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Full paper

Tokishakuyakusan ameliorates spatial memory deficits induced by ovariectomy combined with β-amyloid in rats☆

Nobuaki Egashira [a, b, *], Yuki Akiyoshi [a], Hikari Iba [a], Takashi Arai [a], Izzettin Hatip-Al-Khatib [c], Kenichi Mishima [d], Katsunori Iwasaki [a, e]

[a] Department of Neuropharmacology, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka, 814-0180, Japan
[b] Department of Pharmacy, Kyushu University Hospital, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan
[c] Department of Pharmacology, Division of Internal Medicine, Faculty of Medicine, Pamukkale University, Denizli, 20070, Turkey
[d] Department of Pharmacology, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka, 814-0180, Japan
[e] A.I.G. Collaborative Research Institute for Aging and Brain Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka, 814-0180, Japan

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ABSTRACT

Previously, we reported that ovariectomy (OVX) combined with β-amyloid peptide (Aβ) impaired spatial memory by decreasing extracellular acetylcholine (ACh) levels in the dorsal hippocampus. Here, we investigated the effect of tokishakuyakusan (TSS), a *Kampo* medicine, on the impairment of spatial memory induced by OVX combined with Aβ in rats. Repeated administration of TSS (300 mg/kg, p.o.) significantly decreased the number of errors in the eight-arm radial maze test. Though TSS had no effect on extracellular ACh levels at baseline, TSS significantly increased extracellular ACh levels in the dorsal hippocampus. These results suggest that TSS improves the impairment of spatial memory induced by OVX combined with Aβ by (at least in part) increasing extracellular ACh levels in the dorsal hippocampus.

Introduction

Alzheimer's disease (AD) is characterized by: clinical evidence of cognitive failure in association with cerebral amyloidosis; cerebral intraneuronal neurofibrillary disease; neuronal and synaptic loss; and neurotransmitter deficits.[1] β-Amyloid peptide (Aβ) aggregate is one of the major constituents of the senile plaques in the brain regions serving memory and cognition, and plays a causative part in the neuronal degeneration and memory loss seen in AD patients.[2] In transgenic mice, a 14-fold increase in $A\beta_{(1-42/43)}$ has been reported to accompany the appearance of behavioral deficits.[3] Moreover, $A\beta_{1-42}$ is neurotoxic,[4] and intracerebroventricular infusion of $A\beta_{1-42}$ impairs learning and memory deficits in rats.[5]

AD burden falls more heavily on women than men. It has been hypothesized that plummeting levels of circulating estrogens after the menopause increase a woman's risk for this disorder.[6] In animals, prolonged ovariectomy (OVX) has been shown to result in uterine atrophy and reduced serum levels of 17β-estradiol, and to be associated with a pronounced increase in brain levels of Aβ. Total brain Aβ in ovariectomized (OVX) guinea pigs has been shown to increase by 1.5-fold on average as compared with intact controls.[7] Yamada et al.[8] reported Aβ-induced working memory deficits to be potentiated significantly in OVX rats compared with sham-operated rats. We also investigated the effects of OVX and $A\beta_{1-42}$, separately and in combination, on performance in the radial arm maze. We found that OVX combined with Aβ impaired spatial memory by decreasing extracellular acetylcholine (ACh) levels and alpha7 nicotinic acetylcholine receptor expression without inducing apoptosis in the dorsal hippocampus.[9] Thus, OVX seems to potentiate the Aβ-induced impairment of spatial memory and neurotransmitter in rats.

Decades ago, postmenopausal hormone replacement was considered a panacea for middle-aged women. Prevention of age-related cognitive decline was among the major alleged benefits of this therapy. However, a recent systematic review and prospective cohort study showed that postmenopausal hormone therapy is not associated with a risk of all-cause dementia or AD.[10,11]

☆ Subject categories: Natural medicine materials (NMM) with significant biological activities; Central nervous system pharmacology.

* Corresponding author. Department of Pharmacy, Kyushu University Hospital, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan. Fax: +81 92 642 5937.
E-mail address: n-egashi@pharm.med.kyushu-u.ac.jp (N. Egashira).

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Tokishakuyakusan (TSS) is a *Kampo* medicine, which are Japanese traditional medicines. TSS has been used for the treatment of mainly gynecologic symptoms and cognitive disorders in elderly women due to its estrogen-secreting qualities. TSS can be recommended as anti-dysmenorrhea therapy for women with endometriosis or adenomyosis who wish to become pregnant.[12] TSS has also been used in AD treatment, especially in postmenopausal women.[13] We have reported that TSS: improves scopolamine-induced impairment of spatial memory in rats[14]; protects $A\beta_{25-35}$-induced neuronal damage and lipid peroxidation in cultured rat cortical neurons[15]; prevents impairment of spatial memory induced by repeated cerebral ischemia in rats.[16] However, the effect of TSS on the impairment of spatial memory induced by OVX combined with Aβ has not been explored.

The present study was conducted to investigate the effect of TSS on the impairment of spatial memory induced by OVX combined with Aβ in rats. We also investigated the effects of TSS on extracellular ACh levels in the dorsal hippocampus, which is an important brain site with regard to spatial memory.

Materials and methods

Animals

Experiments were undertaken on 110 female Wistar rats (230–270 g; Kyudo, Tosu, Japan). Rats were housed in groups of 5 per cage (30 × 35 × 17 cm) in a room with controlled temperature (23 ± 2 °C), relative humidity (60 ± 2%) and a 12-h light–dark cycle with the light period starting at 7 am. Animals scheduled to undergo the eight-arm radial maze test (8ARMT) were placed on restricted (10–12 g per day) food (CE-2; Clea Japan, Tokyo, Japan) intake and maintained at ≈80% of their free-feeding body weight (which was determined during the experimental period). All animals had free access to drinking water in their home cages. All animal care and use procedures were performed in compliance with the regulations established by the Experimental Animal Care and Use Committee of Fukuoka University followed the Guidelines of the Science Council of Japan (approved no. 205460 of institutional review board).

OVX. Rats were anesthetized with halothane (Takeda Chemical Industries, Osaka, Japan) using a small animal anesthetizer (TK-4; Bio Machinery, Tokyo, Japan) and laid on a ventral surface with the tail pointing towards the experimenter. The skin was incised (2 cm) midline halfway between the hump and base of the tail, and retracted to the side where the ovary was to be removed. The abdominal muscle two-thirds of the way down the side was incised. The ovary was pulled out through the muscle incision by grasping the periovarian fat and removed.[17] An identical procedure was done on the other side to remove the other ovary. These rats were referred to as “OVX rats”. Rats subjected to the same procedure but where only skin and muscles were cut, but the ovaries were spared, were referred to as “sham rats”.

8ARMT. The behavioral experiment was started on postoperative day (POD)1. The behavioral testing was conducted as reported previously[9] using a modified version of the 8ARMT (Neuroscience, Tokyo, Japan) of the original maze developed by Olton and Samuelson.[18] The trial continued until the rat had entered all eight-arms or 10 min had elapsed.

The performance of each animal in every trial was assessed using three parameters: (i) the number of correct choices in the initial eight chosen arms; (ii) the number of errors defined by choosing arms that had been visited already; (iii) the time elapsed before the animal consumed all eight pellets. One training trial was given per day, Monday to Saturday. Only the rats that made no errors, or only one error for 3 consecutive days, were selected for the study.

Stereotaxic procedure. Rats were anesthetized (sodium pentobarbital, 50 mg/kg, i.p.; Tokyo Kasei, Tokyo, Japan) and placed in a stereotaxic frame (Narishige Scientific Instruments, Tokyo, Japan) with the upper incisor bar 3.4-mm below the level of the interaural line. Guide cannulae were implanted according to the stereotaxic coordinates employed by Paxinos and Watson.[19]

In microdialysis studies, guide cannulae (length = 13 mm; outer diameter (o.d.) = 0.85 mm; inner diameter (i.d.) = 0.75 mm; Eicom, Kyoto, Japan) were implanted into the dorsal hippocampus at the following coordinates (mm): anteroposterior (AP) = −4.0 posterior to the bregma; lateral (L) = 3.3 from the midsagittal line; dorsoventral (DV) = 1.8 relative to the skull surface. For intracerebroventricular infusion, guide cannulae (o.d. = 0.71 ± 0.02 mm; i.d. = 0.41 ± 0.02 mm; length = 13 mm) were implanted bilaterally into the lateral cerebral ventricles at AP = −0.8, L = 1.3 and H = 3.3.

Drugs and experimental paradigm. $A\beta_{1-42}$ HCl (Anaspec, San Jose, CA, USA) was dissolved in sterile distilled water, and allowed to aggregate for 7 days at 37 °C. $A\beta_{1-42}$ was injected once daily for 7 days starting 3 weeks after OVX. $A\beta_{1-42}$ was administered bilaterally (300 pmoL/10 μL) using an injection cannula (o.d. = 0.35 ± 0.01 mm; i.d. = 0.17 ± 0.02 mm; length = 14 mm) connected by polyethylene tubing (o.d. = 1.09 mm; i.d. = 0.38 mm; Intramedic; Becton Dickinson, Franklin Lakes, NJ, USA) to a perfusion pump (CMA/100; Microdialysis, Stockholm, Sweden) driven at a rate of 1 μL/min.

TSS (Lot. No. 2010023010) was a generous gift from Tsumura & Co (Tokyo). TSS was a dried extract of the following raw materials: *Alismatis* tuber (*Alismataceae*, *Alisma orientale* Juzepczuk, 4.0 g), *Angelicae* acutilobae radix (*Umbelliferae*, *Angelica acutiloba* Kitagawa, 3.0 g), *Atractylodis lanceae* rhizoma (*Composite*, *Atractylodes lancea* De Candolle, 4.0 g), *Cnidii* rhizoma (*Umbelliferae*, *Cnidium officinale* Makino, 3.0 g), *Paeoniae* radix (*Paeoniaceae*, *Paeonia lactiflora* Pallas, 4.0 g), and Poria (*Polyporaceae*, *Poria cocos* Wolf, 4.0 g). Each plant material was authenticated by identification of external morphology and marker compounds of plants specimens, according to the methods of the Japanese Pharmacopoeia and Tsumura & Co's standard. The six medical herbs were extracted with purified water at 95 °C for 1 h, and the extraction solution was separated from the insoluble waste and concentrated by removing water under reduced pressure. Spray drying was used to produce a dried extract powder. The yield of the extract was about 17.5%. They were manufactured in compliance with the Japanese Pharmacopoeia (Seventeenth Edition, JP17) under Good Manufacturing Practice (GMP). The product information can be acquired in “KCONSORT” (http://kconsort.umin.jp).

Donepezil hydrochloride (DPZ) was obtained from Eisai, Tokyo, Japan. DPZ and TSS were dissolved in distilled water. TSS was orally administered using a plastic syringe with a stainless-steel tube immediately after injection of $A\beta_{1-42}$ for 7 days. DPZ was administered (p.o.) immediately before the last injection of $A\beta_{1-42}$. DPZ and TSS doses were chosen based on reports.[9,16,20] Control animals received oral injections with the drug vehicle (distilled water). The injection volume of vehicle, DPZ or TSS was 1 mL/kg. The 8ARMT was carried out 60 min after the final injection of $A\beta_{1-42}$. Different groups were used in HPLC study. All experiments in this study were carried out between 7 am and 7 pm.

Brain microdialysis. On day-7 of Aβ injection, we carried out brain microdialysis as described previously.[9] On POD3, extracellular levels of ACh were measured in the dorsal hippocampus of non-anesthetized, freely moving rats by microdialysis. Dialyzate aliquots (20 μL) were collected every 20 min, injected directly into a HPLC system and assayed further for ACh. After a settling period (at least 2–3 h), samples were collected over a 40-min period (baseline, BL-1 and BL-2) before Aβ injection for the following 120 min.

ACh levels in the dialyzates were assayed directly by HPLC (EP-300; Eicom). ACh was separated on a reverse-phase analytical column (Eicompak AC-GEL; 2.0∅ × 150 mm; Eicom). A guard (pre) column (diameter = 3.0 mm × 4 mm with a CH-GEL filter; Eicom) was placed before the analytical column external to the HPLC. An enzyme column (AC-Enzympak; diameter = 3.0 mm; Eicom) in which acetylcholinesterase and choline oxidase were immobilized on controlled-pore glass (diameter of pore and 200/400 mesh glass beads = 546 Å) was placed after the analytical column within the HPLC system.

After its separation on the analytical column, ACh was hydrolyzed by acetylcholinesterase to acetate and choline in a post-column enzyme reactor, and choline was oxidized by choline oxidase to produce betaine and hydrogen peroxide. The analytical and enzyme columns were maintained at 33 °C by a column oven (ATC-300; Eicom). Detection was undertaken using an electrochemical detector (ECD-300; Eicom) with a platinum electrode (WE-PT) set at + 450 mV *vs*. an Ag/AgCl reference electrode (Ag/AgCl RE-100). The mobile phase (50 mM $Na_2HPO_4.12H_2O$, 50 mM H_3PO_4, 1.23 mM sodium 1-decanesulfonate and 0.013 mM EDTA2Na, pH 8.2) was degassed (DG-300; Eicom) and pumped at a flow rate of 0.15 mL/min. An internal standard (0.05 pmol/μL isopropylhemicholine) was injected simultaneously with the perfusion Ringer solution into the autoinjector at a rate of 1 μL/min.

Peaks were recorded using a PowerChrom integrator (Eicom). To evaluate the amount of ACh in the samples, a linear regression curve was constructed for the standards (1 pmol of choline, isopropylhemicholine and ACh standards). The peak heights of ACh in the samples were compared with the standards using a data processor (EPC-300; Eicom). The limit of detection, based on the signal-to-noise ratio, was 10 fmol ACh. The HPLC standards, ACh perchlorate and choline ([2-Hydroxyethyl] trimethylammonium) were purchased from Sigma–Aldrich. Isopropylhomocholine was from Eicom.

At the end of the experiment, the rats were decapitated. Their brains were removed rapidly, sectioned at a thickness of 50 μm, and placement of the microdialysis probes in the dorsal hippocampus was verified.

Statistical analyses. 8ARMT results (number of correct choices and errors) were evaluated for statistical significance using a non-parametric analysis of variance (Kruskal–Wallis test) followed by the Scheffe's test. The data for running time and ACh levels at baseline were evaluated for significance using one-way ANOVA followed by the Tukey–Kramer *post hoc* test. Data for brain microdialysis were expressed as percentages of the baseline concentration, and were analyzed by two-way repeated ANOVA. Data were analyzed using StatView J 5.0 (Abacus Concepts, Berkeley, CA, USA). The criterion for statistical significance was considered to be $p < 0.05$. Values are the mean ± SEM.

Results

Effect of DPZ on the impairment of spatial memory induced by OVX combined with Aβ

OVX combined with Aβ significantly decreased the number of correct choices [$H^2 = 8.187$, $p < 0.05$ by the Kruskal–Wallis test; $p < 0.05$ by the Scheffe's test] and increased the number of errors compared with the sham group [$H^2 = 19.361$, $p < 0.0001$ by the Kruskal–Wallis test; $p < 0.001$ by the Scheffe's test], thereby indicating impairment of spatial memory (Fig. 1a). At first, the validity of this rat model was evaluated by analyzing the effects of DPZ, an acetylcholinesterase inhibitor widely used for the treatment of AD. Single administration of DPZ (3 mg/kg, p.o.) significantly decreased the number of errors ($p < 0.01$ by the Scheffe's test), indicating improvement of spatial memory impairment. Conversely, there was

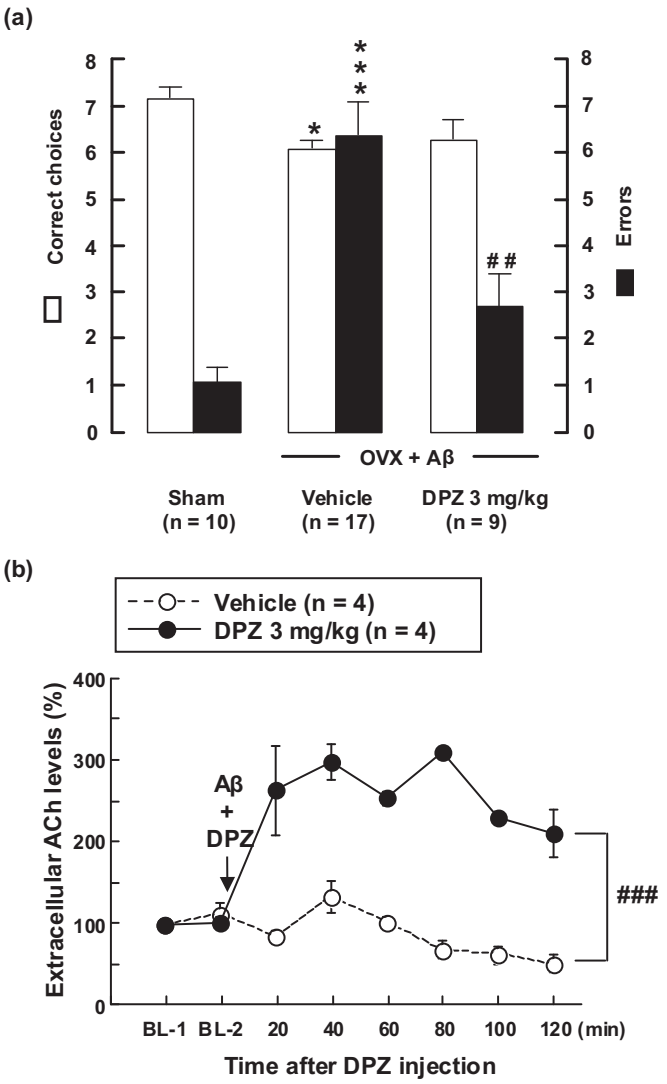

Fig. 1. Effects of DPZ on the impairment of spatial memory and decrease in extracellular ACh levels induced by OVX combined with Aβ (OVX + Aβ). Aβ was injected once daily for 7 days starting 3 weeks after OVX. DPZ (3 mg/kg, p.o.) was administered immediately before the last injection of Aβ. (a) The number of correct choices (open bars) and errors (closed bars) in the eight-arm radial maze test. The eight-arm radial maze test was carried out 60 min after the last injection of Aβ. Values are the mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$ compared with sham, $^{\#\#}p < 0.01$ compared with vehicle (Scheffe's test). (b) Time-course of changes in extracellular ACh levels in dialyzates from the dorsal hippocampus of vehicle- and DPZ-treated OVX + Aβ rats. Extracellular ACh levels were measured in the dorsal hippocampus of non-anesthetized, freely moving rats by microdialysis on the seventh day. After a settling period (at least 2–3 h), samples were collected over a 40-min period (baseline, BL-1 and BL-2) before Aβ injection and for the following 120 min. Values are expressed as percentages (mean ± SEM) of the baseline concentration (BL-1). $^{\#\#\#}p < 0.001$ compared with vehicle (two-way repeated ANOVA).

no significant difference in running time in the 8ARMT [mean running time of sham rats was 139.0 ± 24.9 s, that of vehicle-treated rats was 215.6 ± 41.4 s and that of DPZ 3 mg/kg-treated rats was 232.2 ± 54.8 s; $F(2,35) = 1.131$, $p = 0.335$ by one-way ANOVA].

Effect of DPZ on the decrease in extracellular ACh levels induced by OVX combined with Aβ in the dorsal hippocampus

Fig. 1b shows the time-course of changes in extracellular ACh levels in the dorsal hippocampus. Two-way repeated ANOVA revealed a significant effect of DPZ ($F_{1,6} = 85.564$, $p < 0.001$), no

significant time difference, and a significant TSS × time interaction ($F_{7,42} = 14.289$, $p < 0.001$).

Effect of TSS on the impairment of spatial memory induced by OVX combined with Aβ

Repeated administration of TSS (300 mg/kg, p.o.) significantly decreased the number of errors [$H^3 = 14.671$, $p < 0.01$ by the Kruskal–Wallis test; $p < 0.05$ by the Scheffe's test], indicating improvement of spatial memory impairment (Fig. 2). Conversely, there was no significant difference in running time in the 8ARMT [mean running time of sham rats was 123.4 ± 22.8 s, that of vehicle-treated rats was 133.3 ± 26.2 s, that of TSS 100 mg/kg-treated rats was 124.9 ± 30.0 s and that of TSS 300 mg/kg-treated rats was 81.2 ± 9.9 s; $F(3,39) = 0.942$, $p = 0.430$ by one-way ANOVA].

OVX combined with Aβ significantly reduced uterus weight compared with the sham group, yet repeated treatment with TSS (100 and 300 mg/kg, p.o.) had no effect on the reduction of uterus weight [mean uterus weight of sham rats was 1.028 ± 0.095 g, that of vehicle-treated rats was 0.117 ± 0.013 g, that of TSS 100 mg/kg-treated rats was 0.168 ± 0.048 g and that of TSS 300 mg/kg-treated rats was 0.131 ± 0.010 g; $F(3,37) = 47.019$, $p < 0.0001$ by one-way ANOVA; vehicle, TSS 100 and 300 mg/kg: $p < 0.01$ by the Tukey–Kramer *post hoc* test].

Effect of TSS on the decrease in extracellular ACh levels induced by OVX combined with Aβ in the dorsal hippocampus

OVX combined with Aβ significantly decreased extracellular ACh levels at baseline (BL-1 and BL-2) compared with the sham group, and repeated administration of TSS (300 mg/kg, p.o.) did not affect the decrease in extracellular ACh levels in the dorsal hippocampus [$F(2,31) = 6.687$, $p < 0.01$ by one-way ANOVA; $p < 0.05$ and $p < 0.01$ by the Tukey–Kramer *post hoc* test; Fig. 3a].

Fig. 3b shows the time-course of changes in extracellular ACh levels in the dorsal hippocampus. Two-way repeated ANOVA revealed a significant effect of TSS ($F_{1,9} = 7.067$, $p < 0.05$), no significant time difference, and a significant TSS × time interaction ($F_{7,63} = 2.885$, $p < 0.05$).

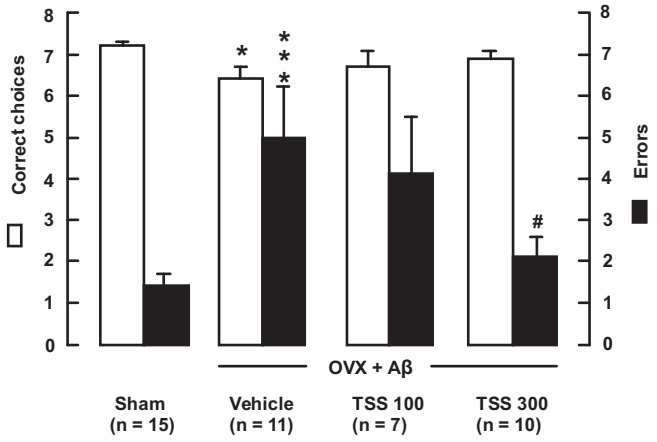

Fig. 2. Effect of TSS on the impairment of spatial memory induced by OVX combined with Aβ (OVX + Aβ) in the eight-arm radial maze test. Aβ was injected once daily for 7 days starting 3 weeks after OVX. TSS (100 and 300 mg/kg, p.o.) was administered immediately after Aβ injection for 7 days. The eight-arm radial maze test was carried out 60 min after the last injection of Aβ. The number of correct choices (open bars) and errors (closed bars) was assessed. Values are the mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$ compared with sham, $^{\#}p < 0.05$ compared with vehicle (Scheffe's test).

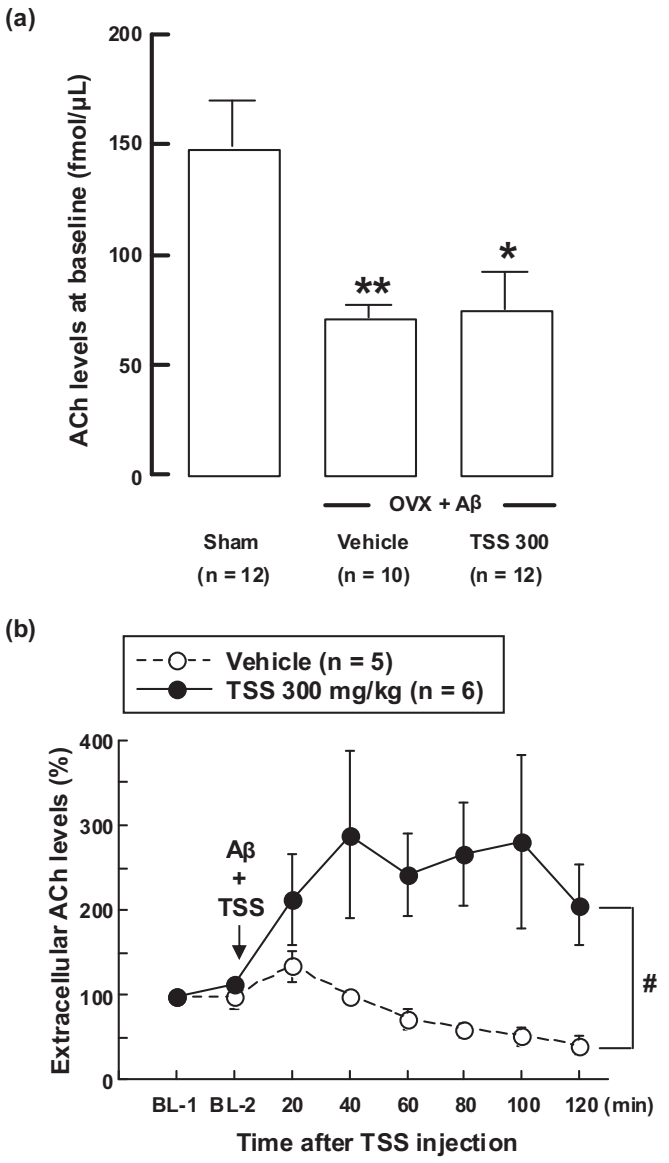

Fig. 3. Effect of TSS on the decrease in extracellular ACh levels induced by OVX combined with Aβ (OVX + Aβ) in the dorsal hippocampus. Aβ was injected once daily for 7 days starting 3 weeks after OVX. TSS (300 mg/kg, p.o.) was administered immediately after Aβ injection for 7 days. Extracellular ACh levels were measured in the dorsal hippocampus of non-anesthetized, freely moving rats by microdialysis on the seventh day. After a settling period (at least 2–3 h), samples were collected over a 40-min period (baseline, BL-1 and BL-2) before Aβ injection and for the following 120 min. (a) Extracellular ACh levels (fmol/μL) at BL-1 and BL-2. Values are the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with sham (Tukey–Kramer *post hoc* test). (b) Time-course of changes in extracellular ACh levels in dialyzates from the dorsal hippocampus of vehicle- and TSS-treated OVX + Aβ rats. Values are expressed as percentages (mean ± SEM) of the baseline concentration (BL-1). $^{\#}p < 0.05$ compared with vehicle (two-way repeated ANOVA).

Discussion

In the present study, single administration of DPZ, an acetylcholinesterase inhibitor widely used for the treatment of AD, improved the impairment of spatial memory and increased extracellular ACh levels in this model. These results suggest that DPZ improves the impairment of spatial memory *via* increasing extracellular ACh levels.

Similarly, TSS (300 mg/kg, p.o.) could reverse the impairment of spatial memory induced by OVX combined with Aβ in rats. This

finding suggests that TSS could attenuate the memory deficits induced by Aβ in postmenopausal women. Conversely, there was no significant difference in running time in the 8ARMT. In addition, TSS at the same dose had no effect on the reduction of uterus weight induced by OVX. Therefore, it is unlikely that the ameliorative effect of TSS on the impairment of spatial memory can be attributed to changes in motor activity or estradiol replacement.

Moreover, we investigated involvement of the cholinergic system in the ameliorative effect of TSS. OVX combined with Aβ decreased extracellular ACh levels at baseline in the dorsal hippocampus. This result was consistent with our previous observation.[9] Hippocampal cholinergic function has been implicated in learning and memory.[21] Farr and colleagues[22] reported that estrogen may interact with cholinergic function in the hippocampus to modulate learning and memory. Therefore, one of the causes of impairment of spatial memory induced by OVX combined with Aβ is thought to be a decrease in extracellular ACh levels in the dorsal hippocampus. We also found that TSS (300 mg/kg, p.o.) did not affect this decrease in extracellular ACh levels at baseline, whereas TSS administration at the same dose increased extracellular ACh levels transiently. These results suggest that TSS increases extracellular ACh levels transiently rather than preventing the baseline decrease in extracellular ACh levels induced by OVX combined with Aβ. Previously, we reported that single administration of TSS (300 mg/kg, p.o.) increases extracellular ACh levels in the dorsal hippocampus in intact rats.[20] Results from the present study are essentially consistent with our previous report. We have also reported that single administration of TSS improves scopolamine-induced impairment of spatial memory in rats.[14] Furthermore, we have reported that single administration of TSS enhances tremors induced by the muscarinic M_1 receptor agonist oxotremorine.[23] These tremors are antagonized completely by scopolamine hydrobromide but not by the peripheral muscarinic receptor antagonist scopolamine methyl bromide,[24] suggesting that tremor enhancement is induced by increasing central cholinergic activity. Hence, TSS may ameliorate the impairment of spatial memory by enhancing the activity of cholinergic neurons.

We investigated the effects of various components and fractions of TSS on scopolamine-induced impairment of spatial memory in the 8ARMT in rats. We found that *Toki*, a medicinal herb in TSS, was the most effective among its component herbs.[25] Moreover, investigation of the effects of fractions isolated from *Toki* revealed that its activity is mainly resided in the butanol layer and its contents of 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (Ang-S-1), which is similar to the structure of *N*-methyl-β-carboline-3-carboxamide (FG7142). A partial inverse agonist of benzodiazepine, FG7142, improved scopolamine-induced memory deficits in the same test,[25] and could indirectly increase the mnemonic function of cholinergic neurons by reducing gamma-aminobutyric acid (GABA)ergic inhibition. Taken together with these findings, our studies suggest that Ang-S-1 is an active compound of TSS that can improve impairment of spatial memory, and that its mechanism of action involves enhancement of cholinergic function. Hence, TSS may increase extracellular ACh levels through benzodiazepine receptors. TSS has also been reported to suppress the decrease of choline acetyltransferase (ChAT) activity in the cerebral cortex and the dorsal hippocampus of OVX mice.[26] Therefore, suppression of decreased ChAT activity might also be involved in the increase of extracellular ACh levels.

In conclusion, TSS improved not only the impairment of spatial memory induced by OVX combined with Aβ, but also the decrease in extracellular ACh levels in the dorsal hippocampus. Our data suggest that the ameliorative effect of TSS on the impairment of spatial memory is mediated (at least in part) by enhancing cholinergic activity in the dorsal hippocampus. Therefore, TSS may be useful for the treatment of memory deficits induced by Aβ, such as AD in postmenopausal women.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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