

Agmatine Improves Cognitive Dysfunction and Prevents Cell Death in a Streptozotocin-Induced Alzheimer Rat Model

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Purpose: Alzheimer's disease (AD) results in memory impairment and neuronal cell death in the brain. Previous studies demonstrated that intracerebroventricular administration of streptozotocin (STZ) induces pathological and behavioral alterations similar to those observed in AD. Agmatine (Agm) has been shown to exert neuroprotective effects in central nervous system disorders. In this study, we investigated whether Agm treatment could attenuate apoptosis and improve cognitive decline in a STZ-induced Alzheimer rat model. **Materials and Methods:** We studied the effect of Agm on AD pathology using a STZ-induced Alzheimer rat model. For each experiment, rats were given anesthesia (chloral hydrate 300 mg/kg, ip), followed by a single injection of STZ (1.5 mg/kg) bilaterally into each lateral ventricle (5 μ L/ventricle). Rats were injected with Agm (100 mg/kg) daily up to two weeks from the surgery day. **Results:** Agm suppressed the accumulation of amyloid beta and enhanced insulin signal transduction in STZ-induced Alzheimer rats [experimental control (EC) group]. Upon evaluation of cognitive function by Morris water maze testing, significant improvement of learning and memory dysfunction in the STZ-Agm group was observed compared with the EC group. Western blot results revealed significant attenuation of the protein expressions of cleaved caspase-3 and Bax, as well as increases in the protein expressions of Bcl2, PI3K, Nrf2, and γ -glutamyl cysteine synthetase, in the STZ-Agm group. **Conclusion:** Our results showed that Agm is involved in the activation of antioxidant signaling pathways and activation of insulin signal transduction. Accordingly, Agm may be a promising therapeutic agent for improving cognitive decline and attenuating apoptosis in AD.

Key Words: Agmatine, streptozotocin, Alzheimer's disease, cognitive dysfunction, apoptosis, insulin signal transduction

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INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia and is a neurodegenerative disorder characterized by the degeneration of neurons, as well as by the

progressive decline of cognitive function. AD exhibits the hallmarks of both senile plaques derived from amyloid beta ($A\beta$) and neurofibrillary tangles, especially in the hippocampus or cerebral cortex, relevant to learning and memory.^{1,2} When $A\beta$ exists in high concentrations, it forms insoluble and fibrillar $A\beta$ plaques, which activate ion channels in the cell membrane to induce membrane depolarization and destabilization of intracellular calcium homeostasis.³⁻⁵ In particular, $A\beta$ oligomers cause intracellular Ca^{2+} overload, leading to neuronal death, which can be prevented by N-methyl-D-aspartate (NMDA) receptor antagonists.^{6,7}

Streptozotocin (STZ) is a glucosamine-nitrosourea compound that, when metabolized, generates a cytotoxic product that preferentially destroys pancreatic β cells.⁸ The alkylating properties of STZ metabolites generate reactive oxygen species and cause oxidative stress.⁸ Previously, intracerebroventricular streptozotocin (STZ-icv) administration was shown to induce oxidative stress,⁹⁻¹¹ neuronal cell damage,^{12,13} and dysfunctions in learning and memory.^{8,14,15} Accordingly, STZ-icv models have been used to assess the therapeutic potential of various drugs, as well as other non-drug therapeutic strategies.¹⁶ Additionally, STZ-induced learning and memory dysfunction is associated with oxidative stress in animal models.¹⁷ Therefore, to attenuate the death of neuronal cells caused by a variety of neurodegenerative diseases, antioxidants have been spotlighted in potential treatments of neurodegenerative diseases such as AD.^{17,18}

Agmatine (Agm) is an endogenous peptide synthesized by arginine decarboxylase, and is reported to be present in glia and neuronal cells. Several researchers have investigated the potential of Agm to improve cognitive function and neuronal cell death in various animal models.¹⁹⁻²³ Agm, a neurotransmitter or neuromodulator, exerts neuroprotective effects in various central nervous system injury models, including neurotrauma and neonatal ischemia animal models.²⁴⁻²⁸ Agm, as an NMDA receptor antagonist, plays a crucial role in regulating the production of nitric oxide (NO). Since NO can enhance a cell's survival rate under oxidative stress, Agm also could protect against damage to cells under oxidative stress.^{29,30} As well, nuclear factor-erythroid 2 related factor 2 (Nrf2) protects the cell against various stresses and regulates the expression of antioxidant genes, including superoxide dismutase, NAD(P)H, and γ -GCS.³¹⁻³⁶ Previous studies demonstrated that Nrf2 is related to cognitive decline.³⁷⁻³⁹ Accordingly, this study attempted to investigate whether Agm promotes Nrf2 mediated antioxidant signaling. In particular, we aimed to determine the potential benefits of Agm

in improving cognitive dysfunction and preventing cell death in a STZ-induced Alzheimer rat model.

MATERIALS AND METHODS

Animal model

Male Sprague-Dawley rats (n=50, weighing 250-330 g) were used in this study. Rats were maintained under controlled hygienic conditions with a 12 hr light/dark reverse cycle at a constant temperature with free access to food and water. All animal experiments were performed in accordance with the Korean Food and Drug Administration guidelines. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Yonsei Laboratory Animal Research Center (Permit #: 10-115).

Drug treatments

For each experiment, rats were given anesthesia [chloral hydrate 300 mg/kg, intraperitoneal (i.p)], followed by a single injection of STZ (1.5 mg/kg, dissolved in a vehicle consisting of 0.05 M citrate buffer) bilaterally into each lateral ventricle (5 μ L/ventricle). Control animals were given an equal volume of intracerebroventricular (icv) vehicle via the same procedure. Agm was purchased from Sigma (Sigma, St. Louis, MO, USA), dissolved in normal saline (pH 7.4), and administered to rats via an intra-peritoneal route. Rats were injected with Agm (100 mg/kg) daily for up to two weeks after surgery. The concentration of Agm (100 mg/kg) was determined based upon the results of our previous study.²⁵

Experimental design

The sham group (n=15) received bilateral icv injection of saline, 5 μ L in each rat. The rats in the EC group (n=17) were given injection of STZ-icv (1.5 mg/kg, 1st and 3rd days after surgery) (Sigma, St. Louis, MO, USA) bilaterally.⁴⁰ Rats in the STZ-Agm group (n=18) were given 5 μ L injection of STZ-icv (1.5 mg/kg, 1st and 3rd days after surgery) (Sigma, St. Louis, MO, USA) bilaterally and treated with Agm [100 mg/kg, intraperitoneal (i.p.) daily for two weeks from the 1st day after surgery]. The heads of the rats were positioned in a stereotactic frame (coordinates of 1.5 mm posterior to the bregma, 1.5 mm lateral to the sagittal suture, 2.5 mm ventral to the surface of the brain).

Cognition assessment tests

Cognitive evaluation of rats was tested using a Morris wa-

ter maze.⁴¹ Before conducting the Morris water maze test, we conducted pre-training. For all trials, a single rat was placed in a pool, facing the wall, at a different starting point (NW, NE, SW, or SE), and was then allowed to swim for a maximum of 60 seconds or until it reached the platform. If the rat failed to find the platform during the trial, it was manually guided to the platform by the investigator and placed on top of it for 15 seconds. Next, an apparatus consisting of a circular pool (200 cm diameter, 60 cm high) filled with water (depth 30 cm; 24±1°C) was placed in a room with consistently positioned spatial cues. An escape platform (15 cm diameter) was placed in the middle of one of the quadrants, 1.5 cm below the water surface, equidistant from the sidewall and the middle of the pool. The time required to reach the platform (escape latency) was measured in each trial. After the acquisition stage, a probe test was performed after removing the platform. We measured the latency up to the point when the rat traversed the location where the platform used to be as a measure of memory.

Hematoxylin and eosin (H&E) staining

Rats were anesthetized and perfused as previously mentioned.⁴² Rat brains were cut into coronal slices of 2 mm thickness using a rat brain matrix (Ted Pella, Redding, CA, USA). Next, the brain slices were fixed with 4% paraformaldehyde (pH 7.4) for 3 days and subsequently embedded in a paraffin block. The paraffin blocks containing the hippocampus and cortex were deparaffinized and re-hydrated with different concentrations of alcohol and xylene. After hematoxylin and eosin (H&E) staining, stained hippocampus and cortex sections were examined under a microscope.

Immunohistochemistry

Five µm thick frozen brain sections were harvested onto clean glass slides (Thermo Scientific, Bremen, Germany), air-dried, and fixed in cold acetone for 10 minutes at -20°C. The slides were washed first in Tris-buffered saline (TBS) and then incubated with 0.3% H₂O₂ in methanol to quench endogenous peroxidase activity. Followed by a series of washes (three times with distilled water), the sections were blocked with 10% normal rabbit serum. Frozen brain sections (20 µm) were fixed in ice-cold acetone for 20 minutes. To block specific staining, sections were incubated in 5% bovine serum albumin (BSA) (Sigma-Aldrich, Sigma, St. Louis, MO, USA) diluted in PBS for 30 minutes before addition of primary and secondary antibodies. Primary antibodies for phosphorylated insulin receptor substrate-1 (p-

IRS-1) (1:200, Santa Cruz, CA, USA), 8-oxo-2'-deoxyguanosine (8-OHdG) (1:200, Chemicon, Billerica, MA, USA), and anti-Aβ (1:200, Millipore, Billerica, MA, USA) were applied to the samples for 24 hours at 4°C, followed by a 90-minute incubation with appropriate fluorescence secondary antibody (1:100, Invitrogen, Carlsbad, CA, USA) and three washings in PBS for 10 minutes each. After three washes in 0.1% phosphate buffered saline with Tween-20 (PBST), the sections were incubated with rhodamine-conjugated sheep anti-rabbit or sheep anti-mouse secondary antibody that was diluted to 1:200 with 5% BSA fraction V in 0.1% PBST for 2 h in the dark at room temperature. After three washing in PBS, all sections were incubated with 1 µg/mL of 4',6-diamidino-2-phenylindole (Sigma-Aldrich, Sigma, St. Louis, MO, USA) and 2 µg/mL of propidium iodide (Sigma-Aldrich, USA) for a counter staining. Tissues were then visualized under a confocal microscope (Zeiss LSM 700, Carl Zeiss, Thornwood, NY, USA).

Western blot analysis

For Western blot analysis, rats from all groups were sacrificed and their brains were perfused with saline through the heart aorta to rinse away traces of blood. Next, the portion of the brain comprising the hippocampus and cortex was dissected for extraction of proteins and treated with lysis buffer (1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM Ethylene-di-amine tetra acetic acid, 1 mM ethylene glycol tetraacetic acid (pH 8.0), 0.2 mM sodium orthovanadate, 0.2 mM phenyl methyl sulfonyl fluoride, and protease inhibitor cocktail). Isolated proteins were centrifuged at 12000 rpm at 4°C. Equal amounts of protein (20 µg) from the supernatants were separated on a 10% acrylamide gel and proteins were electrophoretically transferred onto nitrocellulose membranes. After blocking with 5% skim milk for 2 hrs, the membranes were incubated with primary antibodies against Bcl2 (1:1000, Santa Cruz, San Jose, CA, USA), Bax (1:1000, Santa Cruz, San Jose, CA, USA), cleaved caspase-3 (1:1000, Cell signaling, Billerica, MA, USA), PI3K (1:2000, Millipore, Billerica, MA, USA), Nrf2 (1:200, Santa Cruz, San Jose, CA, USA), γ-GCS (1:500, Thermo Scientific, Bremen, Germany), and β-actin (1:1000, Millipore, Billerica, MA, USA) at 4°C overnight. Later the membranes were washed three times for 5 min each with TBST. The detection of secondary anti-rabbit and anti-mouse antibodies (1:3000, New England Bio labs, USA) was conducted for 1 hour at room temperature. After washing with PBST (0.05% with Tween 20) three times,

immunoreactive signals were detected by chemiluminescence with an ECL detection system (Amersham Life Science, London, UK) using the LAS 4000 program.

Statistical analysis

Statistical analyses were carried out using SPSS 18.0 software (IBM Portsmouth, IBM North Harbour, Portsmouth, UK). All data are expressed as means±S.E.M. Statistical significance in intergroup differences was determined by one-way analysis of variance, followed by Scheffe's post hoc multiple comparison test. Each experiment included at least three replicates per condition. Differences with a *p* value less than 0.05 were considered statistically significant.

RESULTS

Agmatine treatment attenuates A β accumulation and improves cognitive dysfunction in STZ-icv rats

To determine the accumulation of A β , on the 21st day following STZ injection, A β staining was performed in the sham, EC, and STZ-Agm groups. The expression of A β was considerably decreased in the STZ-Agm group compared to the EC group (Fig. 1A). To confirm phosphorylation of IRS-1 by Agm treatment in STZ-icv rats, we conducted immunohistochemistry using phospho-IRS-1 antibody, because IRS-1 plays a key role in transmitting signals from the insulin receptors to intracellular pathways. In the STZ-Agm group, both hippocampus (Fig. 1B) and cortex regions (Fig. 1C) showed an increase in IRS-1 phosphorylation compared to the EC group. These data indicated that Agm treatment could promote IRS-1 phosphorylation in STZ-icv rat models.

In previous studies, Morris water maze tests have been commonly applied for the assessment of cognition and memory functions. To confirm the enhancement of memory function in our STZ-induced Alzheimer rat model upon treatment with Agm, we conducted Morris water maze tests. The swimming times of four trials per day for 5 days in each group are shown in Fig. 1. Escape latency time (days 1-5) (to find a hidden platform) was significantly prolonged in the EC group compared to the sham group (Fig. 1D). Fig. 1D shows that the STZ-Agm group animals presented a significantly lower latency to find the platform than the EC group. The animals of the STZ-Agm group demonstrated improved Morris water maze acquisition performance.

Our findings suggest that Agm treatment could improve learning and memory dysfunction in STZ-induced Alzheimer rats.

Agmatine treatment attenuates histological abnormalities in hippocampus and cortex regions of STZ-icv rats

To investigate morphological differences in cells, we conducted H&E staining. The H&E staining of Cornu Ammonis (CA)1, CA2, and CA3 regions of the hippocampus revealed a large number of degenerated cells in the EC group compared with STZ-Agm group (Fig. 2A), which were evidenced by a decrease in the number of H&E stained cells. The EC group exhibited greater shrinkage of the nuclei of cells than those in the STZ-Agm group. Fig. 2B shows that the number of abnormal cells in the cortical region were higher in the EC group than the STZ-Agm group. Fig. 2 indicates that Agm treatment reduces STZ-induced histological abnormalities in hippocampus and cortex regions, compared with the EC group.

Agmatine treatment inhibits the cell death pathway in STZ-icv rats

To confirm apoptotic cell death in the EC group and the STZ-Agm group, western blot analysis was conducted to check expression of apoptotic proteins. Fig. 3A indicates that the presence of cleaved caspase-3, an active form of caspase-3, was attenuated in the STZ-Agm group compared with the EC group. Additionally, in the present study, the anti-apoptotic effect of Agm was investigated by checking the expression of proteins, such as Bax, Bcl2, and PI3K, using western blotting. Among the quantitative western blot results, the expression of Bax, known as a pro-apoptotic protein, was higher in the EC group than the STZ-Agm group (Fig. 3C). In contrast, Bcl2, known as an anti-apoptotic protein, was expressed more in the STZ-Agm group than the EC group (Fig. 3B). Also, the STZ-Agm group showed increased expression of PI3K, known to be related to survival pathways and insulin signal transduction, compared with the EC group (Fig. 3D). Fig. 3 suggests that Agm treatment inhibits cell death signaling in STZ-induced Alzheimer rats.

Agmatine treatment promotes the Nrf2-mediated antioxidant pathway in STZ-icv rats

To detect the generation of Reactive Oxygen Species (ROS), which causes DNA damage to the cells, we conducted

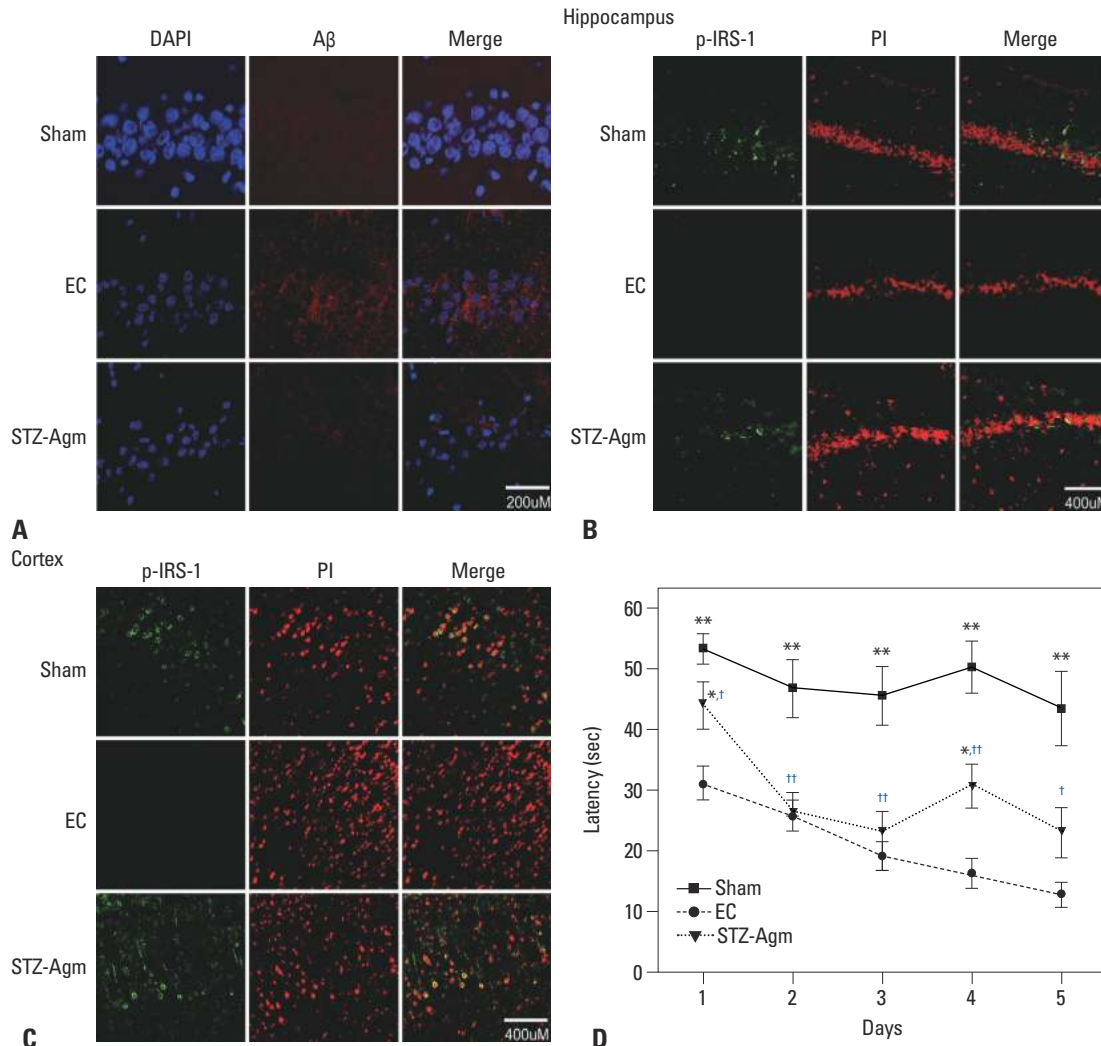


Fig. 1. Agmatine attenuated A β accumulation and promoted phosphorylation of IRS-1 in the STZ-icv rat model. (A) The expression of A β accumulation in the sham group, EC group, and STZ-Agm group. The image was shown at the magnification of 400. Scale bar: 200 μ M. (B) The expression of phosphorylated IRS-1 in the sham, EC, and STZ-Agm groups in hippocampus sections. The image was shown at the magnification of 200. Scale bar: 400 μ M. (C) The expression of phosphorylated IRS-1 in the sham, EC, and STZ-Agm groups in cortex sections. The image was shown at the magnification of 200. Scale bar: 400 μ M. (D) The latency time of Morris water maze was measured in the sham, EC, and STZ-Agm groups. The time required to reach the platform (escape latency) was measured on each day (1-5 days). Data were expressed as mean \pm SEM, and were analyzed statistically using one-way ANOVA, followed by Scheffe's post hoc (* p <0.05, ** p <0.001 compared to Sham group, $^{\dagger}p$ <0.05, $^{\ddagger}p$ <0.01 compared to EC group with STZ-Agm group). DAPI, 4',6-diamidino-2-phenylindole; PI, propidium iodide; p-IRS-1, phosphorylated IRS-1; STZ, streptozotocin; EC, experimental control.

8-OHdG staining. The immunohistochemical localization of 8-OHdG revealed a decreased number of 8-OHdG positive cells in the STZ-Agm group compared to the EC group (Fig. 4A). Fig. 4A indicates that ROS generation is decreased in the STZ-Agm group compared with the EC group. The western blot data for Nrf2 (known as an antioxidant transcription factor) (Fig. 4B) and γ -GCS (an important enzyme in glutathione synthesis) (Fig. 4C) show that Agm treatment promotes Nrf2-mediated antioxidant pathway signaling. Fig. 4 indicates that Agm treatment prevents cell death and promotes Nrf2-mediated antioxidant signaling in STZ-induced Alzheimer rats.

DISCUSSION

AD is one of the most prevalent degenerative diseases in the elderly. AD is characterized by progressive memory loss and cognitive impairment.⁴³ The administration of STZ to the central nervous system (CNS) generates similar pathology to AD.⁴⁴⁻⁴⁶ Previously, STZ-icv administration was shown to induce oxidative stress, neuronal cell damage, and dysfunction in learning and memory.^{8,14,15} Accordingly, STZ-icv models have been used to assess the therapeutic potential of various drugs. Hence, we employed a STZ-induced Alzheimer rat

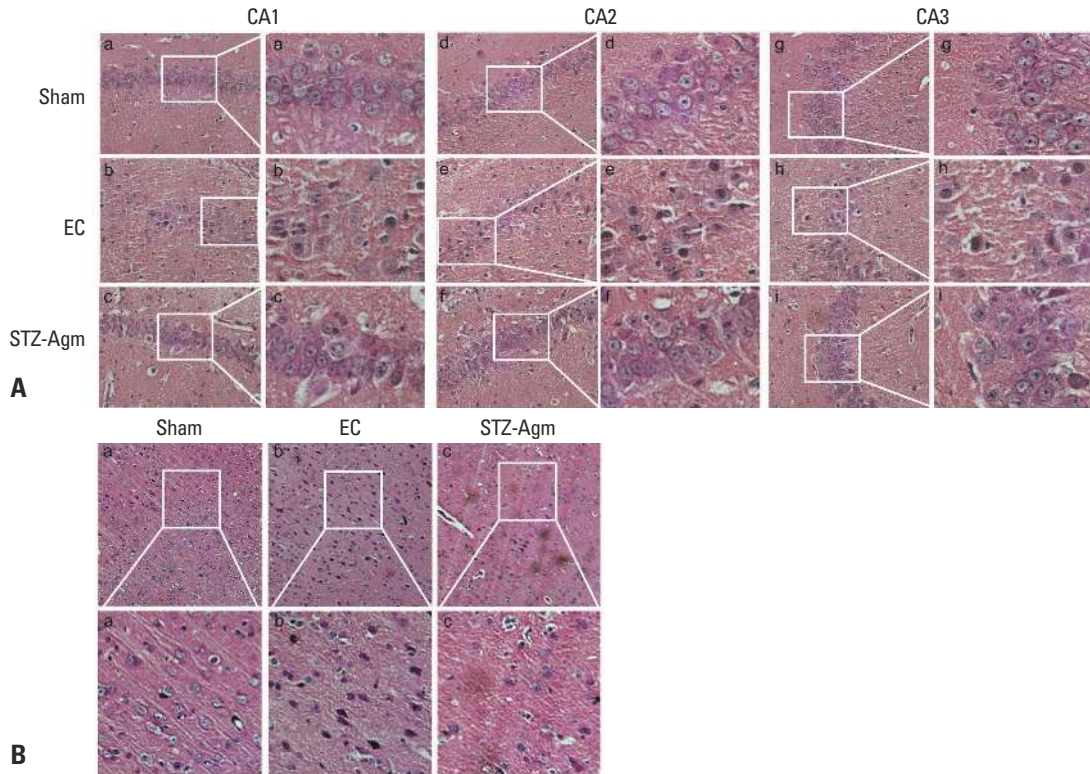


Fig. 2. Histological analysis of the hippocampus and cortex regions in STZ-icv rats. (A) Hippocampus sections from the sham group (a, b, c), EC group (d, e, f), and STZ-Agm group (g, h, i). CA1 (a, d, g), CA2 (b, e, h), CA3 (c, f, i). Scale bars were indicated. (B) Cortex sections from the sham group (a), EC group (b), and STZ-Agm group (c). All slides were stained by hematoxylin and eosin (H&E). STZ, streptozotocin; EC, experimental control.

model to investigate AD like pathologies.

In previous studies, the administration of STZ in the CNS generated similar pathology to AD, mainly accumulation of A β .^{46,47} In regards to the pathologic status of AD, the hyper activation of glutamate receptor and continuous Ca²⁺ influx by A β results in neuronal damage and cognitive dysfunction.⁴⁸⁻⁵⁰ Additionally, a previous study showed that memantine, as a glutamate NMDA receptor channel antagonist, blocks NMDA overstimulation upon excitotoxicity; accordingly, memantine was suggested to be of potential use in the treatment of AD.⁵¹ Several studies reported the neuroprotective effects of Agm in a variety of neurodegenerative pathologies through possible blockade via NMDA channels.^{24,25,30,52-54} As shown in our data, A β accumulation in damaged brain regions was decreased in the STZ-Agm group compared to the EC group.

Also, STZ-icv in rats can induce brain insulin system dysfunction and induce progressive deficits in learning, memory, and cognitive behavior like sporadic AD.^{12,46} As shown in our data, STZ inhibits phosphorylation of IRS-1 in rats. IRS-1 plays a key role in transmitting signals from insulin receptors to intracellular pathways.⁵⁵ Accordingly, several studies demonstrated that IRS-1 signaling pathways

are associated with cognitive function in CNS diseases.⁵⁶ Insulin dysregulation contributes to AD pathologies by reducing brain glucose utilization.⁵⁷⁻⁵⁹ Insulin affects neuronal cognition and memory by regulating ion channels and neurotransmitter receptors in AD brains.^{60,61} Considering that insulin signal transduction is important for cognitive function,^{59,62-64} our data indicated that Agm could induce the activation of brain insulin signal transduction and improve learning and cognitive decline in STZ-induced Alzheimer rat model.

In addition, Agm inhibits cell death by regulating the production of NO,^{29,30,65} and also attenuates neuronal cell death in neurodegenerative animal models.^{19-23,66-68} During the onset of AD, there is increased oxidative stress leading to the retardation of cognitive ability.⁶⁹⁻⁷¹ In the present study, water maze test results highlighted significant improvements in test scores in the STZ-Agm group, compared with EC group. These functional outcomes may because Agm, a NMDA antagonist, improves memory function in EC rats.^{72,73} Additionally, our H&E staining data indicated that the excessive production and accumulation of ROS by A β can cause functional and structural changes in critical macromolecules leading to lipid peroxidation, protein oxidation, and

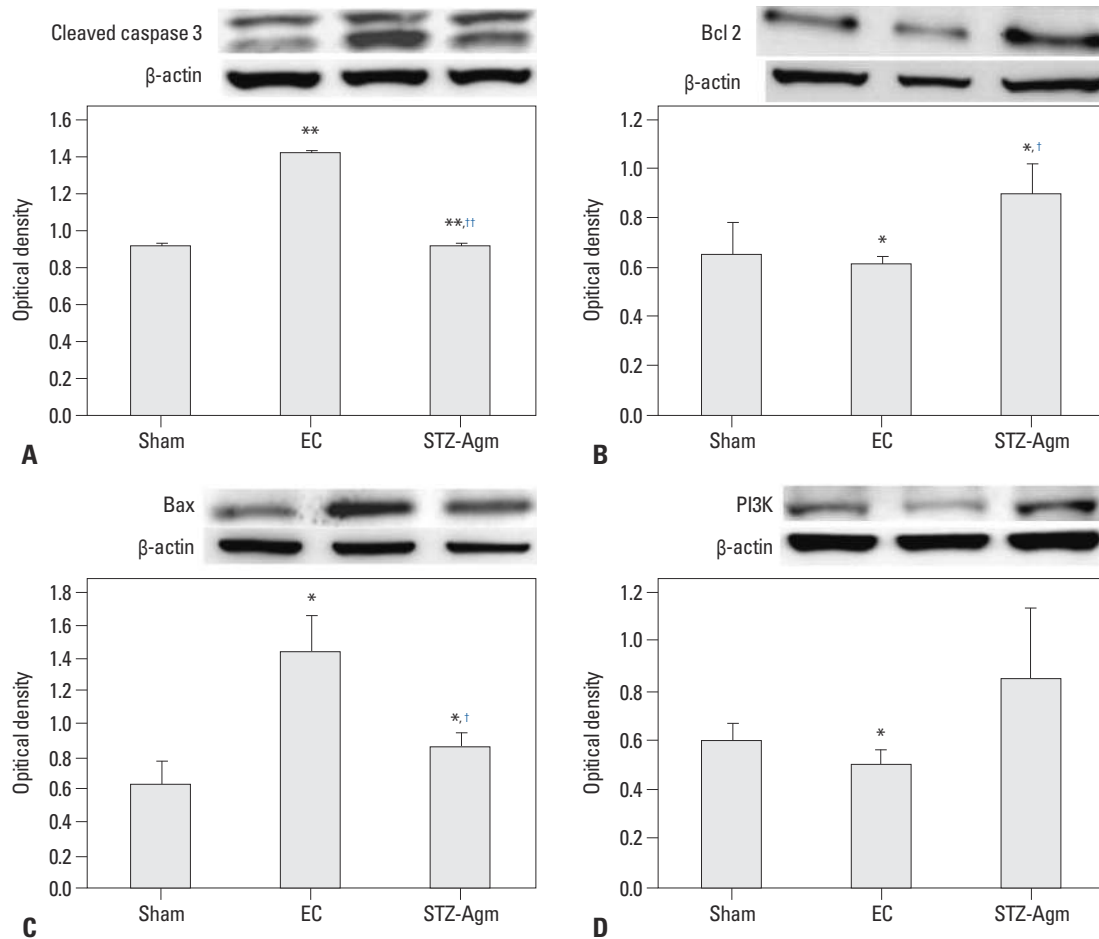


Fig. 3. Agmatine treatment decreased the expression of apoptotic proteins in STZ-icv rats. (A) Representative blots showed the protein levels of cleaved caspase-3 from the total protein extracts prepared from the hippocampus regions of each group. Bars represented the relative protein quantification of active cleaved caspase-3 on the basis of β -actin, respectively. Representative blots showed the protein levels of Bcl2 (B) and Bax (C) from the total protein extracts prepared from the hippocampus regions of each group. (D) Representative blots showed the protein levels of PI3K from the total protein extracts prepared from the hippocampus regions of each group (* p <0.05, ** p <0.01 compared to Sham group, † p <0.05, †† p <0.01 compared to EC group). STZ, streptozotocin; EC, experimental control.

DNA cleavage. Earlier studies have suggested that antioxidant treatment could therapeutically cure and prevent neurodegenerative diseases, especially sporadic AD.^{17,18} Moreover, previous researchers reported that antioxidants such as melatonin, vitamin E, and selegiline can be used to cure AD.⁷⁴⁻⁷⁷

Also, STZ-induced learning and memory dysfunction is considerably associated with oxidative stress in animal models.⁷⁸⁻⁸⁰ The levels of molecular markers for DNA (particularly 8-OHdG) are reported to be elevated in the brains of patients with AD.⁷² Bcl2 is neuroprotective against apoptotic cell death caused by A β .⁸¹ Accordingly, overexpression of Bcl2 could attenuate the processing of amyloid precursor protein and tau and reduce extracellular deposits of A β .⁸² Bcl2 protects neuronal cells by inhibiting the activation of caspase-3.^{81,82} Previously, Bcl-2 expression was shown to be upregulated, while Bax and caspase-3 were down regu-

lated in AD models.⁸³ Bax also plays an essential role in oligomeric A β -induced neuronal cell death.⁸⁴ In the present study, Bax and Bcl2^{85,86} were measured. Agm treatment significantly decreased the expression of 8-OHdG, cleaved caspase-3, and Bax, and increased the expression of Bcl2 in STZ-induced Alzheimer rats.

PI3K signaling cascade promotes NO generation through the activation of endothelial nitric oxide synthase.^{63,87} In addition, the PI3K/Akt signaling pathway plays crucial roles in cell survival, growth, gene expression, apoptosis, metabolism,⁸⁸ and also, neuronal survival.⁸⁹ Liu, et al.⁶⁴ demonstrated that the insulin-PI3K/Akt signaling pathway is reduced in AD brain. As IRS-1 plays a key role in transmitting signals from the insulin receptors to intracellular pathways including the PI3K pathway, the phosphorylation of IRS-1 by Agm treatment could improve cognitive decline and protect against cell death by activating the PI3K pathway.

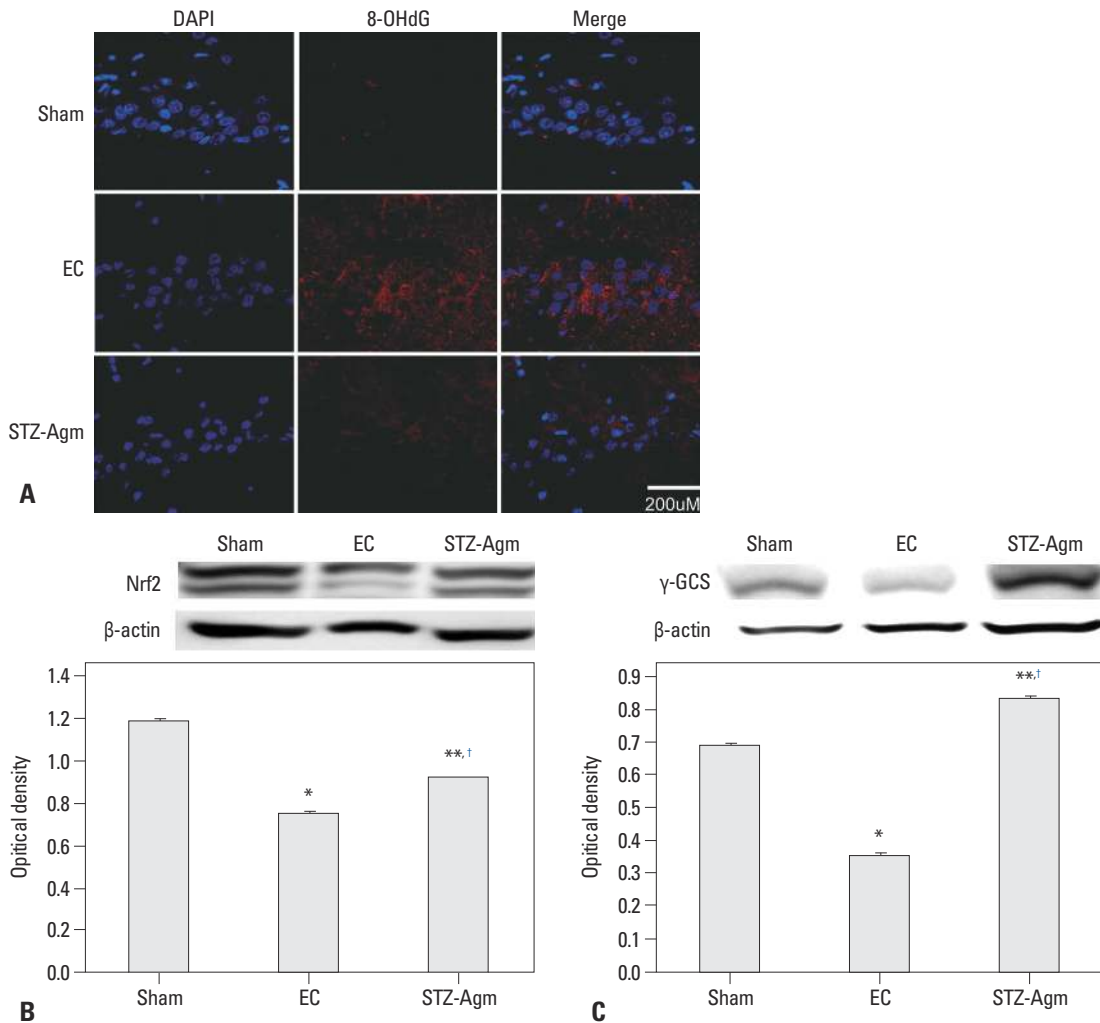


Fig. 4. Agmatine treatment decreased the immunoreactivity of 8-OHdG and increased the expression of Nrf2 and γ -GCS in STZ-icv rats. (A) Immunohistochemistry images showed immunostaining of 8-OHdG (red) in the sham group, EC group and STZ-Agm group. The image was shown at the magnification of 400. Scale bar: 200 μ M. (B) Western blot showed the amount of Nrf2 protein from the total protein extracts prepared from the hippocampus regions of each group. Bar graph showed the quantification of Nrf2 protein levels in all groups. (C) Western blot showed the expression levels of γ -GCS from the total protein extracts prepared from the hippocampus regions of each group. Bar graph showed the quantification of γ -GCS protein levels (* p <0.05, ** p <0.01 compared to the sham group, † p <0.05 compared to the EC group). DAPI, 4',6-diamidino-2-phenylindole; STZ, streptozotocin; GCS, glutamyl cysteine synthetase; EC, experimental control.

Nrf2 plays an important role in regulating cellular oxidative stress and controls the expression of many detoxifying genes such as catalase, superoxide dismutase, heme oxygenase-1 (HO-1), NAD(P)H, and γ -GCS.³¹⁻³⁴ Nrf2 activation can induce the antioxidant pathway and protect cells against oxidative stress.^{35,36} In addition, Nrf2 ameliorates cognitive impairment.³⁷⁻³⁹ Our western blot data for Nrf2 and γ -GCS indicated that Agm could promote Nrf2-mediated antioxidant pathways in STZ-induced Alzheimer rats.

In conclusion, Agm could improve cognitive decline by decreasing accumulation of A β and ameliorating insulin signal transduction. Also, Agm could protect against damage to cells by activating Nrf2-mediated antioxidant signaling. Hence, Agm may be a promising therapeutic agent

for alleviating neuronal cell apoptosis and cognitive decline in AD.

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REFERENCES

- Hyman BT, Damasio H, Damasio AR, Van Hoesen GW. Alzheimer's disease. *Annu Rev Public Health* 1989;10:115-40.
- Van Hoesen GW, Augustinack JC, Dierking J, Redman SJ, Thangavel R. The parahippocampal gyrus in Alzheimer's disease. Clinical and preclinical neuroanatomical correlates. *Ann N Y Acad Sci* 2000;911:254-74.
- Arispe N, Rojas E, Pollard HB. Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminum. *Proc Natl Acad Sci U S A* 1993; 90:567-71.
- Furukawa K, Abe Y, Akaike N. Amyloid beta protein-induced irreversible current in rat cortical neurones. *Neuroreport* 1994;5: 2016-8.
- Mattson MP, Barger SW, Cheng B, Lieberburg I, Smith-Swintosky VL, Rydel RE. beta-Amyloid precursor protein metabolites and loss of neuronal Ca²⁺ homeostasis in Alzheimer's disease. *Trends Neurosci* 1993;16:409-14.
- Alberdi E, Sánchez-Gómez MV, Cavaliere F, Pérez-Samartín A, Zugaza JL, Trullas R, et al. Amyloid beta oligomers induce Ca²⁺ dysregulation and neuronal death through activation of ionotropic glutamate receptors. *Cell Calcium* 2010;47:264-72.
- Kelly BL, Ferreira A. beta-Amyloid-induced dynamin 1 degradation is mediated by N-methyl-D-aspartate receptors in hippocampal neurons. *J Biol Chem* 2006;281:28079-89.
- Lester-Coll N, Rivera EJ, Soscia SJ, Doiron K, Wands JR, de la Monte SM. Intracerebral streptozotocin model of type 3 diabetes: relevance to sporadic Alzheimer's disease. *J Alzheimers Dis* 2006;9:13-33.
- Butterfield DA. Proteomics: a new approach to investigate oxidative stress in Alzheimer's disease brain. *Brain Res* 2004;1000:1-7.
- Lannert H, Hoyer S. Intracerebroventricular administration of streptozotocin causes long-term diminutions in learning and memory abilities and in cerebral energy metabolism in adult rats. *Behav Neurosci* 1998;112:1199-208.
- Gibson GE, Huang HM. Oxidative stress in Alzheimer's disease. *Neurobiol Aging* 2005;26:575-8.
- Grünblatt E, Salkovic-Petrisic M, Osmanovic J, Riederer P, Hoyer S. Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. *J Neurochem* 2007;101:757-70.
- Weinstock M, Shoham S. Rat models of dementia based on reductions in regional glucose metabolism, cerebral blood flow and cytochrome oxidase activity. *J Neural Transm* 2004;111:347-66.
- Dröge W, Kinscherf R. Aberrant insulin receptor signaling and amino acid homeostasis as a major cause of oxidative stress in aging. *Antioxid Redox Signal* 2008;10:661-78.
- Hoyer S, Lannert H. Inhibition of the neuronal insulin receptor causes Alzheimer-like disturbances in oxidative/energy brain metabolism and in behavior in adult rats. *Ann N Y Acad Sci* 1999; 893:301-3.
- Hoyer S. Glucose metabolism and insulin receptor signal transduction in Alzheimer disease. *Eur J Pharmacol* 2004;490:115-25.
- Ahmad M, Saleem S, Ahmad AS, Yousuf S, Ansari MA, Khan MB, et al. Ginkgo biloba affords dose-dependent protection against 6-hydroxydopamine-induced parkinsonism in rats: neurobehavioural, neurochemical and immunohistochemical evidences. *J Neurochem* 2005;93:94-104.
- Ansari MA, Ahmad AS, Ahmad M, Salim S, Yousuf S, Ishrat T, et al. Selenium protects cerebral ischemia in rat brain mitochondria. *Biol Trace Elem Res* 2004;101:73-86.
- Arteni NS, Lavinsky D, Rodrigues AL, Frison VB, Netto CA. Agmatine facilitates memory of an inhibitory avoidance task in adult rats. *Neurobiol Learn Mem* 2002;78:465-9.
- Liu P, Collie ND. Behavioral effects of agmatine in naive rats are task- and delay-dependent. *Neuroscience* 2009;163:82-96.
- Lu W, Dong HJ, Gong ZH, Su RB, Li J. Agmatine inhibits morphine-induced memory impairment in the mouse step-down inhibitory avoidance task. *Pharmacol Biochem Behav* 2010;97:256-61.
- McKay BE, Lado WE, Martin LJ, Galic MA, Fournier NM. Learning and memory in agmatine-treated rats. *Pharmacol Biochem Behav* 2002;72:551-7.
- Zarifkar A, Choopani S, Ghasemi R, Naghdi N, Maghsoudi AH, Maghsoudi N, et al. Agmatine prevents LPS-induced spatial memory impairment and hippocampal apoptosis. *Eur J Pharmacol* 2010;634:84-8.
- Feng Y, Piletz JE, Leblanc MH. Agmatine suppresses nitric oxide production and attenuates hypoxic-ischemic brain injury in neonatal rats. *Pediatr Res* 2002;52:606-11.
- Kim JH, Yenari MA, Giffard RG, Cho SW, Park KA, Lee JE. Agmatine reduces infarct area in a mouse model of transient focal cerebral ischemia and protects cultured neurons from ischemia-like injury. *Exp Neurol* 2004;189:122-30.
- Regunathan S, Dozier D, Takkalapalli R, Phillips WJ. Agmatine levels in the cerebrospinal fluid of normal human volunteers. *J Pain Palliat Care Pharmacother* 2009;23:35-9.
- Reis DJ, Regunathan S. Agmatine: a novel neurotransmitter? *Adv Pharmacol* 1998;42:645-9.
- Regunathan S, Youngson C, Raasch W, Wang H, Reis DJ. Imidazoline receptors and agmatine in blood vessels: a novel system inhibiting vascular smooth muscle proliferation. *J Pharmacol Exp Ther* 1996;276:1272-82.
- Halaris A, Plietz J. Agmatine: metabolic pathway and spectrum of activity in brain. *CNS Drugs* 2007;21:885-900.
- Reis DJ, Regunathan S. Is agmatine a novel neurotransmitter in brain? *Trends Pharmacol Sci* 2000;21:187-93.
- Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, Cook JL. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J Biol Chem* 1999;274:26071-8.
- Motohashi H, Yamamoto M. Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol Med* 2004; 10:549-57.
- Ramprasath T, Selvam GS. Potential impact of genetic variants in Nrf2 regulated antioxidant genes and risk prediction of diabetes and associated cardiac complications. *Curr Med Chem* 2013;20: 4680-93.
- Wild AC, Gipp JJ, Mulcahy T. Overlapping antioxidant response element and PMA response element sequences mediate basal and beta-naphthoflavone-induced expression of the human gamma-glutamylcysteine synthetase catalytic subunit gene. *Biochem J* 1998;332(Pt 2):373-81.
- Tomobe K, Shinozuka T, Kuroiwa M, Nomura Y. Age-related changes of Nrf2 and phosphorylated GSK-3 β in a mouse model of accelerated aging (SAMP8). *Arch Gerontol Geriatr* 2012;54:e1-7.
- Venugopal R, Jaiswal AK. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proc Natl Acad Sci U S A* 1996;93:14960-5.

37. Kanninen K, White AR, Koistinaho J, Malm T. Targeting Glycogen Synthase Kinase-3 β for Therapeutic Benefit against Oxidative Stress in Alzheimer's Disease: Involvement of the Nrf2-ARE Pathway. *Int J Alzheimers Dis* 2011;2011:985085.
38. Li XH, Li CY, Xiang ZG, Hu JJ, Lu JM, Tian RB, et al. Allicin ameliorates cardiac hypertrophy and fibrosis through enhancing of Nrf2 antioxidant signaling pathways. *Cardiovasc Drugs Ther* 2012;26:457-65.
39. Yang Y, Zhang J, Liu H, Zhang L. Change of Nrf2 expression in rat hippocampus in a model of chronic cerebral hypoperfusion. *Int J Neurosci* 2013. [Epub ahead of print]
40. Rodrigues L, Dutra MF, Ilha J, Biasibetti R, Quincozes-Santos A, Leite MC, et al. Treadmill training restores spatial cognitive deficits and neurochemical alterations in the hippocampus of rats submitted to an intracerebroventricular administration of streptozotocin. *J Neural Transm* 2010;117:1295-305.
41. Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods* 1984;11:47-60.
42. Paul CA, Beltz B, Berger-Sweeney J. Perfusion of brain tissues with fixative. *CSH Protoc* 2008;2008.pdb.prot4802.
43. Flynn BL. Pharmacologic management of Alzheimer disease, Part I: Hormonal and emerging investigational drug therapies. *Ann Pharmacother* 1999;33:178-87.
44. Sharma M, Gupta YK. Chronic treatment with trans resveratrol prevents intracerebroventricular streptozotocin induced cognitive impairment and oxidative stress in rats. *Life Sci* 2002;71:2489-98.
45. Selkoe DJ. Alzheimer's disease results from the cerebral accumulation and cytotoxicity of amyloid beta-protein. *J Alzheimers Dis* 2001;3:75-80.
46. Salkovic-Petrisic M, Hoyer S. Central insulin resistance as a trigger for sporadic Alzheimer-like pathology: an experimental approach. *J Neural Transm Suppl* 2007:217-33.
47. Maffei M, Thurm F, Schnack C, Tumani H, Otto M, Elbert T, et al. Increased levels of antigen-bound β -amyloid autoantibodies in serum and cerebrospinal fluid of Alzheimer's disease patients. *PLoS One* 2013;8:e68996.
48. Selkoe DJ. Alzheimer's disease: a central role for amyloid. *J Neuropathol Exp Neurol* 1994;53:438-47.
49. Varadarajan S, Yatin S, Aksenova M, Butterfield DA. Review: Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity. *J Struct Biol* 2000;130:184-208.
50. Varadarajan S, Kanski J, Aksenova M, Lauderback C, Butterfield DA. Different mechanisms of oxidative stress and neurotoxicity for Alzheimer's A beta(1--42) and A beta(25--35). *J Am Chem Soc* 2001;123:5625-31.
51. Kornhuber J, Bormann J, Retz W, Hübers M, Riederer P. Memantine displaces [3H]MK-801 at therapeutic concentrations in post-mortem human frontal cortex. *Eur J Pharmacol* 1989;166:589-90.
52. Piletz JE, Aricioglu F, Cheng JT, Fairbanks CA, Gilad VH, Haenisch B, et al. Agmatine: clinical applications after 100 years in translation. *Drug Discov Today* 2013;18:880-93.
53. Salloway S, Mintzer J, Weiner MF, Cummings JL. Disease-modifying therapies in Alzheimer's disease. *Alzheimers Dement* 2008;4:65-79.
54. Wang WP, Iyo AH, Miguel-Hidalgo J, Regunathan S, Zhu MY. Agmatine protects against cell damage induced by NMDA and glutamate in cultured hippocampal neurons. *Brain Res* 2006;1084:210-6.
55. O'Neill C, Kiely AP, Coakley MF, Manning S, Long-Smith CM. Insulin and IGF-1 signalling: longevity, protein homeostasis and Alzheimer's disease. *Biochem Soc Trans* 2012;40:721-7.
56. Freude S, Hettich MM, Schumann C, Stöhr O, Koch L, Köhler C, et al. Neuronal IGF-1 resistance reduces Abeta accumulation and protects against premature death in a model of Alzheimer's disease. *FASEB J* 2009;23:3315-24.
57. Craft S, Asthana S, Newcomer JW, Wilkinson CW, Matos IT, Baker LD, et al. Enhancement of memory in Alzheimer disease with insulin and somatostatin, but not glucose. *Arch Gen Psychiatry* 1999;56:1135-40.
58. Park CR, Seeley RJ, Craft S, Woods SC. Intracerebroventricular insulin enhances memory in a passive-avoidance task. *Physiol Behav* 2000;68:509-14.
59. van der Heide LP, Ramakers GM, Smidt MP. Insulin signaling in the central nervous system: learning to survive. *Prog Neurobiol* 2006;79:205-21.
60. Wang YT, Salter MW. Regulation of NMDA receptors by tyrosine kinases and phosphatases. *Nature* 1994;369:233-5.
61. van der Heide LP, Kamal A, Artola A, Gispen WH, Ramakers GM. Insulin modulates hippocampal activity-dependent synaptic plasticity in a N-methyl-D-aspartate receptor and phosphatidylinositol-3-kinase-dependent manner. *J Neurochem* 2005;94:1158-66.
62. Zhao WQ, Chen H, Quon MJ, Alkon DL. Insulin and the insulin receptor in experimental models of learning and memory. *Eur J Pharmacol* 2004;490:71-81.
63. Park CR. Cognitive effects of insulin in the central nervous system. *Neurosci Biobehav Rev* 2001;25:311-23.
64. Liu Y, Liu F, Grundke-Iqbal I, Iqbal K, Gong CX. Deficient brain insulin signalling pathway in Alzheimer's disease and diabetes. *J Pathol* 2011;225:54-62.
65. Ahn SK, Hong S, Park YM, Choi JY, Lee WT, Park KA, et al. Protective effects of agmatine on lipopolysaccharide-injured microglia and inducible nitric oxide synthase activity. *Life Sci* 2012;91:1345-50.
66. Bokara KK, Kwon KH, Nho Y, Lee WT, Park KA, Lee JE. Retroviral expression of arginine decarboxylase attenuates oxidative burden in mouse cortical neural stem cells. *Stem Cells Dev* 2011;20:527-37.
67. Seo SK, Yang W, Park YM, Lee WT, Park KA, Lee JE. Overexpression of human arginine decarboxylase rescues human mesenchymal stem cells against H₂O₂ toxicity through cell survival protein activation. *J Korean Med Sci* 2013;28:366-73.
68. Liu P, Bergin DH. Differential effects of i.c.v. microinfusion of agmatine on spatial working and reference memory in the rat. *Neuroscience* 2009;159:951-61.
69. Frölich L, Riederer P. Free radical mechanisms in dementia of Alzheimer type and the potential for antioxidative treatment. *Arzneimittelforschung* 1995;45:443-6.
70. Launer LJ, Kalmijn S. Anti-oxidants and cognitive function: a review of clinical and epidemiologic studies. *J Neural Transm Suppl* 1998;53:1-8.
71. Mikati MA, Abi-Habib RJ, El Sabban ME, Dbaibo GS, Kurdi RM, Kobeissi M, et al. Hippocampal programmed cell death after status epilepticus: evidence for NMDA-receptor and ceramide-mediated mechanisms. *Epilepsia* 2003;44:282-91.
72. Brouillette J. The Effects of Soluble A β Oligomers on Neurodegeneration in Alzheimer's Disease. *Curr Pharm Des* 2013. [Epub ahead of print]
73. Teixidó L, Martín-Satué M, Alberdi E, Solsona C, Matute C. Amyloid β peptide oligomers directly activate NMDA receptors. *Cell Calcium* 2011;49:184-90.

74. Feng Z, Zhang JT. Protective effect of melatonin on beta-amyloid-induced apoptosis in rat astrogloma C6 cells and its mechanism. *Free Radic Biol Med* 2004;37:1790-801.
75. Montiel T, Quiroz-Baez R, Massieu L, Arias C. Role of oxidative stress on beta-amyloid neurotoxicity elicited during impairment of energy metabolism in the hippocampus: protection by antioxidants. *Exp Neurol* 2006;200:496-508.
76. Pavlik VN, Doody RS, Rountree SD, Darby EJ. Vitamin E use is associated with improved survival in an Alzheimer's disease cohort. *Dement Geriatr Cogn Disord* 2009;28:536-40.
77. Sano M, Ernesto C, Thomas RG, Klauber MR, Schafer K, Grundman M, et al. A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. *N Engl J Med* 1997;336:1216-22.
78. Ishrat T, Khan MB, Hoda MN, Yousuf S, Ahmad M, Ansari MA, et al. Coenzyme Q10 modulates cognitive impairment against intracerebroventricular injection of streptozotocin in rats. *Behav Brain Res* 2006;171:9-16.
79. Pathan AR, Viswanad B, Sonkusare SK, Ramarao P. Chronic administration of pioglitazone attenuates intracerebroventricular streptozotocin induced-memory impairment in rats. *Life Sci* 2006;79:2209-16.
80. Sharma M, Gupta YK. Intracerebroventricular injection of streptozotocin in rats produces both oxidative stress in the brain and cognitive impairment. *Life Sci* 2001;68:1021-9.
81. Ferreira E, Eufrásio A, Pereira C, Oliveira CR, Rego AC. Bcl-2 overexpression protects against amyloid-beta and prion toxicity in GT1-7 neural cells. *J Alzheimers Dis* 2007;12:223-8.
82. Rohn TT, Vyas V, Hernandez-Estrada T, Nichol KE, Christie LA, Head E. Lack of pathology in a triple transgenic mouse model of Alzheimer's disease after overexpression of the anti-apoptotic protein Bcl-2. *J Neurosci* 2008;28:3051-9.
83. Kong J, Ren G, Jia N, Wang Y, Zhang H, Zhang W, et al. Effects of nicorandil in neuroprotective activation of PI3K/AKT pathways in a cellular model of Alzheimer's disease. *Eur Neurol* 2013;70:233-41.
84. Kudo W, Lee HP, Smith MA, Zhu X, Matsuyama S, Lee HG. Inhibition of Bax protects neuronal cells from oligomeric A β neurotoxicity. *Cell Death Dis* 2012;3:e309.
85. Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* 2006;443:787-95.
86. Slee EA, Adrain C, Martin SJ. Serial killers: ordering caspase activation events in apoptosis. *Cell Death Differ* 1999;6:1067-74.
87. Montagnani M, Chen H, Barr VA, Quon MJ. Insulin-stimulated activation of eNOS is independent of Ca²⁺ but requires phosphorylation by Akt at Ser(1179). *J Biol Chem* 2001;276:30392-8.
88. Scheid MP, Woodgett JR. PKB/AKT: functional insights from genetic models. *Nat Rev Mol Cell Biol* 2001;2:760-8.
89. Brunet A, Datta SR, Greenberg ME. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr Opin Neurobiol* 2001;11:297-305.