate statistically significant difference ($P < 0.05$).

Next we investigated if scopolamine-induced impairment in spatial memory could be ameliorated by nacre extract using the Barnes maze test (Figure 4). Control rats learned the location of the darkened escape hole more quickly compared to scopolamine-injected rats. The latency time to enter the darkened escape hole shortened from day 2 of training in the control rats, but scopolamine-injected rats increased latency time as compared to the control rats. Treatment with nacre extract (100 mg/kg) suppressed the scopolamine-induced increase of latency time on days 2-4. This result suggested that the scopolamine-induced impairment in spatial memory could be ameliorated with the nacre extract. Hence, a probe test was performed. The darkened escape hole was removed and time searching around the space (about 20 cm) where the darkened escape hole had been situated was evaluated. Control rats searched around the space for approximately 40 s during 60-s probe trial, but scopolamine-treated rats searched only for about 10 s. However, treatment with nacre extract significantly increased the time spent searching around the space. This result further supported the hypothesis that nacre extract could improve scopolamine-induced impairment in spatial memory.

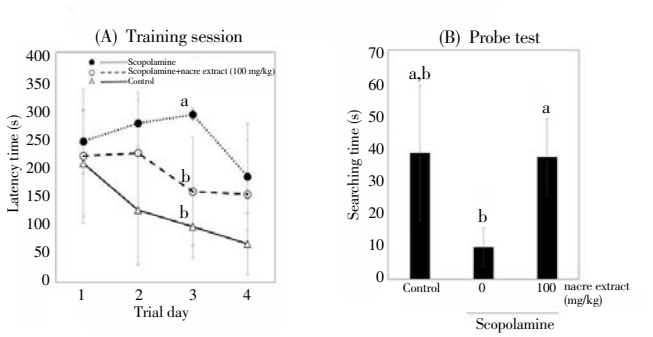

Figure 4. Effects of nacre extract on scopolamine-induced memory impairment of rats in Barnes maze test.
(A) Latency time in training session. Rats were injected intraperitoneally with PBS and scopolamine (closed circle, dotted line) or nacre extract and scopolamine (open circle, dashed line). In control rats, PBS only was injected (triangle, solid line). (B) Searching time in a probe test. Data were expressed as mean ± SD. Different letters between groups indicate statistically significant difference ($P < 0.05$).

Furthermore, we investigated the effect of nacre extract using the Y-maze test (Figure 5). Alternation in Y-maze test is believed to reflect spatial working memory and short-term memory. The scopolamine injection significantly decreased the percent alternation compared to the control rats by about 70% and the decrease showed a tendency to recover from scopolamine-induced memory impairment by treatment with the nacre extract. Nacre extract significantly suppressed the increase in arm entries, showing that nacre extract could suppress the scopolamine-induced increase in locomotor activity.

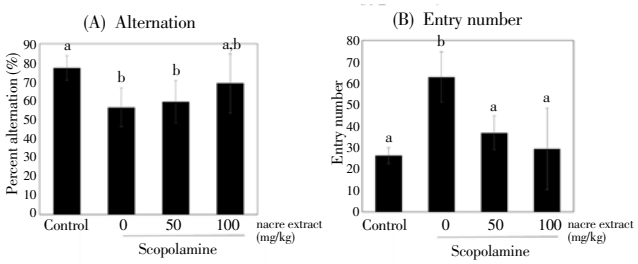

Figure 5. Effects of nacre extract on scopolamine-induced memory impairment of rats in Y-maze test.

(A) The percent alternation; (B) the total number of arm entries. Data were expressed as mean ± SD. Different letters between groups indicate statistically significant difference ($P < 0.05$).

3.3. Improvement of memory and cognitive impairments by nacre extract in mice

Next we performed similar experiments using ICR mice to confirm that nacre extract can improve scopolamine-induced memory impairment. As in the case with rats, treatment with nacre extract showed a tendency to improve the scopolamine-induced memory impairment in the novel object recognition test (Table 1). In addition, treatment with nacre extract significantly improved the decrease of the percent alternation with scopolamine treatment in Y-maze test. These results showed that nacre extract was effective in rescuing, both rats and mice, from scopolamine-induced memory impairment.

Table 1

Effects of intraperitoneal administration of nacre extract on scopolamine-induced memory impairment in novel object recognition test and Y-maze test in mice.

Groups	Y-maze test		Object recognition test (%)	
	Percent alternation (%)	Entry number	Relative exploration time in training session	Relative exploration time in test session
Control	69.3±8.9[a]	44.0±18.7	52.9±2.7	61.3±5.9[a]
Scopolamine	45.9±7.0[b]	80.7±31.2	51.3±1.9	50.1±2.4[b]
Scopolamine+ nacre extract (50 mg/kg)	63.5±1.5[a]	48.0±19.1	49.4±4.6	56.2±8.9[a,b]

Different letters between groups indicate statistically significant difference ($P < 0.05$).

3.4. Effect of oral administration of nacre extract in mice

We established that intraperitoneal administration of nacre extract plays a role in the protection against scopolamine-induced memory impairment. However, pearl powder, traditional Chinese medicine, is administered orally. When nacre extract is administered orally, the components in nacre extract may be degraded in gastrointestinal tract. Therefore, we investigated the effect of oral administration of the nacre extract using mice. Oral administration of the nacre extract also showed similar results as those of intraperitoneal administration. The nacre extract rescued the animals from scopolamine-induced short-term memory and object recognition impairments (Table 2), showing that the nacre extract was also effective when administered orally.

Table 2

Effect of oral administration of nacre extract on scopolamine-induced memory impairment in the novel object recognition test and Y-maze test in mice.

Groups	Y-maze test		Object recognition test (%)	
	Percent alternation (%)	Entry number	Relative exploration time in training session	Relative exploration time in test session
Control	65.2±3.8[a]	28.3±4.5[a]	49.3±4.7	57.5±1.6[a]
Scopolamine	49.7±2.9[b]	67.3±26.7[b]	48.7±3.6	46.8±2.4[b]
Scopolamine +nacre extract (50 mg/kg)	76.7±7.2[a,c]	10.7±2.9[c]	53.9±5.3	51.2±3.7[a,b]
Scopolamine +nacre extract (100 mg/kg)	60.3±3.7[a]	36.7±7.4[a]	50.8±5.0	53.2±6.2[a]

Different letters between groups indicate statistically significant difference ($P < 0.05$).

3.5. Effect of nacre extract on mRNA expression in hippocampus

To investigate the mechanism of action of nacre extract in the brain, we examined the mRNA expression levels of Homer-1a, BDNF, Arc and Egr-1, which are genes associated with memory, using semi-quantitative RT-PCR analysis (Table 3). Hippocampal mRNA expressions of BDNF and Homer-1a were significantly decreased in scopolamine-treated animals as compared to the controls. These decreases were significantly restored by treatment with nacre extract (100 mg/kg). The scopolamine treatment also decreased the mRNA expressions of Arc and Egr-1; treatment with nacre extract showed a tendency to increase the expressions of both mRNAs. These results suggested that nacre extract could restore the scopolamine-induced decrease of mRNA expression associated with memory impairment.

Table 3

Expressions of *Homer-1a*, *BDNF*, *Arc*, and *Egr-1* in hippocampus.

Groups	Relative intensity			
	BDNF/actin	*Homer-1/actin*	*Arc/actin*	*Egr-1/actin*
Control	1.00±0.27[a]	1.00±0.55[a]	1.00±0.13	1.00±0.67
Scopolamine	0.54±0.13[b]	0.18±0.27[b]	0.65±0.07	0.54±0.13
Scopolamine +nacre extract (50 mg/kg)	1.46±0.81[a]	1.82±2.36[a,b]	1.94±1.05	0.50±0.14
Scopolamine +nacre extract (100 mg/kg)	1.32±0.63[a]	1.93±0.44[a]	3.10±3.46	1.19±0.47

Different letters between groups indicate statistically significant difference ($P < 0.05$).

4. Discussion

Pearls have been used as traditional Chinese medicine for treating epilepsy, convulsion, myopia and promoting wound healing[1-4]. Zhang *et al*. showed that oral administration of water-soluble proteins from nacre decreased locomotor activity and prolonged the latency time of pentylenetetrazol-induced convulsion by decreasing 5-hydroxytryptamine and increasing γ-aminobutyric acid in the brain[1], showing that nacre extract can affect brain function. Our results also showed that intraperitoneal and oral administration of nacre extract could attenuate scopolamine-induced memory impairment, suggesting that nacre extract can affect brain function even through oral administration.

The nacre extract was extracted using 5% acetic acid and dialyzed in deionized water using a dialysis membrane with a molecular weight cutoff of 10 kDa for removing acetic acid, and was then lyophilized. Therefore, the nacre extract we used comprised acidic and water-soluble components with a molecular weight of >10 kDa. Many studies have shown that peptides and polysaccharides can improve memory impairment in rodents treated with scopolamine or other drugs[23-25]. Nacre extract contained proteins and saccharides, suggesting that proteins or saccharides or their degradation products may play a role in the protection against scopolamine-induced memory impairment.

Mice and rats are often used in behavioral pharmacology. However, differences between rats and mice in behavioral performance have been reported[26]. Therefore we performed similar experiments using both rats and mice to confirm that nacre extract can improve scopolamine-induced memory impairment. Our results showed that nacre extract is effective in both rats and mice.

We found that nacre extract significantly mitigated the downregulation of *Homer-1a* and *BDNF* mRNAs, induced by scopolamine administration. *BDNF* plays an essential role in the potentiation and storage of memory by facilitating synapse plasticity and transmission[27,28]. Scopolamine has been reported to induce memory impairments through the dysfunction of the CREB-BDNF signaling pathway. Studies using some bioactive substances and extracts have shown that scopolamine-induced memory impairment is rescued by increasing the expression of *BDNF*[25,28-31]. Chen *et al*. suggested that augmentation of the CREB-BDNF signaling pathway may be important for memory impairments[32]. Nacre extract may also attenuate scopolamine-induced memory impairment through activation of the same CREB-BDNF signaling pathway.

Homer proteins act as scaffolding support for the group 1 metabotropic glutamate receptors[33,34]. Brouillette *et al*. reported that the glutamatergic components, Homer 1 and γ-aminobutyric acid B receptors in the dorsal hippocampus are involved in scopolamine-induced memory impairment[35]. Nacre extract restores the scopolamine-induced decrease of *Homer-1a* mRNA expression, suggesting that nacre extract may protect against the dysfunction in glutamate neurotransmission associated with memory impairment.

Some studies showed that scopolamine-induced memory impairment is induced by an increase in oxidative stress. We measured the levels of malondialdehyde, a lipid peroxidation product, and glutathione content in the hippocampus (data not shown). Foyet *et al*. reported that consecutive daily treatments with scopolamine increased brain levels of malondialdehyde and decreased glutathione[36]. However, under our experimental condition (single-dose administration of scopolamine), we did not observe any changes in brain levels of malondialdehyde and glutathione. Consecutive daily administration or higher dose of scopolamine may be necessary to investigate the action of nacre extract on oxidative stress in the brain.

Currently, little is known about the bioactive components in nacre extract. We are trying to identify these substances and their mechanisms of action.

Our results show that nacre extract attenuates scopolamine-induced memory impairment. The results of this study support that pearl powder, which has been employed as a traditional Chinese medicine, may be used to ameliorate cognitive impairment.

Conflict of interest statement

We declare that we have no conflict of interest.

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