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Improving Effect of Silk Peptides on the Cognitive Function of Rats with Aging Brain Facilitated by $_D$-Galactose

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Abstract

In order to develop silk peptide (SP) preparations possessing cognition-enhancing effect, several candidates were screened through *in vitro* assays, and their effectiveness was investigated in facilitated brain aging model rats. Incubation of brain acetylcholinesterase with SP-PN (1-1,000 μg/ml) led to inhibition of the enzyme activity up to 35%, in contrast to a negligible effect of SP-NN. The expression of choline acetyltransferase (ChAT) mRNA of neural stem cells expressing ChAT gene (F3.ChAT) was increased by 24-hour treatment with 10 and 100 μg/ml SP-NN (1.35 and 2.20 folds) and SP-PN (2.40 and 1.34 folds). Four-week subcutaneous injections with D-galactose (150 mg/kg) increased activated hippocampal astrocytes to 1.7 folds (a marker of brain injury and aging), decreased acetylcholine concentration in cerebrospinal fluid by 45-50%, and thereby impaired learning and memory function in passive avoidance and water-maze performances. Oral treatment with SP preparations (50 or 300 mg/kg) for 5 weeks from 1 week prior to D-galactose injection exerted recovering activities on acetylcholine depletion and brain injury/aging as well as cognitive deficit induced by D-galactose. The results indicate that SP preparations restore cognitive functions of facilitated brain aging model rats by increasing the release of acetylcholine, in addition to neuroprotective activity.

Key Words: Brain aging, $_D$-Galactose, Cognitive function, Learning and memory, Silk peptides, Choline acetyltransferase

INTRODUCTION

Brain function decreases following aging and central nervous system diseases such as Alzheimer disease (AD), which is one of the most devastating neurodegenerative diseases. Deterioration of cognitive functions including learning and memory loss is a characteristic feature in aging and AD patients (Terry and Davies, 1980). In AD patients, the dysfunction of presynaptic cholinergic system is one of the causes of cognitive deficit (Whitehouse *et al.*, 1982; Coyle *et al.*, 1983), in which decreased activity of enzyme choline acetyltransferase (ChAT) responsible for acetylcholine (ACh) synthesis is observed (Kasa *et al.*, 1997; Terry and Buccafusco, 2003).

Meanwhile, it is well known that cognitive dysfunction is progressive along with aging (Barnes, 1979; Rosenzweig and Barnes, 2003). Several hypotheses have been presented to explain the process of aging (Miquel *et al.*, 1980), in which oxidative stress was demonstrated to play a key role of senescence (Floyd and Hensley, 2002). Increased oxidative stress causes mitochondrial dysfunction that produces high-level reactive oxygen species (ROS), accelerating neurodegeneration (Zeevalk *et al.*, 2005; Reddy, 2007).

$_D$-Galactose ($_D$-Gal) is a nutrient derived from lactose in milk. Lactose is hydrolyzed to monosaccharides glucose and galactose. However, excessive $_D$-Gal undergoes abnormal conversion to galactitol, causing osmotic stress and generation of ROS (Kaplan and Pesce, 1996; Hsieh *et al.*, 2009). $_D$-Gal, a reduced form of sugar, reacts with free amines of amino acids in proteins and peptides to form advanced glycation end products which in turn cause activation of their own receptors and facilitation of oxidative damage linked to the pathogenesis of degenerative diseases (Song *et al.*, 1999). Especially, $_D$-Gal makes cellular constituents vulnerable to oxidative reaction by decreasing various antioxidant components and enzymes, and reduces mitochondrial energy production (Kumar *et al.*, 2010). In addition, $_D$-Gal not only accelerates

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inflammatory brain injury through activation and degeneration of astrocytes, but also causes cognitive deficits by activating acetylcholinesterase (AChE), an ACh-degrading enzyme (Cui *et al.*, 2006; Lei *et al.*, 2008; Kumar *et al.*, 2010). Accordingly, in the present study, we adopted $_D$-Gal as a model compound for facilitated brain aging.

To date, AD therapy is largely based on compounds to increase ACh concentration, including AChE inhibitors, ACh precursors and cholinergic receptor agonists (Terry and Buccafusco, 2003; Musial *et al.*, 2007). It was also demonstrated that choline-uptake enhancers were effective for improvement of cognitive function (Terry and Buccafusco, 2003; Bessho *et al.*, 2008; Takashina *et al.*, 2008). Furthermore, enhancers of ChAT mRNA expression recovered impaired learning and memory function (Wang *et al.*, 2000; Egashira *et al.*, 2003; Karakida *et al.*, 2007). In our studies, it was found that transplantation of stem cells encoding ChAT gene markedly recovered cognitive function of animal models of AD and aging (unpublished results).

Silks from silkworm (*Bombyx mori*) are composed of 2 major peptides, sericin and fibroin (Lee *et al.*, 2003), which are well known to have pharmacological activities including anti-diabetic effect (Park *et al.*, 2002; Lee *et al.*, 2007b). Hydrolysis of silk proteins leads to different sizes of peptide, while enzymatic degradation results in specific sizes or compositions of silk peptides (SP) exerting diverse bioactivities including anti-diabetic, hypocholesterolemic and antioxidative actions (Kato *et al.*, 1998; Lee *et al.*, 2002; 2007b; Zhaorigetu *et al.*, 2003; Kim *et al.*, 2008). Furthermore, silk amino acids (SAA) enhanced physical stamina by preventing tissues from oxidative injures (Shin *et al.*, 2009a; 2009b). Interestingly, it was reported that silkworm extract inhibited monoamine oxidase-B (MAO-B), a dopamine-degrading enzyme (Kang *et al.*, 2006, 2010), and that SAA improved Parkinson disease (PD) via dopaminergic neuroprotection (Park *et al.*, 2010). Especially, brain factor-7 (BF-7), a peptide obtained by enzymatic degradation of silk proteins, was found to increase cognitive function in both animal and human (Lee *et al.*, 2005; Kim *et al.*, 2009).

In order to develop SP preparations possessing cognition-enhancing effect, we obtained several SPs by treating silk proteins with various enzymes, and selected SP-NN and SP-PN based on their activities on AChE inhibition and ChAT mRNA expression *in vitro. In vivo* cognition-improving effects of SP-NN and SP-PN were investigated through passive avoidance and Morris water-maze performances in rats with aging brain facilitated by repeated administration of $_D$-Gal.

MATERIALS AND METHODS

Peptides

Silk peptide-NN (SP-NN; World Way code: SPF-B6) and SP-PN (World Way code: SPF-B5) were prepared by 12-hour incubation with Protease N (150,000 U/g; Amano G from *Bacillus subtilis*) plus Neutrase (160,000 U/g; *Bacillus amyloliquefaciens*) and Protease P (60,000 U/g; Amino 6 G from *Aspergillus melleus*) plus Neutrase, respectively. In brief, cocoon was dissolved at 115°C for 6 hours in $CaCl_2$ solution, and sterilized at 90°C for 30 min. After cooling to 50°C, the proteins were degraded with the above enzymes at pH 5.8, and autoclaved at 90°C for 30 min to inactivate the enzymes. GPC-RI analyses revealed that peak molecular weights of SP-NN and SP-PN were 962 and 886, respectively (Fig. 1). Freeze-dried SP-NN and SP-PN were obtained from Worldway Co., Ltd. (Jeoneui, Korea).

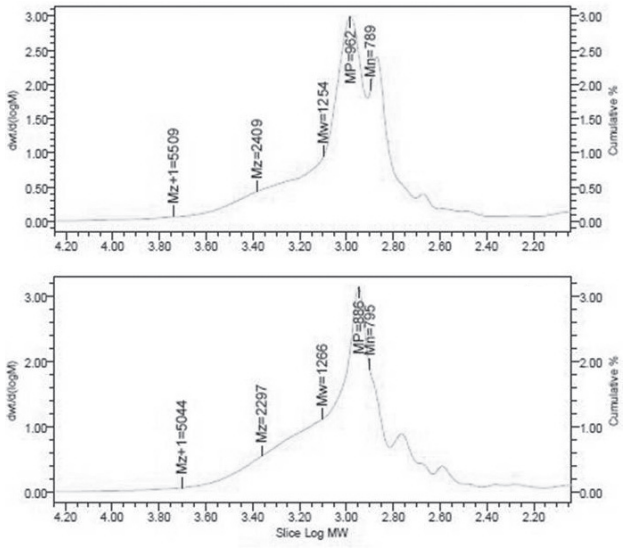

Fig. 1. Size analysis of SP-NN (A) and SP-PN (B).

Animals

Five-week-old male Sprague-Dawley rats were procured from the Daehan-Biolink (Eumseong, Korea). The animals (n=10/group) were maintained at a constant temperature (23 ± 2°C), relative humidity of 55 ± 10%, and 12-hour light/dark cycle. The animals were fed with standard rodent chow and purified water *ad libitum*. All experimental procedures were approved and carried out in accordance with the Institutional Animal Care and Use Committee of Laboratory Animal Research Center at Chungbuk National University, Korea.

Acetylcholinesterase assay

Rat brain was quickly removed after transcardial perfusion with cold saline under anesthesia and homogenized in 19 volumes of cold phosphate-buffered saline (PBS; 100 mM NaCl and 100 mM sodium phosphate, pH 7.8) to prepare the 5% homogenate. Assay for cholinesterase activity was performed at 25°C by a slight modification of the method of Ellman *et al.* (1961). Briefly, assay mixture (3.0 ml PBS) contained 1.5 mM acetylcholine iodide, 1.0 mM 5.5'-dithiobis-(2-nitrobenzoic acid). Fifty μl SP preparations (1-1,000 μg/ml) and brain homogenate (50 μl) were added to the assay mixture, and the change in absorbance at 412 nm during 5 min was monitored (Kim *et al.*, 1998). The relative enzyme activity (inhibited by SP preparations) was calculated based on the absorbance change (full activity: 100%) of assay mixture containing 50 μl PBS without SP.

Expression of ChAT mRNA

For the evaluation of the effects of SP preparations on neuronal ChAT mRNA expression, human neural stem cells encoding ChAT gene (F3.ChAT) was used (Kim, 2004; Matsuo *et al.*, 2005; Lee *et al.*, 2007a). F3.ChAT cells (1×10^6 cells/ml) were incubated with 10 or 100 μg/ml of SP-NN or SP-PN at 37°C for 24 hours in a 5% CO_2 incubator. For reverse

transcriptase-polymerase chain reaction (RT-PCR) analysis of ChAT mRNA expression, total RNA was extracted from F3.ChAT cultures using the Trizol method (Invitrogen, USA). Complimentary DNA templates from each sample were prepared from 1 μg of total RNA primed with oligo dT primers using 400 U of Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, USA) followed by 30 PCR cycles, and RT-PCR products were separated electrophoretically on 1.2% agarose gel containing ethidium bromide and visualized under UV light. The primers used for the RT-PCR of ChAT are followings;

Sense: 5'-CTCTGACCTGTCAGAAGAAT-3'
Antisense: 5'-GACGCTGACACTTACAGAAT-3'

Aging model and treatment

Rats were orally administered with 50 or 300 mg/kg of SP-NN or SP-PN in a volume of 5 ml/kg purified water for 5 weeks from 1 week before initiation of $_D$-Gal injection. $_D$-Gal (150 mg/kg) was subcutaneously administered in 10% solution (in physiological saline) for 4 weeks 30 min after administration of SP preparations (Lu *et al.*, 2007; Chen *et al.*, 2008; Lei *et al.*, 2008; Kumar *et al.*, 2010). From 30 min after $_D$-Gal injection on the 25th day, learning and memory function was evaluated for 4 days as 4 acquisition trials. Fifth retention trial was performed 1 week later. One day after the final administration and memory acquisition trials, ACh concentration in cerebrospinal fluid (CSF) was analyzed.

Measurement of cognitive function

Passive avoidance performances: For the evaluation of memory acquisition, the rats were subjected to a passive avoidance Shuttle box once a day for 4 days from the 25th day of experiment. Fifth trial was performed 1 week after the 4th acquisition trial, for the evaluation of retention of the acquired memory. The Shuttle box apparatus consists of two compartments equipped with a lamp and a steel-grid floor for electric shock. On the trials, electric shock (1 mA for 2 sec) was delivered when rats entered the dark compartment from the light room through a guillotine door. The latency time of stay in the light room from light-on was recorded. The end-point was set at 300 sec, denoting full acquisition of memory.

Morris water-maze performances: For the evaluation of spatial memory, the rats were subjected to Morris water-maze test. Water-maze trials were performed in a circular water bath (180 cm in diameter) filled with water (27 cm in depth) maintained at 22 ± 2°C. The bath was divided into 4 quadrants and a hidden escape platform (10 cm in diameter, 25 cm in height) was submerged in the center of one quadrant, 2 cm below the surface of the water. The rats were trained to learn to find the platform hidden by spreading white styrofoam granules (5 mm in diameter) on the surface of water, based on several cues external to the maze. The position of the cues remained unchanged throughout the experiments. Escape latency, i.e., the time spent to escape onto the platform during trials, was recorded.

Analysis of ACh in CSF

By puncturing atlantooccipital membrane after sacrifice of rats with deep ether anesthesia, CSF was collected carefully not to be contaminated with blood (Kim *et al.*, 1999). ACh concentration in CSF was measured with an Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit (Molecular Probes, Eugene, USA) according to the manufacturer's instructions. In this assay, ACh is hydrolyzed by AChE to release choline, which is then oxidized by choline oxidase to betaine and H_2O_2. H_2O_2 interacts with Amplex Red (7-dihydroxyphenoxazine) in the presence of horseradish peroxidase to generate the highly-fluorescent resorufin. The resulting fluorescence was measured in a fluorescence microplate reader (Bio-Tek Flx800; Bio-Tek Instruments Inc., Winooski, USA) using excitation in the range of 530-560 nm and emission at -590nm.

Evaluation of brain injury/aging

At the end of behavioral testing, each animal was sacrificed under deep anesthesia, and transcardially perfused with cold saline, followed by post-fixation in neutral formalin solution for 48 hours and in 30% sucrose for additional 72 hours. Coronal sections at -3.80 mm distal from bregma, exhibiting full hippocampal formations (CA1-CA4 and dentate gyrus), were cut at a thickness of 30 μm with a cryomicrotome.

To demonstrate activated astrocytes as a marker of brain injury and aging, immunohistochemical staining to glial fibrillary acidic proteins (GFAP), a cytoskeletal protein of astrocytes, was performed. Brain sections were incubated overnight at 4°C with rabbit polyclonal antibody (1:200; Chemicon, Temecula, USA). The sections were rinsed with PBS and incubated with biotinylated goat anti-rabbit antibody (1:150; Vector, Burlingame, USA) for 1 hour at room temperature, followed by incubation for 1 hour with avidin-biotin-peroxidase complex (ABC Elite Kit, Burlingame, USA). The reaction product was visualized by catalysis of diaminobenzidine, and counterstained with hematoxylin. Activated astrocytes in the 3 hippocampal regions (mean numbers in CA1, CA2 and CA3) were counted under the field of ×400 of a light microscope.

Statistical analysis

The results are presented as means±standard deviation. The significance of differences of all results was analyzed by one-way analysis of variance followed by the Tukey's test correction. Statistical significance was set a priori at $p<0.05$.

RESULTS

Approved AD therapeutics for the improvement of cognitive function are principally AChE inhibitors, we measured the direct AChE-inhibitory potential of SP preparations. SP-NN (1-1,000 μg/ml) exerted a moderate inhibitory activity (15-20%) on brain AChE, although the inhibition rate reached approximately 35% at 320 μg/ml, while the AChE-inhibitory effect of SP-PN was negligible (Fig. 2).

To evaluate the stimulating potential of SP preparations on ACh synthesis, we analyzed ChAT mRNA expression in neural stem cells encoding ChAT gene. Twenty four-hour treatment with SP-NN facilitated ChAT mRNA expression of F3.ChAT cells to 1.35 and 2.40 folds of vehicle control at 10 μg/ml and 100 μg/ml, respectively (Fig. 3). A low concentration (10 μg/ml) of SP-PN also remarkably enhanced (2.20 folds) the ChAT mRNA expression, while a high concentration (100 μg/ml) increased only 1.34 times the control level.

After confirmation of *in vitro* activities, *in vivo* cognition-enhancing effects of SP preparations were investigated in an aging-facilitated memory deficit model. Four-week subcutaneous injection of $_D$-Gal (150 mg/kg) led to profound impairment

of cognitive tasks in both passive avoidance and Morris water-maze tests (Fig. 4). In the passive avoidance performances, SP-NN significantly improved the memory acquisition in a dose-dependent manner (Fig. 4A). Maintenance of the acquired memory was confirmed via an additional trial performed 1 week later to evaluate memory retention. Such improving effects of SP-NN on memory acquisition and retention were also achieved in the water-maze performances (Fig. 4B). In addition, SP-PN exerted cognition-improving activities in both learning and memory tests, which were comparable to those of SP-NN.

To confirm the relationship between memory deficit and the decrease in ACh release, we analyzed ACh concentration. Repeated administration of $_D$-Gal decreased ACh concentration in CSF by 45-50% (Fig. 5), which was significantly reversed by SP-NN (50 and 300 mg/kg). Notably, a high dose (300 mg/kg) of SP-PN near-fully recovered ACh concentration to normal control level, although a low dose (50 mg/kg) of SP-PN was ineffective.

Next, we examined astrocytic response by staining GFAP which has been used as a specific marker of brain injury and aging. GFAP immunostaining revealed that activated hippocampal astrocytes increased to 1.7 times the control level (Fig. 6A and 7) following 4-week injection of $_D$-Gal (Fig. 6B and 7). The $_D$-Gal-induced increase in activated astrocytes were markedly attenuated by both doses (50 and 300 mg/kg) of SP-NN (Fig. 6C and Fig. 7). SP-PN also exerted remarkable suppressive activities on the astrocyte activation related to brain aging, similar to SP-NN (Fig. 6D and Fig. 7).

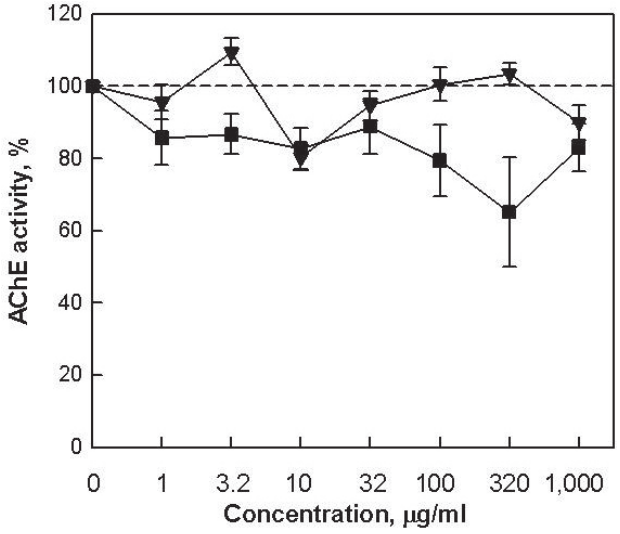

Fig. 2. Inhibition of rat brain acetylcholinesterase (AChE) by SP-NN (▼) and SP-PN (■) (n=6). The control (100%) activity means the absorbance change of assay mixture containing vehicle without SP preparations.

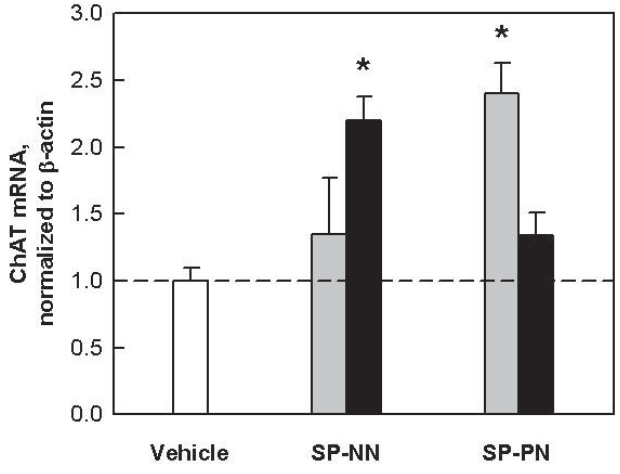

Fig. 3. Facilitation of choline acetyltransferase (ChAT) mRNA expression in F3.ChAT neural stem cells by SP-NN and SP-PN (n=6). Grey: 10 μg/ml, Black: 100 μg/ml. *p<0.05 vs. vehicle control.

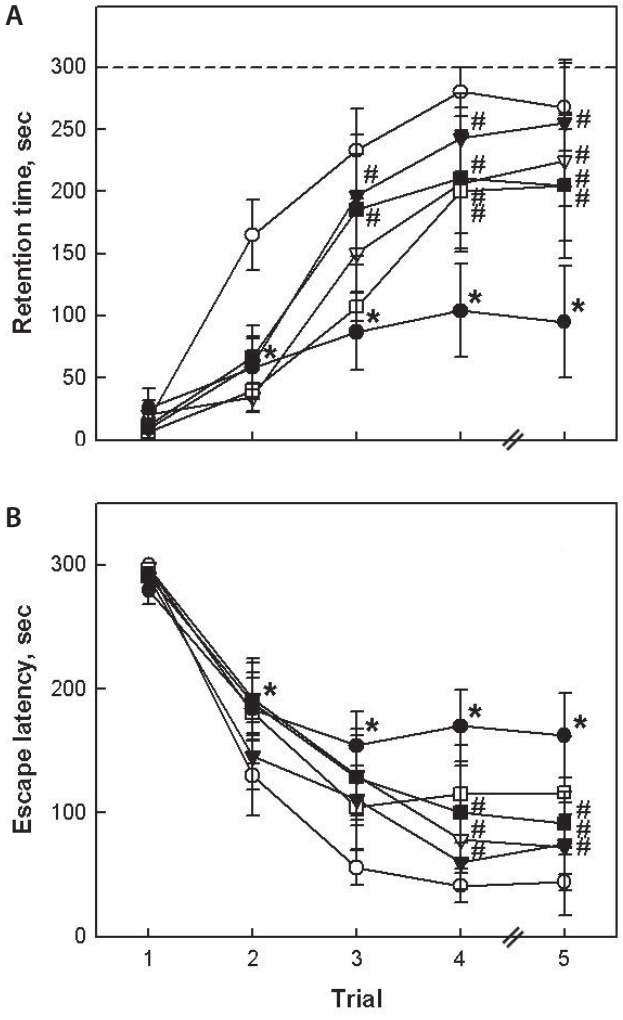

Fig. 4. Effects of SP-NN and SP-PN on the passive avoidance (A) and water-maze (B) performances of $_D$-galactose ($_D$-Gal)-induced aging model rats. Memory acquisition was evaluated through 4 daily trials (trials 1-4) (n=10), and 5th trial was performed 1 week after the 4th acquisition trial to assess memory retention (n=4). ○: Vehicle control, ●: $_D$-Gal alone, ▽: $_D$-Gal+50 mg/kg SP-NN, ▼: $_D$-Gal+300 mg/kg SP-NN, □: $_D$-Gal+50 mg/kg SP-PN, ■: $_D$-Gal+300 mg/kg SP-PN. *p<0.05 vs. vehicle control. $^{\#}p$<0.05 vs. D-Gal alone.

DISCUSSION

Cognitive function is composed of acquisition (learning) of information and retention of acquired memory. Acquired memory is preserved only for a short time called short-term memory, which is formed by ACh in cholinergic nervous system. Long-term memory is produced through the consolidation of acquired information during long-term potentiation of glutamatergic nervous system mediated by nitric oxide (NO), which is produced by NO synthase following activation of *N*-methyl-$_D$-aspartate receptors (Terry and Buccafusco, 2003; Musial *et al.*, 2007). Therefore, AD patients and aging are characterized by cognitive deficit resulting from deterioration of cholinergic system. Accordingly, recent standard treatment of dementia have been focused on increasing ACh concentration by inhibiting AChE to enhance memory acquisition (Terry and Buccafusco, 2003; Musial *et al.*, 2007).

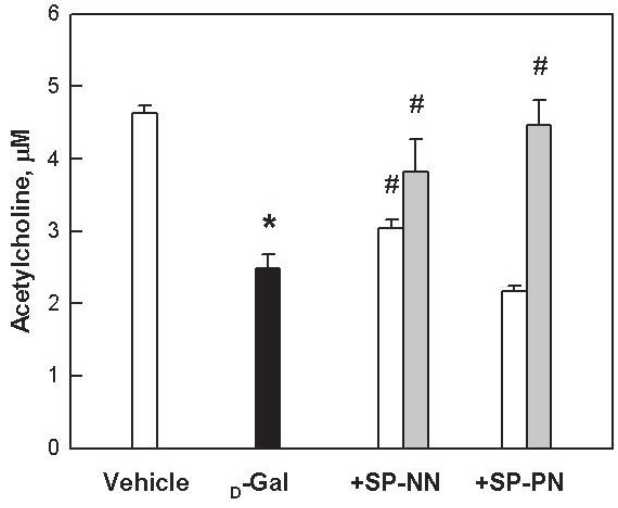

Fig. 5. Effects of SP-NN and SP-PN on the acetylcholine concentration in cerebrospinal fluid of $_D$-galactose ($_D$-Gal)-induced aging model rats (n=6). white, 50 mg/kg; grey, 300 mg/kg. $^*p<0.05$ vs. vehicle control, $^\#p<0.05$ vs. $_D$-Gal alone.

In animal models of AD, i.e., β-amyloid protein-expressing transgenic and AF64A-injected mice, 25-30% decrease in brain ACh resulted in severe impairment of learning and memory function (Yamazaki *et al.*, 1991; Abe *et al.*, 1993; Tsai *et al.*, 2007; Bessho *et al.*, 2008). During aging, brain atrophy and malfunction of cholinergic nervous system occur, leading to cognitive deficit. Facilitated brain aging by only 4-week injection of $_D$-Gal (150 mg/kg) caused reduction of ACh concentration in CSF to lower than 55% of control level, brain damage/aging (increase in activated astrocytes to 1.7 times), and severe learning and memory dysfunction (Cui *et al.*, 2006; Lei *et al.*, 2008; Kumar *et al.*, 2010).

Although it has been known that silkworm and SP improve diabetes and lipid metabolism, protective effects of SAA against tissue injury were observed in a forced swimming stress model (Shin *et al.*, 2009a; 2009b). Especially, SAA exerted *in vitro* and *in vivo* neuroprotective effects on 6-hydroxydopamine-induced dopaminergic neurotoxicity, and thereby improved movement functions of PD animals (Park *et al.*, 2010). In the present study, it was also confirmed that SP preparations inhibit GFAP production in astrocytes, a marker of brain injury and aging, in $_D$-Gal-facilitated aging model rats.

In addition to a direct cytoprotective activity, it was reported that an extract of silkworm prevented reduction of dopamine concentration by inhibiting MAO-B in 1-methyl-4-

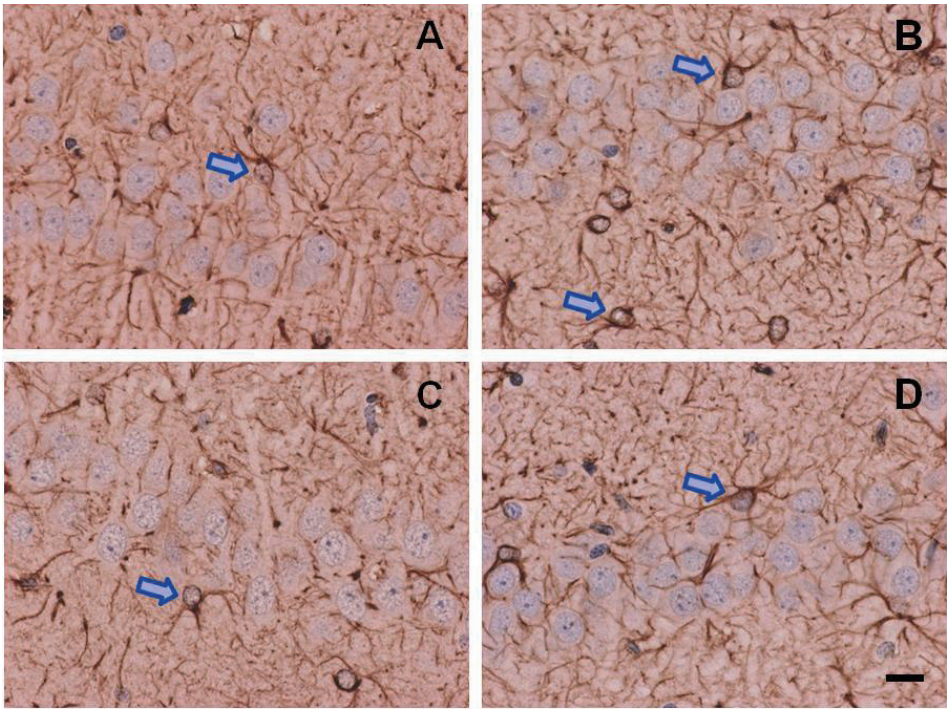

Fig. 6. Representative microscopic findings of gilal fibrillary acidic protein-positive (brown-stained) astrocytes (arrows) in hippocampal CA1 region of $_D$-galactose-induced aging model rats (n=6). (A) vehicle control, (B) $_D$-Gal alone, (C) $_D$-Gal+300 mg/kg SP-NN, (D) $_D$-Gal+300 mg/kg SP-PN. Scale bar=50 μm.

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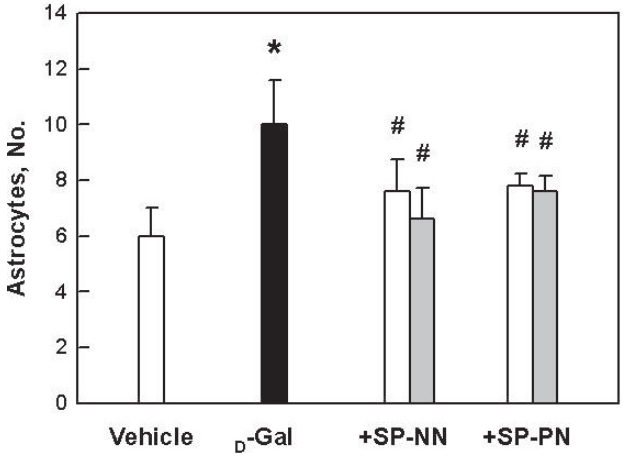

Fig. 7. Effects of SP-NN and SP-PN on the number of hippocampal astrocytes of $_{D}$-galactose ($_{D}$-Gal)-induced aging model rats (n=6). white, 50 mg/kg; grey, 300 mg/kg. *p<0.05 vs. vehicle control, $^{\#}p$<0.05 vs. $_{D}$-Gal alone.

phenyl-1,2,3,6-tetrahydropyridine-induced PD animals (Kang *et al.*, 2006; 2010). In the present study, it was observed that SP-NN and SP-PN, degradation products of silk proteins by Neutrase and Protease N or P, facilitated expression of ChAT gene in neurons. Such a ChAT expression-stimulating effect of SP preparations might be associated with the marked ACh recovery in the brain and improvement of cognitive function, as a similar effect achieved with Onji (Egashira *et al.*, 2003; Karakida *et al.*, 2007).

In spite of the different inhibitory effects on AChE activity, SP-NN and SP-PN greatly enhanced the expression of ChAT mRNA *in vitro* and increased ACh concentration in vivo. The SP preparations also suppressed astrocytic activation following brain aging, in parallel with neuroprotective efficacy of SAA in PD models (Park *et al.*, 2010). Therefore, it is suggested that SP-NN and SP-PN improve cognitive function of aging model animals by increasing ACh production as well as by protecting host cells against aging process.

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