The Seed of *Zizyphus jujuba* var. *spinosa* Attenuates Alzheimer’s Disease-Associated Hippocampal Synaptic Deficits through BDNF/TrkB Signaling

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*Zizyphus jujuba* is a plant, which bears fruits and seeds that are used for medicinal purposes in Traditional oriental medicine. The seed of *Zizyphus jujuba* var. *spinosa* (EZJ) has been also traditionally used for psychiatric disorders in Chinese and Korean medicines. Recent findings have indicated that EZJ improves memory impairment, a common symptom of various neurological diseases. However, the effects of EZJ on amyloid β (Aβ) toxicity, which is a main cause of Alzheimer’s disease (AD), remain to be elucidated. To illuminate the potential anti-AD effect and mechanism in the mouse hippocampal tissue, we examined the effect of standardized EZJ on Aβ-induced synaptic long-term potentiation (LTP) deficit in the hippocampal tissue. EZJ blocked Aβ-induced LTP deficits in a concentration-dependent manner. Moreover, EZJ increased brain-derived neurotrophic factor (BDNF) level in naïve hippocampal slices. The finding that the blockade of BDNF receptor reduced the effect of EZJ suggests that EZJ ameliorates the Aβ-induced LTP deficit through BDNF/topomyosin receptor kinase B (TrkB) signaling. However, transcription or translation inhibitors failed to block the effect of EZJ, suggesting that BDNF synthesis is not required for the action of EZJ on LTP. Finally, we found that EZJ stimulates plasmin activity. In contrast, plasmin inhibitor blocked the effect of EZJ on the Aβ-induced LTP deficit. Our findings indicate that EZJ ameliorates Aβ-induced LTP deficits through BDNF/TrkB signaling. This phenomenon is induced by a regulatory effect of EZJ on the post-translation modification of BDNF.

**Key words** Zizyphus jujuba var. spinosa; amyloid β (Aβ); long-term potentiation; brain-derived neurotrophic factor (BDNF); plasmin

Alzheimer’s disease (AD) is a typical neurodegenerative disorder showing progressive memory decline. Learning and memory deficits and the loss of higher cognitive function are observed in the early stage of AD. AD is characterized by memory deficits and the loss of higher cognitive function are disorders showing progressive memory decline. Learning and memory deficits and the loss of higher cognitive function are observed in the early stage of AD. Although the specific initiators of AD still remain unknown, mounting evidence suggests that amyloid β (Aβ) plays an important role in the progress of the AD pathology. Extensive research revealed that soluble Aβ oligomers block hippocampal long-term potentiation (LTP), the cellular model of learning and memory. These indicated that hippocampal LTP could be a target for developing an anti-AD drug.

Brain-derived neurotrophic factor (BDNF) is an endogenous neurotrophin family important for the structural and functional plasticity of the brain. In AD patients, BDNF level is decreased in the several brain regions even in pre-clinical stages (very early time) of AD. Because BDNF is critical for neuronal survival, neuronal function, synaptic plasticity, and cognition, BDNF deficiency may result from AD pathology. Recent findings have shown that BDNF has protective effects against the toxic effects of Aβ peptides. Therefore, BDNF and its signaling activators are being investigated for application as new therapeutic targets for AD.

*Zizyphus jujuba* var. *spinosa* (Bunge) Hu ex HF Chow (Rhamnaceae) (English names: spine date seed, sour Chinese date seed, sour jujube seed, sour date seed) (EZJ) has been used in Asian traditional medicine as a treatment for psychiatric disorders. EZJ has showed anti-fidget and anti-insomnia effects. Moreover, it has also been used for amnesia in prescriptions. Recent studies have revealed that the ethanol extract of EZJ and its constituents produce hypnotic effects through the modulation of neurotransmitter systems including serotoninergic and γ-aminobutyric acid (GABA)ergic systems. Further, numerous brain pathology models have indicated memory-enhancing effects of EZJ. These findings demonstrated that EZJ can modulate brain function and may show anti-AD actions. To clear this hypothesis, we examined the action of EZJ on Aβ-induced LTP impairment.

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MATERIALS AND METHODS

Materials A standardized EZJ was donated by DAEHWA Pharmaceutical Co., Ltd. (Pangyo, Korea). The seed of Z. jujuba var. spinosa were crushed with blender. Extraction was conducted two times with 50% ethanol under reflux (80–85°C) for 3 h. Evaporation was conducted under reduced pressure and the extract was dried using spray dryer (yield, 13.8%). EZJ was standardized with spinosin (molecular weight (MW), 608.55, >0.5%). And the chemical profile of EZJ is presented in supplemental Fig. 1. Herbarium of the Traditional Herb Research Center, Korea Food and Drug Administration has a voucher specimen (No.11E-1001). Plasmin activity assay kit, actinomycin D, and anisomycin were purchased from Abcam Biochemicals (Cambridge, U.K.). ANA-12, gebexate mesylate and 6-aminocaproic acid were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).
Animals We obtained male CD-1 mice (26–28 g, 6 weeks old) from the SAMTAKO Biokorea (Osansi, Korea). Mice were housed in Animal facility for 1 week for adapting new environment. Four mice were kept in a cage and allowed free access to water and food (temperature: 23 ± 1°C, humidity: 60 ± 10%). The lights were on from 07:00 to 19:00. Institutional Animal Care and Use Committee of Dong-A University approved protocols of animal experiments.

Preparation of Acute Hippocampal Slices Artificial cerebrospinal fluid (ACSF) was comprised with 124 mM of NaCl; 3 mM of KCl; 26 mM of NaHCO3; 1.25 mM of NaH2PO4; 2 mM of CaCl2; 1 mM of MgSO4; 10 mM of d-glucose. We rapidly moved in ice-chilled Tris–HCl buffer [20 mM, pH 7.4, sucrose (0.32 m), ethylenediaminetetraacetic acid (EDTA) (1 mM), ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (1 mM)]. Debris was removed using microcentrifugation (4200 × g, 20 min). A mixture of 50 µL of sample (10 µg of protein) and 50 µL of reaction mix (48 µL plasmin assay buffer + 2 µL of plasmin substrate) was made. Measure output on a fluorescent microplate reader at Ex/Em=360/450 nm in a kinetic mode, every 2–3 min, for 10–20 min at 37°C protected from light.

RT-PCR Total RNA isolation and first-strand cDNA synthesis were performed as described previously.23) PCR amplification was performed with a PC-818A Program Temp Control System (Astec, Fukuoka, Japan), with 1 cycle for 5 min at 95°C and 30 cycles consisting of denaturation at 95°C for 40 s, annealing at 60°C for 40 s, and extension for 45 s at 72°C, followed by incubation at 72°C for 5 min. PCR products were analyzed by 1% agarose gel electrophoresis. Quantitation of the intensity of the amplified bands was performed using a Scion Image Instrument (Scion Corp., Frederick, MD, U.S.A.).

Statistical Analysis The values are expressed as the means±standard error of the mean (S.E.M). The results of all experiments were analyzed with one-way ANOVA followed by Turkey’s test for multiple comparisons. The statistical significance was set at p<0.05.

RESULTS EZJ Blocked Aβ-Induced LTP Deficit First, to test whether EZJ could modulate Aβ toxicity, we monitored LTP deficits by Aβ. HFS induced LTP in the Schaffer-collateral pathway of control slices (132 ± 5, n=7, Fig. 1A). However, HFS failed to induce LTP in Aβ-treated slices (109 ± 3, n=7, Fig. 1B). EZJ treatment blocked the Aβ-induced LTP deficit in a concentration-dependent manner (EZJ (10): 111 ± 9, n=7, Fig. trification (4200×g, 20 min). Proteins from whole-cell lysates were quantified using a BCA protein assay kit according to the manufacturer’s instructions. Samples (30 µg of protein) were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel) under reducing conditions. Proteins were transferred to PDVF membranes using transfer buffer [25 mM Tris–HCl (pH 7.4) containing 192 mM glycine and 20% (v/v) methanol] at 400 mA for 2 h (4°C). Next, blots were incubated for 2 h with blocking solution (5% skimmed milk) and then placed at 4°C overnight with 1:1000 dilutions of either anti-BDNF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or anti-phosphor-TrkB (tyrosine 706) antibody (Santa Cruz Biotechnology). After serial washing, blots were incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Blots were then incubated with anti-tubulin (1:5000, Santa Cruz Biotechnology) or TrkB (1:1000, Santa Cruz Biotechnology). BDNF expression levels were normalized to tubulin levels in the same membrane. Phosphor-TrkB expression levels were normalized to TrkB levels in the same membrane.

Plasmin Activity Assay Plasmin activity was measured using commercial plasmin activity assay kit (Abcam, ab204728). All procedures were followed to protocol presented from Abcam. Hippocampal slices were incubated with EZJ containing ACSF for 2 h. After then hippocampal slices were homogenized in ice-chilled Tris–HCl buffer [20 mM, pH 7.4, sucrose (0.32 m), ethylenediaminetetraacetic acid (EDTA) (1 mM), ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (1 mM)]. Debris was removed by microcentrifugation (4200×g, 20 min). A mixture of 50 µL of sample (10 µg of protein) and 50 µL of reaction mix (48 µL plasmin assay buffer + 2 µL of plasmin substrate) was made. Measure output on a fluorescent microplate reader at Ex/Em=360/450 nm in a kinetic mode, every 2–3 min, for 10–20 min at 37°C protected from light.
Donepezil (DNZ), a positive control, also blocked Aβ-induced LTP deficit (134±3, n=7, Fig. 1F). These results indicate that EZJ blocked Aβ-induced synaptic toxicity.

**EZJ Increased BDNF in the Hippocampus** To know the mechanism of EZJ effect on Aβ synaptotoxicity, we measured BDNF levels in the hippocampal slices. BDNF is known to exert neuroprotective effects in various neurodegenerative diseases. In this experiment, we found that EZJ increased BDNF levels (t₁=1.964, p<0.05, n=7/group). This result indicated that EZJ activate BDNF signaling (Fig. 2).

**TrkB Inhibition Blocked the Effect of EZJ on Synaptotoxicity of Aβ** We tested if the activation of BDNF signaling is required for the effect of EZJ on Aβ-induced LTP deficit.

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**Fig. 3. TrkB Inhibition Blocks the Action of EZJ on Aβ-Induced Synaptotoxicity**

Hippocampal slices were incubated in ANA-12 (100µM) and EZJ (100μg/mL) containing aCSF for 30 min. And then, the slices were further incubated in Aβ+EZJ+EAN-12 for 2h. fEPSP was recorded in stratum pyramidale of the hippocampus. Stimulating electrode was located on Shaffer collateral–commissural pathway. To induce LTP, HFS (100Hz, 100 pulses, 2 trains) was introduced at 20 min of stable baseline. LTP of control (A), Aβ (B), Aβ+EZJ (100µg/mL) (C), Aβ+EZJ (100µg/mL)+ANA-12 (100µM) (D), Aβ+ANA-12 (100µM) (E), groups were presented. (F) Bar chart for the normalized fEPSP slope at 80 min time points. *p<0.05 vs. control group. #p<0.05 vs. Aβ group. $p<0.05 vs. Aβ+EZJ (100µg/mL) group.
For this purpose, we used Tropomyosin receptor kinase B (TrkB) antagonists ANA-12. TrkB is a BDNF receptor. HFS could induce LTP in control slices, but not in Aβ-treated slices (control, 139 ± 11, n = 7, Fig. 3A; Aβ, 95 ± 7, n = 7, Fig. 3B). Although EZJ alleviated the Aβ-induced LTP deficit (129 ± 6, n = 7, Fig. 3C), it failed to block the Aβ-induced LTP deficit in the presence of ANA-12 (101 ± 4, n = 7, Fig. 3D). It was noted that ANA-12 alone did not affect the Aβ-induced LTP deficit.
(107±4, n=7, Fig. 3E). Both, inhibition of LTP by Aβ and rescue of LTP by EZJ were statistically significant. The effect of EZJ was blocked by ANA-12 (F<sub>4,30</sub>=7.948, p<0.05, n=7 groups, Fig. 3F). These results suggest that the effect of EZJ on Aβ synaptotoxicity might be mediated by BDNF signaling.

Transcriptional and Translational Regulations Are Not Involved in the Effect of EZJ on Aβ-Induced LTP Deficit

BDNF is a protein regulated by transcription and...
translation. Various neuroprotective agents regulate the synthesis of BDNF. Therefore, because EZJ increased BDNF level in the hippocampus, we tested whether transcription and translation are involved in the synaptoprotective effect of EZJ. HFS induced LTP in control slices, but not in Aβ-treated slices (control, 153±3, n=5, Fig. 4A; Aβ, 103±9, n=5, Fig. 4B). EZJ blocked Aβ-induced LTP deficit (Aβ+EZJ, 135±4, n=5, Fig. 4C). Interestingly, actinomycin D, a transcription inhibitor, and anisomycin, a translation inhibitor, failed to inhibit the effect of EZJ on Aβ-induced LTP deficit (Aβ+EZJ+anisomycin, 136±4, n=5, Fig. 4D; Aβ+EZJ+actinomycin D, 132±7, n=5, Fig. 4E; F_{4, 20}=9.788, p<0.05, n=5/group, Fig. 4F). These results indicated that BDNF transcription and translation are not involved in the effect of EZJ on Aβ synaptotoxicity.

**EZJ Regulate Plasmin Activity** BDNF is also regulated by post-translational modification, including cleavage of proBDNF to mature BDNF (mBDNF). Because plasmin is involved in this process, we first measured plasmin activity in hippocampal slices. We found that plasmin activity was decreased in Aβ-treated slices compared to that in control slices (F_{3, 16}=14.15, p<0.05, n=5/group, Fig. 5A). EZJ blocked this reduction of plasmin activity. Further, EZJ administration alone significantly increased plasmin activity as compared with plasmin activity in the control group. These results suggest that EZJ may exert its synaptoprotective effects through the regulation of plasmin activity. To test this, we conducted an LTP experiment with the plasmin inhibitors, gebexate mesylate and 6-aminocaproic acid. Aβ treatment blocked HFS-induced LTP (control, 147±11, n=7, Fig. 5B; Aβ, 105±5, n=7, Fig. 5C) and EZJ blocked Aβ-induced LTP impairment (Aβ+EZJ, 136±11, n=7, Fig. 5D). Of note, gebexate mesylate and 6-aminocaproic acid blocked the effect of EZJ on Aβ-induced LTP deficit (Aβ+EZJ+gebexate mesylate, 96±9, n=7, Fig. 5E; Aβ+EZJ+6-aminocaproic acid, 95±10, n=7, Fig. 5F). These results suggest that EZJ may exhibit its synaptoprotective effect through regulation of plasmin activity.

Finally, we observed the effect of plasmin inhibitor on EZJ-induced changes of BDNF signaling. We first checked whether EZJ regulates BDNF expression through examining BDNF mRNA expression. EZJ did not affect BDNF mRNA level in the hippocampus (Figs. 6A, B). Next, we examined changes of
BDNF/TrkB signaling with plasmin inhibitor, 6-aminocaproic acid. Aβ showed slightly reduced BDNF and pTrkB level but not significant. EZJ significantly increased BDNF and pTrkB levels compared to Aβ alone group. This increase of BDNF/TrkB signaling by EZJ was blocked by plasmin inhibitor (BDNF: $F_{3,16}=5.645$, $p<0.05$, $n=5$ group; pTrkB: $F_{3,16}=5.949$, $p<0.05$, $n=5$ group, Figs. 6C, D).

DISCUSSION

In the present study, we found that EZJ ameliorated Aβ-induced synaptic dysfunction, which occurs in the early stage of AD. EZJ administration increased BDNF levels in the hippocampus. Because BDNF can protect neurons against various cellular damages, we speculated that BDNF acting as a mediator of EZJ action might be responsible for AD symptoms, including neuronal dysfunctions. Previous reports have shown that a direct or indirect increase of BDNF levels reduces AD-like symptoms. Devi and Ohno recently showed that 7,8-dihydroxyflavone, a small molecular agonist of TrkB, rescued memory impairments in transgenic AD models. Brain and serum levels of BDNF in AD patients and animal model show a decline. Thus, the absence of BDNF action might be responsible for AD symptoms, including neuronal dysfunctions.

In the present study, we show protein transcription and translation are not required for the synaptoprotective effect of EZJ, although EZJ increased BDNF levels and alleviated Aβ-induced synaptic toxicity through BDNF receptor action. These results indicated that EZJ-induced BDNF generation is not involved in the synaptoprotective effect of EZJ. BDNF is generated as a precursor, which is cleaved by proteases and generates mBDNF. proBDNF preferentially binds to the TrkB receptor, whereas mBDNF selectively interacts and stimulates TrkB. Most neuroprotective actions of BDNF are mediated by TrkB receptor; therefore, mBDNF is considered the predominant form that exerts neuroprotective effects on various brain diseases. A previous study indicated that extracellular conversion of proBDNF to mBDNF by the tissue plasminogen activator (tPA)/plasmin protease system is critical for late-phase LTP. Moreover, tPA/plasmin system activation resulted in neuroprotection by increasing mBDNF. In the present study, we found that EZJ increased plasmin activity. Further, blockade of plasmin activity inhibited the effect of EZJ on Aβ-induced synaptotoxicity. Further, TrkB inhibitor also alleviated the synaptoprotective action of EZJ. Therefore, our results suggest that the increase in mBDNF levels by EZJ-induced plasmin activation blocked Aβ-induced synaptic toxicity through TrkB activation.

Many components of EZJ, including jujubosides and spinosin, were reported to exert neurological effects. Jujuboside A protects behavioral disorders in Aβ-induced AD model. Spinosin also reported to show protective effects in scopolamine or Aβ-induced dementia models. Moreover, spinosin facilitates neurogenesis and brain BDNF signaling in the hippocampus. Therefore, these two components could be active components of EZJ regarding the present study.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES


