Fructus Corni Officinalis water extract Ameliorates Memory Impairment and Beta amyloid (Aβ) clearance by LRP-1 Expression in the Hippocampus of a Rat model of Alzheimer's Disease

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This study evaluated the effects of *Fructus Corni Officinalis* water extract (FCE) on congnitive impairment and Aβ clearance induced by beta amyloid Aβ (1-42) injection in the hippocampus of rat. Aβ (1-42) was injected into the hippocampus using a Hamilton syringe and micropump (5 ㎍/5 ㎕, 1 ㎕/min, each hippocampus bilaterally). FCE was administered orally once a day (100, 250, 500 mg/kg) for 4 weeks after the Aβ (1-42) injection. The acquisition of learning and retention of memory were tested using the Morris water maze. Aβ accumulation and Aβ clearance in the hippocampus were observed using immunostaining. Aβ (1-42) level in plasma was confirmed using enzyme-linked immunosorbent assay (ELISA). FCE significantly shortened the escape latencies during acquisition training trials. FCE significantly increased the number of target heading to the platform site and significantly shortened the time for the 1sttargetheadingduringtheretentiontesttrial.FCEsignificantlyattenuatedtheAβ accumulation in the hippocampus produced by Aβ (1-42) injection. FCE significantly increased LRP-1 expression around vessels in the hippocampus and Aβ (1-42) levels in plasma. The results suggest that FCE improved cognitive impairment by ameliorate Aβ clearance and Aβ accumulation in the hippocampus. FCE may be a beneficial herbal formulation in treating cognitive impairment including Alzheimer's disease.

keywords : *Fructus Corni Officinalis* water extract, beta-amyloid clearance, Memory impairment, Alzheimer's disease

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease and the most common form of senile dementia[1,2]. It is characterized by accumulation of beta amyloid protein(Aβ) in brain parenchyma[3]. Aβ (1-40) and Aβ (1-42) are formed by amyloid precusor protein (APP) after sequential cleavage by β- and γ-secretase[4]. The amount of Aβ accumulation is not only caused by the overproduction of Aβ, but also by an impaired clearance of Aβ from the brain[5]. Recent evidence suggests that impaired clearance of Aβ is responsible for the most common type of AD. A relatively small number (<5%) of AD patients (familial cases) might have increased Aβ production in the brain because of inherited mutations in the amyloid protein precursor (APP) gene or presenilins 1 or 2 genes. However, the majority of AD patients (sporadic or late-onset AD) do not have an increased Aβ production or APP overexpression in the brain. The balance in Aβ clearance is crucial for the accumulation of Aβ in AD brains[6].

Lipoprotein receptor related protein 1 (LRP-1) is a member of the LDL receptor family. At the endothelial cells in blood brain barrier (BBB), LRP-1 transport Aβ from the brain into the blood. Subsequently effluxed Aβ has eliminated systemic clearance via liver, spleen, and kidneys. LRP-1 recognizeds in the transcytosis more than 40 different ligands, including apoE, α2-macroglobulin, APP, and Aβ[7,8].

Fructus Corni Officinalis (FC) is widely distributed in Korea and the fruits of this plant have been used in traditional medicine for its tonic, analgesic and diuretic properties in Korea, Japan and China[9]. The majo ringredients of FC are loganin, morroniside and gallic acid. The neuroprotective activity of FC has been reported through a variety of *in vitro* and *in vivo* studies[10]. Morroniside protected SH-SY5Y neuroblastoma cells against hydrogen peroxide-induce dcytotoxicity, and also protected rat brain from damage by focal cerebral ischemia[11]. FC attenuated β-amyloid (25-35)-induced toxicity in PC12 cells and glutamate-induced toxicity in HT22 hippocampal

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cells[10,12]. However, there have been no reports on the Aβ clearance activity of FC against Aβ-induced AD rat model.

This study examined the effect of FC water extract (FCE) on Aβ clearance by LRP-1 Expression in rat induced by intrahippocampal Aβ injection and the restoration of cognitive impairment caused by the Aβ deposition using the Morris water maze

Materials and Methods

1. Preparation of *Fructus Corni Officinalis* (FC)

FC was purchased from Okchundang Herbs (Seoul, Korea). FC was extracted with 300 mL of distilled water (DW) at 100℃ for 3 hours using a reflux heater (Changshin Science, Seoul, Korea). The extracted fluid was filtrated with filter paper (Hyundai Micro Co., Seoul, Korea), and the remaining fluid was evaporated to <300 mL with a rotary evaporator (Sunileyela Co., Gyeonggi, Korea) and lyophilized with a freeze-dryer (Operon™, Seoul, Republic of Korea). The powders were stored at 4℃.

2. HPLC method

FC (20 mg) was dissolved in 10 ml methanol (HPLC reagent, Duksan Chemical, Korea) and ultrapure distilled water (resistivity > 18 MΩ) and filtered through a 0.45 μm syringe filter (PVDF, Advantec, Japan). The standard materials used for the qualitative analysis of FC was Loganin. The standard stock solutions were prepared by dissolving 1 mg samples of Loganin, each in 10 ml methanol. The HPLC apparatus was a Gilson System equipped with a 234 Autosampler, a UV/vis-155 detector and a 321 HPLC Pump (Gilson, Korea). A Luna 3.0 × 250 mm C18 reversed-phase column with 5 μm particles (Phenomenex, Torrance, CA, USA) was used. Two solvents were used: A, acetonitrile (HPLC grade, Duksan Chemical, Korea); and B, water (with 0.01% formic acid). The flow rate was 1 ml/min. The elution profile was 0-70 min, 20-50% B in A (linear gradient), and 5 μl (standard materials) and 20 μl (FC) volumes were analysed. The column eluent was monitored at UV 240 nm and then all solvents were degassed with a micromembrane filter (PTFE, Advantec, Japan).

3. Animals and AD model

Male SD rats (280-300 g, Nara Biotechnology, Korea) were used for this study. All animal protocols were approved by the Ethics Committee for the Care and Use of Laboratory Animals at Kyung Hee University. The animals were housed in plastic cages at constant temperature (22 ± 2℃) and humidity (55 ± 10%) with 12 h-12 h light-dark conditions. The animals were allowed free access to food and water before the experiment.

The Aβ (1-42) (#A9810, Sigma, St. Louis, MO. USA) was dissolved in sterile saline to a concentration of 1 mg/ml and incubated at 37℃ for 4days to allow for fibril formation. The rats were anesthetized with ketamine (80 mg/kg, i.p.) and placed on a stereotaxic instrument (USA). The scalp of each rat was incised, and the skull was adjusted to place the bregma and lambda on the same horizontal plane. Small bone hole was drilled through the skull. Aβ (1-42) (5 μl) was injected into a Hippocampus bilaterally, total 10 μl, (-3.3 mm anteroposterior, ±2.2 mm medial-lateral, -2.8 mm dorsal-ventral from the dura, in relation to the bregma) at a rate of 1 μl/min using a 10ul Hamilton syringe fitted with a 26-gauge stainless steel needle. The hole was blocked with bone wax and scalp was then closed with suture. Normal group rats received the same surgical procedure with injection of identical volume vehicle (sterile saline).The rat was allowed to recover from surgery for 2 days. Saline and FCE (100, 250, 500 mg/kg) were administered intragastrically once daily for 28 days beginning from two days after the Aβ (1-42) injection.

4. Experimental groups

The rats were randomly divided into six groups. The A β inj, FCE, Donepezil groups received intrahippocampal injection with Aβ (1-42) (10 μl). The normal group received the same surgical procedures and was injected with identical volume of vehicle (sterile saline). The FCE groups administered FCE (100, 250, 500 mg/kg, dissolved in normal saline, orally), once a day for 28 days from two days after the Aβ (1-42) injection. The Donepezil group administered donepezil (1mg/kg, disolved in normal saline, orally) The Aβ inj and normal groups received vehicle (normal saline) orally. A total of 48 rats were used.

5. Morris water maze test

The Morris water maze test was performed for 5 days. The acquisition training was performed for 4 days and the retention test on the 5th day. The apparatus consisted of a circular water pool 190 cm in diameter and 40 cm in height. It was filled with 23 ± 1℃ water with a depth of 28 cm and covered a black platform (15 cm in diameter). The platform was submerged approximately 1 cm below the surface of the water. The pool was divided into four equal quadrants: north- east (NE), northwest (NW), southeast (SE),

and southwest (SW). The platform was located in the center of the southwest quadrant. During the first 4 days acquisition test, rats were given 4 trials per day to find the hidden platform. Each rat (7 rat per group) was gently placed into the water facing the wall in the direction of north (N), east (E), south (S), and west (W) in two series of order. The rat was allowed to swim until they reached the hidden platform (maximum swim time was 60 seconds). The escape latency to reach the platform was recorded and they were allowed to remain on the platform for 10 seconds before being removed. The rat which failed to find the platform within 60 seconds was guided to the hidden platform and was then placed on the platform for 10 seconds for reinforcement before being removed. One trial of the retention test without the platform was performed on the 5th day to assess the memory of the correct platform location. The rats were placed into the pool and swam freely for 60 seconds. The swimming paths were recorded by a video camera linked to a computer-based image analyzer (SMART 2.5 video-tracking system, Panlab, Spain). The number of target heading and the Escape latency were analyzed. The rats were sacrificed after the retention test trial.

6. Immunohistochemisty

The brain sections were stained by the free-floating DAB reaction. The sections were rinsed with 0.05 M PBS and incubated for 15 min in 1% hydrogen peroxide PBS at room temperature. The sections were incubated overnight at 4℃ with primary antibody against Aβ(1:200, ab10148, Abcam) then incubated with biotinylated anti-rabbit secondary antibody (1:200, Millipore, Billerica, MA, USA), LRP-1 (1:50, sc-16166, Santa-cruz), then incubated with biotinylated anti-goat secondary antibody (1:200, Millipore, Billerica, MA, USA) for 2 h at room temperature, after which the avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA) method was carried out with peroxidase coupling in a mixture containing 0.05% DAB (Sigma-Aldrich, St. Louis, MO, USA) and 0.03% H_2O_2 for 2-5 min. Images of the DAB-colorized brain sections were captured using a light microscope (BX51, Olympus, Tokyo, Japan) equipped with CCD camera (DP70, Olympus).

7. Image Analysis

Measurement of the relative optical densities of various immuno-labeled cells were analyzed using the ImageJ software (Ver. 1.44p, NIH, Bethesda, MD, USA). The relative optical densities were measured in the CA1 pyramidal cells, dentate gyrus (DG) granular cells, vessels by the mean gray value on an inverted black-white binary image. The images normalized with the squared same area ($10^5 \mu m^2$). In vessel image case, the squared area contained vessel image. The mean values from the four sections analyzed in each rat were used for statistical analysis.

8. ELISA

Blood was collected from the heart of rat with a 22-gauge needle and transferred to cold EDTA-coated tubes. Samples were centrifuged at 1500 rpm for 10 min at 4℃, and the plasma phase was stored at -80℃. Plasma Aβ (1-42) levels were quantified using the Human/Rat Aβ (1-42) High-Sensitive Assay kit (#292-64501, Wako, Japan). Absorbance was read at 450 nm on a spectrophotometer (Rosys 2010, Anthos), and Aβ concentrations were determined from the Aβ (1-42) peptide standard curves after correcting for background absorbance and dilution factors. ELISA preparation and analysis were performed blind to treatment.

9. Statistical Analysis

All data in this study are presented as means ± standard errors and evaluated using the Student's t-test. A probability value of less than 0.05 was used to indicate a significant difference. Differences between the escape latencies between groups in the acquisition trials were evaluated using one-way ANOVA. Following significant ANOVAs, multiple post hoc comparisons were performed using the Duncan test. All tests were performed using SPSS 20.0 for wind windows (SPSS Inc., Chicago, IL, USA).

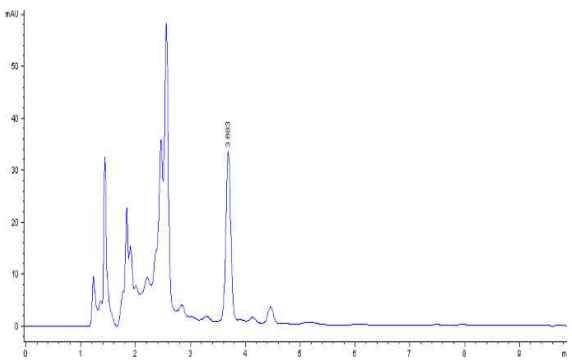

Fig. 1. The chromatographic profile of *Fructus Corni Officinalis* water extract. The numbers indicate retention times. The peaks identified was Loganin (3.683 min).

Results

FCE improved the spatial learning and memory deficits

in a rat model of Alzheimer's Disease.

1. Acquisition trials analysis.

Fig. 2 demonstrates that escape latencies in the traing period for four days. The Aβ inj group had significantly longer acquisition time than the normal group at the 2nd,3rd nd 4th days ($p<0.05$, $p<0.01$, $p<0.01$, respectively). The FCE groups and Donepezil groups howed significantly less acquisition time at the 3rd and 4th days ($p<0.05$, $p<0.01$ respectively) on the 3rd day. There were many trials that had shorter escape latency on the 3rd and 4th day than compared to the 1st and 2nd day(Fig. 2).

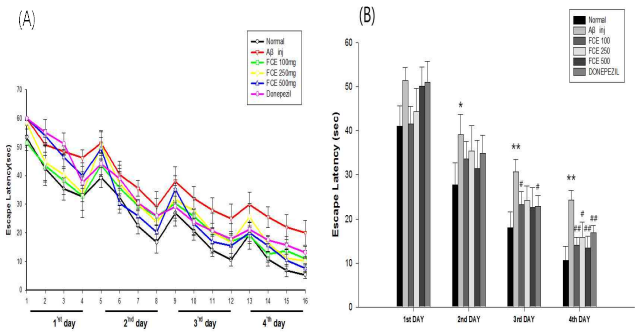

Fig. 2. Effect of *Fructus Corni Officinalis* water extract (FCE) on escape latencies in the acquisition training trials. Aβ(1-42) (5 μg/5 μℓ) or sterile saline (5 μℓ) was injected into the each hippocampus bilaterally, total 10 μl, 24 days before the first training day. A solution of FCE or normal saline was given orally once-daily for 28days after surgery. The training trial was performed four times a day for four days. (A) Escape latency on each trials. (B) Escape latency on each days. Data are represented by mean ± SEM (n=8 in each group). Statistical significances are compared between normal and Aβ inj groups (*, $p<0.05$; **, $p<0.01$) or between Aβ inj and FCE groups (#, $p<0.05$; ##, $p<0.01$).

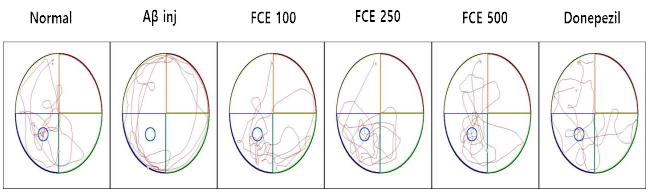

Fig. 3. Shown are representative swim paths in a probe test conducted following the completion of training.

2. Retention trials analysis

Upon completion of the acquisition trials, the escape latency and number of target headings were examined without the platform for 60 seconds on the 5thday.The escape latency of the Aβ inj group was 37.8 ± 6.0 s and was significantly longer than in the normal group that was 21.7 ± 5.0 s ($p<0.01$). The escape latency of the FCE 500 mg/kg group was 25.6 ± 5.3, a significantly shorter time compared to that of the Aβ inj group ($p<0.01$)(Fig. 4(A)).

The number of target heading, for the Aβ inj group was 0.8 ± 0.4. This was significantly smaller compared to the normal group's (1.5 ± 0.5), ($p<0.01$). The number of target heading for the FCE 500 mg/kg group was 1.8 ± 0.3. This was significantly larger compared to that of the Aβ inj group ($p<0.01$)(Fig. 4).

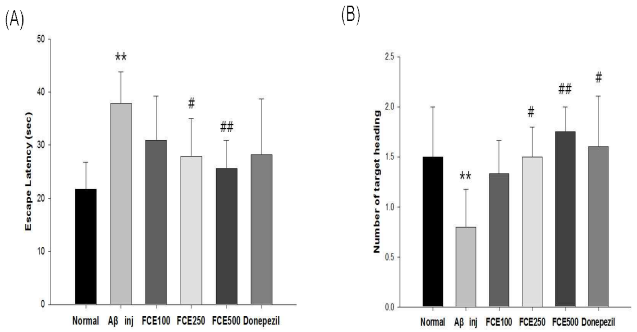

Fig. 4. Effect of *Fructus Corni Officinalis* water extract (FCE) on the retention trial. escape latency and number of target heading in the retention test trial. The retention trial was conducted the day after training trial. (A) Escape latency. (B) Number of target heading. Data are represented by mean ± SEM (n=8 in each group). Statistical significances are compared between normal and Aβ inj groups (*, $p<0.05$; **, $p<0.01$) or between Aβ inj and FCE groups (#, $p<0.05$; ##, $p<0.01$).

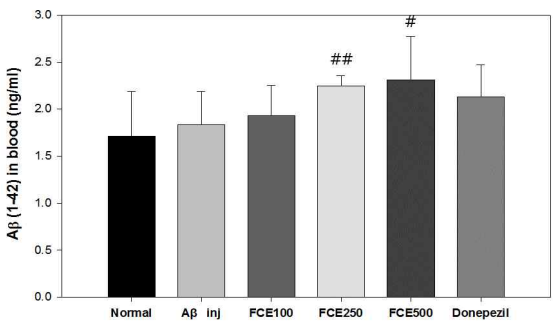

Fig. 5. Effect of *Fructus Corni Officinalis* water extract (FCE) on Aβ clearance in the blood. The expression of Aβ (1-42) was significantly increased by FCE 500 mg/kg dose. Data are represented by mean ± SEM (n=8 in each group). Statistical significances are compared between normal and Aβ inj groups (*, $p<0.05$; **, $p<0.01$) or between Aβ inj and FCE groups (#, $p<0.05$; ##, $p<0.01$).

3. FCE attenuated Aβ (1-42) accumulation in the hippocampus

The optical density of immunostaining of Aβ (1-42) accumulation was observed in hippocampus. The optical density of Aβ (1-42) immunostaining in the CA1 region for the FCE group was 104.6 ± 4.3, which was significantly lower compared to the Aβ inj group ($p<0.05$). And in the DG region, the FCE group was 120.4 ± 3.5, which was significantly lower compared to the Aβ inj group ($p<0.05$) (Fig. 6).

4. FCE increased Aβ (1-42) levels in the blood.

Plasma Aβ (1-42) levels were quantified using ELISA. A β inj group was 1.8 ± 0.4 nm/ml, higher compared to the normal group (1.7 ± 0.5 nm/ml). But there was no significant. The FCE 250, 500 mg/kg groups were higher, (2.2 ± 0.1, 2.3 ± 0.5 nm/ml) compared to the Aβ inj group significantly ($p<0.05$)(Fig. 5).

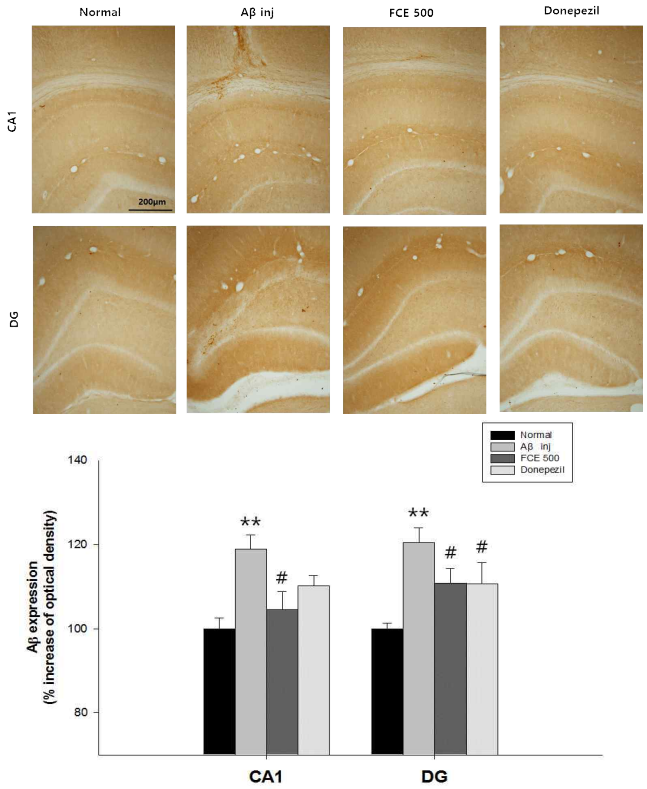

Fig. 6. Effect of *Fructus Corni Officinalis* water extract (FCE) on Aβ (1-42) expression in the hippocampus. (A) Representative photographs show the CA1 and DG regions of the hippocampus immnuno-stained against Aβ (1-42). Scale bar is 200 μm, applicable to all sections. FCE 500 mg/kg group significantly attenuated the upregulation of Aβ (1-42) expression in the CA1 and DG of the hippocampus. (B) The mean of optical density of Aβ (1-42) in CA1 and DG region of the hippocampus. Data are represented by mean ± SEM (n=4 in each group). Statistical significances are compared between normal and Aβ inj groups (*, $p<0.05$; **, $p<0.01$) or between Aβ inj and FCE groups (#, $p<0.05$; ##, $p<0.01$).

5. FCE increased LRP-1 expression around vessels in the hippocampus.

The optical density of immunostaining in the CA1 and vessels showed different tendency. In the hippocampus CA1, Aβ inj group was 110.9 ± 3.2, higher compared to the normal group (100.0 ± 2.7) significantly. The FCE 500 mg/kg group was lower, 105.4 ± 3.2 compared to the Aβ inj group ($p<0.05$). Otherwise, in the vessels, Aβ inj group was 88.1 ± 3.7, lower compared to the normal group (100.0 ± 3.1) significantly. The FCE 500 mg/kg group was 110.4 ± 2.4, higher compared to the Aβ inj group significantly ($p<0.05$)(Fig. 7).

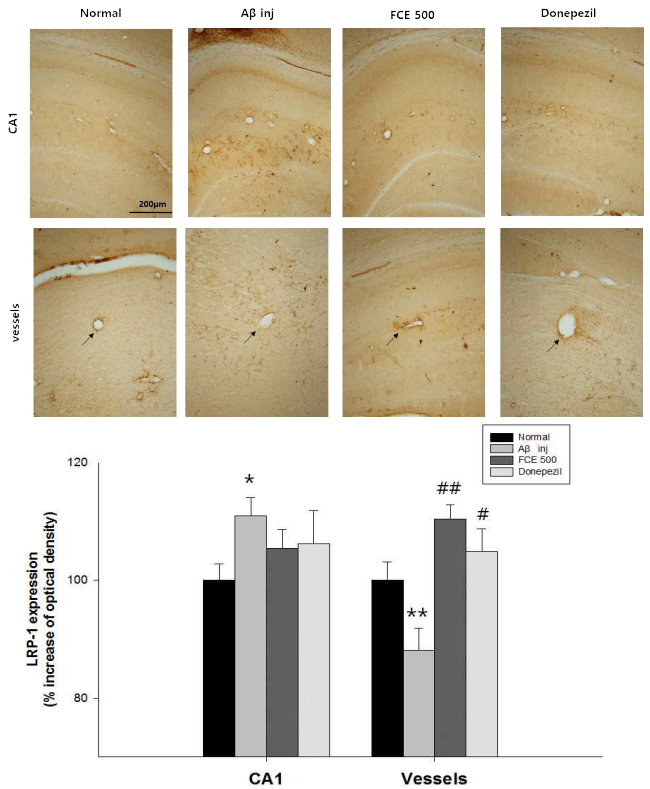

Fig. 7. Effect of *Fructus Corni Officinalis* water extract (FCE) on LRP-1 expression in the hippocampus and vessels. (A) Representative photographs show the hippocampus and vessels of the brain immnunostained against LRP-1. Scale bar is 200 μm, applicable to all sections. FCE 500 mg/kg group showed elevated LRP-1 expression around the vessels (arrows). (B) The mean of optical density of LRP-1 in CA1 and vessels region of the hippocampus. Data are represented by mean ± SEM (n=4 in each group). Statistical significances are compared between normal and Aβ inj groups (*, $p<0.05$; **, $p<0.01$) or between Aβ inj and FCE groups (#, $p<0.05$; ##, $p<0.01$).

Discussion

Alzheimer's disease (AD), neurodegenerative disease, is the most common form of senile dementia. The disease is characterized by histological anomalies, such as senile plaques, neurofibrillary tangles, and granulovacuolar degeneration[13,14]. Beta-amyloid (Aβ) plaques in the extracellular environment cause neuronal apoptosis and activation of neuroglia cells[14]. In the intracellular environment, tau protein produces neurofibrillary tangles that form filamentous shapes. Tau tangles create empty intracellular space surrounded by basophilic granules and granulovacuolar degeneration[15,16].

So far, several possible causes of Alzheimer's disease have been identified. Few of them are the reduction of acetylcholine, increase of acetylcholinesterase[17], amyloid cascade hypothesis[18], inflammatory response by neuroglia cell[19], gene mutation of *presenilin 1* and *presenilin 2*[20], and reactive oxygen species[21]. But the exact cause and

pathogenesis is still unknown and many efforts have been carried out to clear it.

Until recently, the majority of AD drugs elevate neuronal acetylcholine by suppressing acetylcholinesterase. Inhibitors of this enzyme including donepezil, rivastigmine, and galantamine are used to elevate levels of neuronal acetylcholine[22,23]. As the knowledge of AD pathogenesis increases, new treatments are now being developed for Aβ clearance[24]. Aβ clearance involves the efflux of Aβ through BBB to combat AD pathogenesis[25].

LRP-1 in brain capillaries plays a key role in Aβ elimination from the brain by mediating its efflux across the BBB. In a recent study, human Aβ microinjected into the mouse brain was detected in plasma using human specific ELISA for intact Aβ[26]. This study demonstrates transcytosis of intact Aβ from brain interstitial fluid into blood. Direct LRP/Aβ interaction has been observed in vitro with surface plasmon resonance analysis and ELISA assays, Direct binding of Aβ to the abluminal surface of brain capillaries, suggests that the LRP- Aβ interaction is the first step of Aβ transcytosis across the mouse BBB *in vivo*[26,27].

Current studies indicates that FCE is effective at boosting learning, memory, and preventing cognitive impairment[10,12,28]. We evaluated FCE as a potential treatment strategy for Alzheimer's disease. Using an animal model AD, we tested the effects of FCE on Aβ clearance and cognitive impairments.

The Morris water maze test, which assesses spatial reference memory and spatial working memory, was used to assess cognitive ability. The FCE groups exhibited an improvement in advanced learning memory and cognition, which became significant during the 3rd and 4th day of the 4 day training period (Fig 2B). FCE mainly benefited repeated learning and memory. Learning and memory deficits are major problems in Alzheimer's disease and prevent patients from having a normal life. From these findings, it appears that FCE may improve memory deficits and cognitive impairment in diverse conditions. Retention test showed, that the FCE 500 mg/kg group had 84.8% recovery compared to those in the control group in the escape latency. In particular FCE had a greater effect than Donepezil (76.9% recovery), which is a known remedy for dementia. Our results suggest that FCE can improve memory deficits and cognitive impairment.

Aβ (1-42) accumulation in brain tissue was confirmed by immunostaining. The Aβ inj group displayed higher Aβ accumulation in the hippocampal CA1 and DG region than the control group, The FCE 500 mg/kg group displayed significantly lower Aβ accumulation in the hippocampal CA1 region than the Aβ inj group (Fig. 6). Aβ accumulation tends to occur more often in the hippocampal CA1 region than in any other hippocampalus region in patients with AD28). Reduction of Aβ accumulation in CA1 with FCE could be a potentially effective treatment for Alzhemer's disease. We injected human Aβ (1-42) into the rat hippocampus to generate rat models of AD similar to many other studies using animal models of AD[29,30]. In another study, the AD phenotype was induced by intracerebroventricularly (i.c.v.) injecting a solution containing Aβ (1-42) for 4 weeks[31]. Aβ (1-42) was chosen because of its superior aggregating properties and because, it was thought to constitute the core of amyloid plaque. We found endogenous Aβ (1-42) in the control group. Several studies indicate that endogenous Aβ may play a role in controlling synaptic activity[32]. However we could not distinguish between the endogenous and exogenous form of Aβ (1-42) in the Aβ inj group and FCE groups, because we used Aβ (1-42) antibody which has both human and rat species reactivity. In our study, Aβ (1-42) expression was the sum of expression of both endogenous and exogenous Aβ (1-42).

We confirmed Aβ clearance with LPR-1 immunostaining. We hypothesized that LRP-1 expression would decrease in the Aβ inj group, however our data demonstrated that change in LRP-1 expression varied by region. The AB inj group showed increased LRP-1 expression in CA1, but decreased expression in vessels. The likely explanation for these results is that LRP-1 colocalizes with Aβ in senile plaques[33], which suggests a possible relationship between the decrease in vascular LRP-1 expression, the increase in neuronal LRP-1 expression, and Aβ induced toxicity. Therefore, there is a shift in LRP-1 immunoreactivity between neurons and vasculature. In the human tissue component of an LRP-1 study, reduced vascular LRP-1 staining was additionally observed in brains from patient with AD[34]. These studies report that distribution of LRP-1 receptors changes significantly between neurons and vasculature, in the hippocampus.

We additionally confirmed Aβ (1-42) levels in plasma. A β plasma levels were higher in the Aβ inj group than the control group; however the difference was not significant. The FCE 250 and 500 mg/kg groups had significantly higher Aβ plasma levels than Aβ inj group. In another study, both radiolabelled and unlabeled Aβ administered intracerebrally appeared intact in plasma, which indicated Aβ efflux from the brain by cerebrovascular LRP-1[35,36]. Coimmunoprecipitation of "LRP-1 bound Aβ" in normal

human patients indicated that circulating LRP1 can sequester 70-90% of plasma Aβ[37], thereby driving the Aβ gradient in favor of efflux across the BBB. Endogenous peripheral Aβ effluxed by LRP-1 promotes Aβ clearance from the brain into plasma. Our additional data confirmed Aβ (1-42) levels, but not "LRP-1 bound Aβ" levels in the plasma. Futher studies are needed to confirm that "LRP-1 bound Aβ" mediates Aβ efflux via the BBB.

FCE treatment increased LRP-1 expression in the vessels and reduced Aβ accumulation in the hippocampus by facilitation Aβ efflux via the BBB. The improvements in learning memory and cognition appears to be due to the amelioration of Aβ clearance. Our study is the first to confirm that FCE treatment reduces Aβ accumulation through Aβ clearance in an animal model of AD. FCE ameliorated Aβ induced memory impairment and Aβ clearance deficits. Our results suggest that FCE is an effective alternative for treating AD.

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