The possible underlying neuroprotective mechanisms of agmatine in rats injected with streptozotocin

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The possible underlying neuroprotective mechanisms of agmatine in rats injected with streptozotocin

Directed by Professor Jong Eun Lee

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ACKNOWLEDGEMENT

학부를 졸업하고 뇌 공부를 하고 싶어 연세를 찾아 온지가 엊그제 같은데 벌써 3 년이나 흘러 졸업논문에 감사의 글을 씁니다. 신촌으로 가는 지하철 안에서 2010 년 새해 한강을 바라보며, 다짐했던 그날이 새록새록 떠오릅니다.

먼저, 제 2 의 어머니라고 불러드리고 싶을 만큼 지난 3 년동안 지식뿐만 아니라 인생의 지혜를 가르쳐주신 이종은 지도교수님, 온화한 웃음과 카리스마로 기둥이 되시는 정말 닮고 싶은 존경하는 박경아 교수님, 바쁘신 와중에도 저의 논문에 조언과 관심을 주신 김경환 교수님, 수많은 질문에 답해주신 인자하신 이원택 교수님께 깊은 감사를 드립니다. 시끄러운 저를 마주칠 때 인자하게 웃어주시던 이혜연 교수님, 김명희 교수님, 복진웅 교수님께도 감사드립니다.

3 년동안 정이 흠뻑 들어버린 우리 조직학 식구들에게도 감사드립니다. 항상 웃으시며, 반겨주시는 신영호 선생님, 정말 많은 도움을 주시고 조언을 아끼지 않으신 양원석 선생님, 유머와 재치로 실험실을 밝게 해주시는 Kiran 선생님, 시크한 매력으로 실험을 주도하시는 송주현 선생님, 사려 깊게 생각해주시는 최혜송 선생님께도 깊은 감사를 드립니다. 이제 가족이라고 할 만큼 가까운 실험실원, 매번 티격태격 하지만 많은 도움을 주신 재영 선생님, 친언니처럼 때로는 좋은 말로 감싸고 일침으로 혼내주시던 고마운 유미 선생님, 타지에 있지만 학부 때부터 길고 긴 인연으로 많은 도움을 주던 용희 언니, 제일 많이 울고 웃으며 지난 3 년을 함께 보냈던 어른스러운 수경이, 많이 챙겨주지 못해 미안함 반 고마움 반인 자용오빠, 이제 새로 들어올 소망이, 함께 수업을 들으며 좋은 말을 아끼지 않던 혜진 언니, 여러모로 많이 도와주신 소영언니와 경주 선생님, 지혜 선생님까지도 모두 지난 연세 생활을 함께 해 주셔서 감사합니다.

석사학위를 마칠 수 있게 물심양면으로 도와주신 저희 가족에게도 감사의 말을 전하고 싶습니다. 대학원 입학을 함께 걱정해주셨던 친지 분들, 앞으로 나아가는데 밑거름을 마련해주신 아버지, 석사 기간 동안 속 많이 썩여 드린 어머니, 착한 동생 보윤이가 있었기에 저의 석사생활을 무사히 마칠 수 있었습니다.

전부 다 열거 할 수는 없지만, 내 절친들, 송이,도연,수영이와 인애,학부 때부터 꽉 채워 8 년 은영,수지,순미,많은 실험을 알려주시던 혜진,석환 선배, 대학원 생활에 많은 조언을 주신 학술연합 사람들, 그 밖에 나와 인연을 맺고 있는 모든 사람들에게 감사를 돌리고 싶습니다.

말도 많고 탈도 많았지만, 정이 흠뻑 들은 연세에서의 3 년을 가슴 깊숙하게 간직하고, 새로운 곳에서 더 발전하는 허보은이 되도록 하겠습니다.

2013 년 새해, 겨울

허 보은

ABSTRACT

The possible underlying neuroprotective mechanisms of agmatine in rats injected with streptozotocin

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Alzheimer's disease (AD) is a representative neurodegenerative disease which is characterized by the progressive cognitive disability and memory loss commonly observed in the elderly people. Of importance, several studies demonstrate that NMDA receptor antagonists and antioxidant agents are able to inhibit these pathologies of AD.

Agmatine is a polycationic and endogenous amine which is synthesized by arginine decarboxylase (ADC). Recent investigations delineate the possible functions of agmatine as a NMDA receptor antagonist or an antioxidant substance, respectively. Therefore, the present study was undertaken to explore the beneficial effects of agmatine in the brain of rats exposed to streptozotocin.

In particular, it was examined whether and how agmatine relieves amyloid beta (Aβ) and oxidative stress-mediated toxicity in animal model. In the first part, rats were stereotaxically injected with streptozotocin via intra-cerebral route (STZ: 3mg/5 $\mu\ell$, 1st and 3rd day after surgery) and then, intra-peritoneally received agmatine (100mg/kg) for 14 days. Agmatine proved to recuperate the cognitive and memory deficit in rats exposed to STZ as evidenced by behavioral tests such as morris water maze and radial arm maze. As well, it suppressed the induction of amyloid precursor protein (APP) and amyloid beta (Aβ) peptide and reduced the immunoreactivity against caspase-3.

To further investigate the underlying protective effect of agmatine, primary hippocampal neurons were treated with Aβ (20μM), MK801 (NMDA receptor antagonist), or combination of these agents, respectively. Agamtine inhibited the reduction of formazan (cell viability indicator) formation and the increase of Hoechst/PI positivity (cell death indicator) in primary hippocampal neurons treated with STZ. This effect of agmatine was comparable to it of MK801. Therefore, these findings demonstrate that agmatine might exert its protective effect through NMDA receptor antagonism.

In the second part, rats were stereotaxically injected with streptozotocin via intracerebroventricular route (1.5mg/kg, 5μl, 1st and 3rd day after surgery) and then, intra-peritoneally received agmatine (100mg/kg) for 14 days. Like what was observed in rats intra-cerebrally injected with STZ, agmatine improved the cognitive and memory impairment and inhibited caspase 3 as demonstrated by behavioral tests or immunohistochemistry, respectively.

Then, to more unravel the protective effect of agmatine, Bax, Bcl2, PI3K, AKT, Nrf_2, and r-GCS were checked with western blotting. As expected, agmatine reduced Bax and raised the rest proteins mentioned above. It is interesting that agmatine affects protein levels of Nrf2 and r-GCS. These proteins are known to play the pivotal roles in neutralizing oxidative damage. Correspondingly, agmatine declined 8-oxo immunofluorescence in hippocampal neurons of rats injected with STZ, potentiating that it detoxifies oxidative condition within the cells.

Therefore, the current data suggest that agmatine stimulates both cell survival and antioxidant related protein involving pathway to support cognitive function. Taken together, our data propose the idea that agmatine may ameliorate cognitive dysfunction in neurodegenerative diseases such as Alzheimer`s disease (AD), Parkinson`s disease (PD), Huntington`s disease (HD), and Amyotrophic lateral sclerosis (ALS) through the pro-survival and anti-oxidant action.

Key words : agmatine, Alzheimer's disease, cognitive ability, NMDA receptor antagonist, apoptosis pathway, oxidative stress, cell survival

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I. INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the degeneration of neurons as well as by the progressive decline of cognitive function. Histologically, AD has hallmarks including both aggregation of senile plaques derived from amyloid beta peptides and neurofibrillary tangles, especially in hippocampus or cerebral cortex relevant to learning and memory.[1,2] When amyloid beta (Aβ) exists in the high concentration, they form insoluble and fibrillar Aβ plaque or activate ion channels in cell membrane to induce membrane depolarization and destabilization of intracellular calcium homeostasis.[3-5] In particular, Aβ oligomers cause intracellular Ca^{2+} overload followed by neuronal death that can be prevented by NMDA receptor antagonists.[6,7]

Agmatine is an endogenous peptide, mainly synthesized by arginine decarboxylase (ADC) in glia and neuronal cells. Recently, studies have shown that agmatine exhibits protective effects in neurotrauma, neonatal ischemia, and AD like pathology.[8,9] As well, it has been suggested to work as a neurotransmitter or neuromodulator in brain, especially, hippocampus.[10-12] Of interest, several investigations have reported the potential of agmatine to support cognition and memory ability in various animal models. In particular, agmatine is known to antagonize N-methyl-D-aspartate (NMDA) receptor, nicotinic receptor, and voltage gated Ca^{2+} channel to demonstrate its physiological functions. As well, it plays a crucial role in regulating the production of NO via influencing the catalytic activity of nitric oxide synthetase (NOS).[13,14] Because of these pharmacological profiles, several researchers investigated the potential of agmatine to support cognitive and memory function in numerous animal models.[15-20]

Streptozotocin (STZ), a glucosamine-nitrosourea compound, is known to destruct pancreatic β cells, and resulting in diabetes. In another manner, the experimental administration of streptozotocin (STZ) injection into central nervous system of rodents has been reported to produce Alzheimer like pathologies including amyloid beta accumulation, oxidative stress, hippocampal neuronal death and so on. Thus, is steadily employed to reproduce several pathologies of Alzheimer's disease (AD).[21]
In fact, STZ has shown to induce learning and memory deficits, oxidative damage[22], aberrant glucose metabolism, and neuronal damage.[23,24]

Apoptosis is mediated by the sequential activation of Caspases.[25] Among caspases, caspase-3 is a key mediator protease[25] and its activation commonly leads to the occurrence of the apoptoctic death and thus, can be an irreversible step in apoptotic cascade.[25] In significance, caspase 3 activation is known to the increase of Aβ level through BACE1 activation.[26]

Oxidative stress, defined an imbalance between reactive oxygen species (ROS) and antioxidant system, plays a pivotal role in AD occurrenc.[27,28]. Brain tissues have limitation to accept sources of ROS such as sugar, lipids, proteins, and DNA [29,30] , which are leading neuronal dysfunction.[30] In this reason, antioxidants are therapeutically spotlighted to treat a variety of neurodegernative diseases.[31,32]

In the present study, we examined two kinds of the neuroprotective effect of agmatine. First, we investigated the effect of agmatine against amyloid beta implicated toxicity both in the *in vivo* and *in vitro* model exposed to streptozotocin intracerebral (i.c) model. Second, we studied the effect of agmaitne as an antioxidant against AD like pathology induced by streptozotocin in intracerebroventricular (i.c.v) model. In this aim, we did AD like model construction, behavior test, immunohistochemical and immunocytochemical experiments.

Ⅱ. MATERIALS AND METHODS

A. Animals

Male Sprague-Dawley rats (n=50, weighing 250-330g) were used in this study. They were maintained under controlled hygiene system with 12 h light/dark reverse cycle at a constant temperature with free access to food and water. Animal behavior tests were carried out during daylight. All animal experiments were performed in accordance with the Korean Food and Drug Administration (KFDA) guidelines. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Yonsei Laboratory Animal Research Center (YLARC) (Permit #: 10-115). All mice were maintained in the specific pathogen-free facility of the YLARC.

B. Agmatine administration

Agmatine was purchased from Sigma (St. Louis, MO, USA), and dissolved in normal saline (PH 7.4) and administered to rat through intraperitoneal route. Rats were injected with agmatine daily during two weeks from surgery day. Control was treated with normal saline in the same volumes.

C. Intracerebral (I.C) and Intracerebroventricular (I.C.V) administration of streptotozotocin

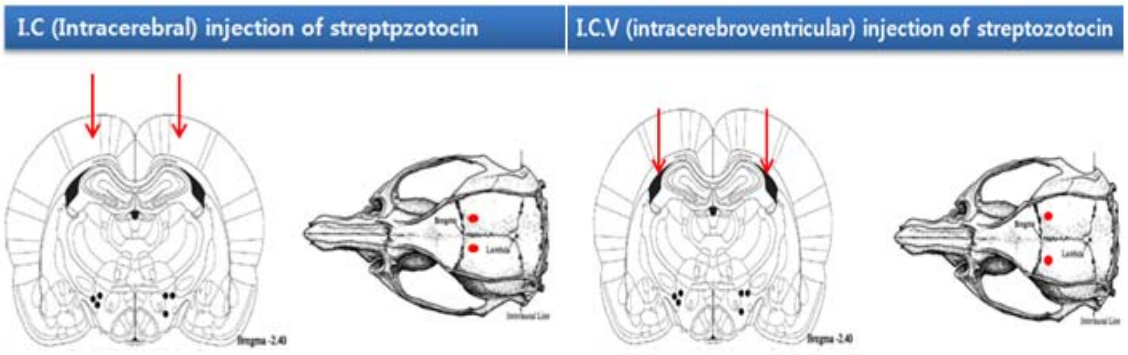

Figure 1. The administration of streptozotocin approach. Intracerebral (i.c.) model : streptozotocin was injected following coordinates modified Paxinos and Watson (1998)[33] : 1.5mm lateral, 1.5mm posterior from bregma and 2.5 beneath from ventricle.

Intracerebroventricular (i.c.v) model : streptozotocin was injected following coordinates Paxinos and Watson (1998)[33] method : 1.5mm lateral, 0.8 posterior from bregma and 3.6 beneath from ventricle.

D. Experimental groups

Rats were divided into nine groups consisting of five rats depending on surgical method and period. In surgical method division, groups were divided into three.

Ⅰ. Sham group : the animals received STZ vehicle (saline) with I.C injection

Ⅱ. STZ group : the animals received STZ (1.5mg/kg, 5μl, 1st and 3rd injection from surgery) with I.C injection.

Ⅲ. STZ-Agmatine group : the animal received STZ(1.5mg/kg, 5μl, 1st and 3rd injection from surgery) with I.C injection and agmatine (100mg/kg, I.P.,for two weeks on a surgery, daily).

In period division, groups were divided into three.

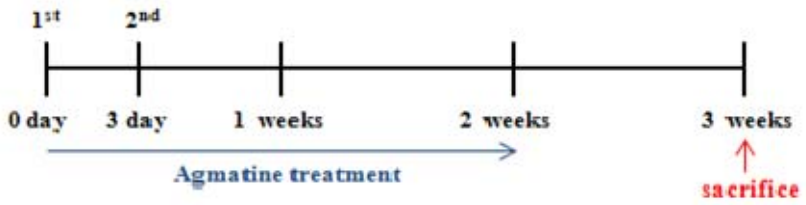

Figure 2. *in vivo* study experimental procedure

E. Behavioral assessment

1) Morris water maze

Cognitive evaluation of animals was tested in Morris water maze.[34] After three weeks later, rats were subjected to training in Morris water maze. The apparatus was circular form of 110cm, 50cm height, filled with depth of 30cm with colored water to hide the platform location and the surround of wall has some cues. After pre-training, rats were placed in water to find out probe within given maximum time (60 seconds). They were performed to four trials per one day at each changed starting points for consecutive five days. All rats were placed on the probe for 30 seconds and had interval of approximately 30 seconds before next trial. If rats failed to reach on the probe, they were gently guided by researchers to location of probe and stayed 30 seconds on the probe. During a session, latency time to reach the platform was recorded by computer tracking system and calculated the mean of each trial latency time.

2) Radial arm maze

The radial arm maze was constructed using black Plexiglas with eight 70 cm long and 9 cm wide arms attached to 6 cm high clear Plexiglas walls, which projected from a central circular platform 34cm in diameter. The end of each arms had a clear Plexiglas food cup and the maze was pivoted so

that it could be rotated around the central axis. The maze was elevated approximately 100 cm above the floor. Chocolate was used as compensation. Animals were habituated in maze by exploring arms before starting tests and restricted diet. Briefly, each animal was placed individually in the center of the maze and subjected to working memory. The training trial conducted consistently until all eight baits in the food cups had been consumed of until 15min had elapsed. The evaluation of cognitive ability, the number of error that they visited again arms baited already. The test was performed during 5 days consistently.

F. Histological examination

Rats were anesthetized and perfused as previously mentioned. Rat brains were cut into coronal slices of 2 mm in thickness using a rat brain matrix (Ted Pella, Redding, CA, USA). With 4% paraformaldehyde (pH 7.4), the brain slices were fixed for 3 days and subsequently embedded paraffin block. Embedded paraffin brain sections were firstly stained with Hematoxylin and Eosin immunostaining.

G. Hematoxylin and Eosin (H&E) staining

The paraffin block embedded hippocampus level was de-paraffined and re-hydrated with different concentration of alcohol and xylen. After Hematoxylin-eosin(H&E) staining, stained hippocampus was examined under microscopy.

H. Immunohistochemistry

Three weeks later, treated brain slices were immunostained with antibodies against amyloid precursor protein, caspase 3 and amyloid beta in i.c. model and after three weeks, treated brain slices were immunostained with cleaved caspase 3 and 8-OHdG in i.c.v. model.

They were washed three times with 1x phosphate buffered saline and fixed with 4% paraformalehyde (PFA), and then washed three times again with PBS.

Brain tissues were permeabilized with 0.025% triton X100 and were blocked with blocking solution for 1 hour at room temperature. APP (1:500, Abcam, Cambridge, MA, USA), amyloid beta (1:250, Abcam, Cambridge, MA, USA), Caspase 3 (1:200, Milipore, Billerica, MA, USA) Cleaved caspase 3 (1:600, cell signaling) and 8-OHdG (1:200, Chemicon) were incubated overninght. After being rince with PBS (0.05% with Tween 20) three times, with appropriate secondary antibody (1:200) prepared with conjugated to fluorescenece for 2 hours at room temperature and counter stained with 4',6-diamidino-2-phenylindole (DAPI) when tissue slides mounted. Tissues were visualized under a confocal microscopy (Zeiss LSM 700)

I. Western analysis

After animals were sacrificed, brains were perfused with saline through heart arota to rinse out blood. The brain portion of hippocampus and cortex was dissected for extraction of proteins, treated with lysis buffer consisting of PBS, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, proteinase inhibitor-PMSF, Aprotinin and Sodium orthovandate and isolated protein from homogenizer (Dramel, Racine WI, USA).

Using the BCA method (PIERCE, Rockford, USA), the determination of protein concentration in the supernatant was conducted.

Western blot analysis was performed with equal amounts of protein, 20g per condition ,separated on appropriate sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PACE) gels and electrotranceferred onto Immobilon-NC membrane (Milipore, Billerica, MA, USA). BSA 5% with TBST was used to blocking. Transferred membrane was probed with primary antibody that recognize Bcl2 (Santa Cruz, Santa Cruz, CA), Bax (Santa Cruz, Santa Cruz, CA), Caspase 3 (1:1000, cell signaling), cleaved caspase 3 (1:1000, cell signaling), PI3K (1:2000, Milipore, Billerica, MA, USA), AKT (1:1000, cell signaling,) , phospho-AKT (1:1000, cell signaling), Nrf_2 (1:200, Santa Cruz, Santa Cruz, CA), γ-GCS (1:500, Thermo scientific, Waltham, MA, USA) and actin (1:1000 Santa Cruz, Santa Cruz, CA) at 4℃ overnight and washed three times for 5 minutes with TBST.

The detection of primary antibody, horseradish peroxidase-conjugated secondary antibody (New England Bio labs, Beverly, NA; 1:3000 diluted in BSA 3% with TBST) was used at room temperature for 1hour at room temperature.

After washing TBS (0.05% with Tween 20) three times, immunoreactive signals were detected by chemiluminescence with ECL detection system (Amersham Life Science, Buckinghamshire, UK) by using LAS 4000 program. Signals of immunoblotting were quantified using a computer program.

J. Statistical analysis

All result data were analyzed with mean ±SEM and Immunohistostatining quantifications were analysed by Student's T-test. Differences with $P < 0.05$ were considered statistically significant.

K. Primary hippocampal neuron culture

Primary cultures of hippocampal neurons were prepared from embryonic (E-14) mice (Koatech, Gyeong Gi-do, South Korea) of ICR strain. The pregnant mice were anesthetized with diethyl ether and were sacrificed by cervical dislocation. The stomach was cut opened and the developing pups were dissected out and the heads were chopped. The brain's were removed carefully from the skull.

The hippocampal tissues were isolated from the brain and placed in HBSS (Hank's balanced salt solution.) on ice. The isolated tissues were centrifuged with 800 rpm for 5min at 4℃. After centrifuging, hippocampi were sorted and treated with trypsin-EDTA (0.09%) for 15min at 37℃. Incubated tissues were centrifuged with 2000 rpm for 5 min at 4℃. The pellet containing the brain tissue was dissociated into monocells in Neuro Basal® Medium with B-27® Supplement and 0.5 mM L-glutamine (Gibco). Hippocampal neuronal cells were seeded at a density of $1x10^5$ cells/ml in poly-D-lysine plus laminin coated 6-well, 12-well, or 24-well plates, respectively.

L. Drug treatment

Aβ peptide 25–35 (sAβ) (Sigma Aldrich, USA) the physical and biological properties and toxicity of the full length of Aβ, is widely used to delineate degenerative changes in neuronal cells. sAβ was suspended in sterile double distilled water (PBS) at a concentration of 1.0 mM, and pre-incubated for 7days at 37℃ prior to addition to the cell cultures. sAβ was added to clean and primary cultured cells at a final concentration of 20 mM. D.I.V.8 primary cultured neuronal cells were changed in fresh medium with Aβ25-35 (20μM) only, Aβ25-35 (20μM) + agmatine (50μM), Aβ25-35 (20μM) + agmatine (100μM) and Aβ25-35 (20μM) + MK801 (10μM). Cultures were continued for additional 48 h.

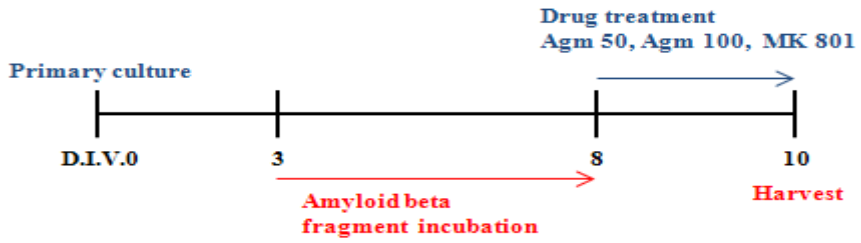

Figure 3. *in vitro* study experimental procedure.

M. Hoechst-PI staining

Cells were stained with propidium idide and Hoechst 33258 (Sigma, ST. Louis, Missouri, USA) to evaluate cell death. This staining expressed the evaluation of membrane integrity and the basis of nuclear morphology on apoptotic cells verses non apoptotic cells. Hoechst dye was added to the culture medium and cultured cells were kept at 37℃ for 30 minutes. Propidium iodide was added just

before observation. Stained cells were observed by using epifluorescence with a UV filter block microscope (Olympus).

N. Immunocytochemistry

After 48hrs from treatment, treated primary hippocampal cells were immunostained with Amyloid Precursor protein , Caspase 3, Amyloid beta.

They were washed three times with 1x phosphate buffered saline and fixed with 4% paraformalehyde (PFA), and then washed three times again with PBS.

Primary hippocampal neuron where permeabilized with 0.025% triton x100 and were blocked with blocking solution for 1 hour at room temperature. Primary antibodies anti-APP (1:750, Abcam, Cambridge, MA, USA), anti-Amyloid beta (1:500, Abcam, Cambridge, MA, USA) and Caspase 3 (1:500, Milipore, Billerica, MA, USA) overninght. After being rince with TBS (0.05% with Tween 20) three times, with appropriate secondary antibody prepared with conjugated to fluorescenece (1:500) for 2 hours at room temperature and counter stained with 4',6-diamidino-2-phenylindole (DAPI) 10µl for 10 min.

Cells were washed again three times for 3 minutes each with PBS visualized under a confocal microscopy (Zeiss LSM 700)

Ⅲ. RESULT

1. The neuroprotective effects of agmatine in rats exposed to streptozotocin via intracerebral route

When 5 days remained for sacrifice, each rat was applied to morris water maze to evaluate learning and memory. Figure 4 (A) presents the average of latency time during 5 consecutive days in each group.

Figure 4 (B) expresses the latency time for 5 days in each group in detail. As shown in data, the latency time of agmatine treated group is lower than EC group. EC group demonstrated the greatest latency time and sham group exhibited the shortest latency time among three groups. Overall, these data suggest agmatine enhances cognitive function in rats treated with streptozotocin.

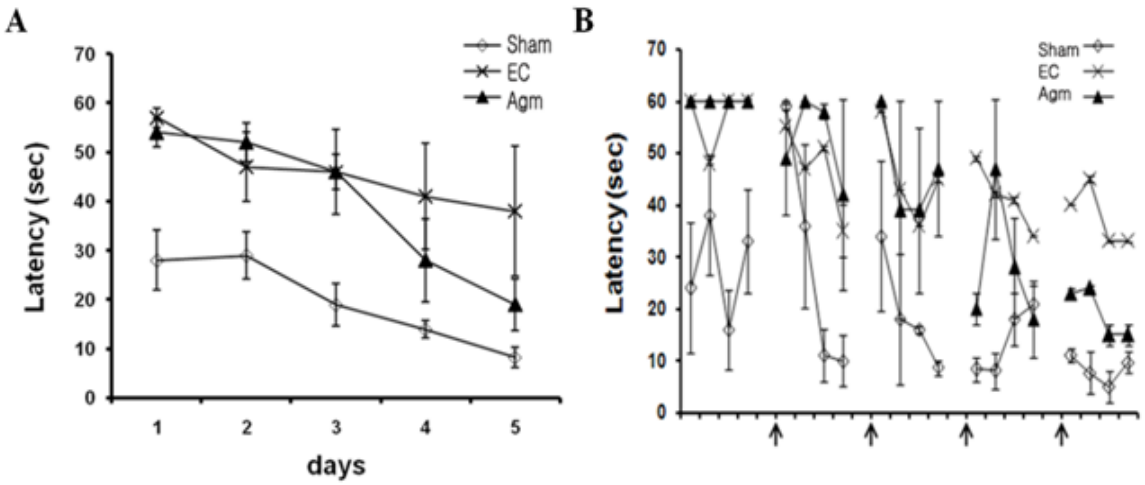

Figure 4. The effect of agmatine treatment on the progressive changes of the behavioral parameters in Morris water maze.

To evaluate cognitive function, we conducted morris water maze (MWM). (A) The latency time of Morris water maze (MWM) among three groups. The average of daily latency time (5 trials) showed on each group three weeks after surgery (STZ i.c. injection). The latency time of agmatine treated group decreased compared to EC group. The longest latency time is EC group. This result shows the effect of agmatine affects learning and memory. (B) Detailed latency time of Morris water maze. Arrow indicates initiation point of each day. EC group has longer latency time than agmatine treated group. Sham : Sham control, EC : experimental control (STZ i.c. injection), Agm : agmatine treated group (Agmatine i.p. injection, daily for 2 weeks following surgery).

Figure 5 (A) and (B) show the expression of APP in cortex and hippocampus. APP is the precursor of amyloid beta and thus it is likely to be amyloid beta. In cortex and hippocampus, agmatine treated group showed less APP immunopositivity of neuronal cells, compared with EC group. Importantly, APP expression in EC group was highest among three groups. Sham showed the lowest expression of APP in both cortex and hippocampus.

These data suggest agmatine may suppress the formation of APP in cortex and hippocampus exposed to stretopzotocin.

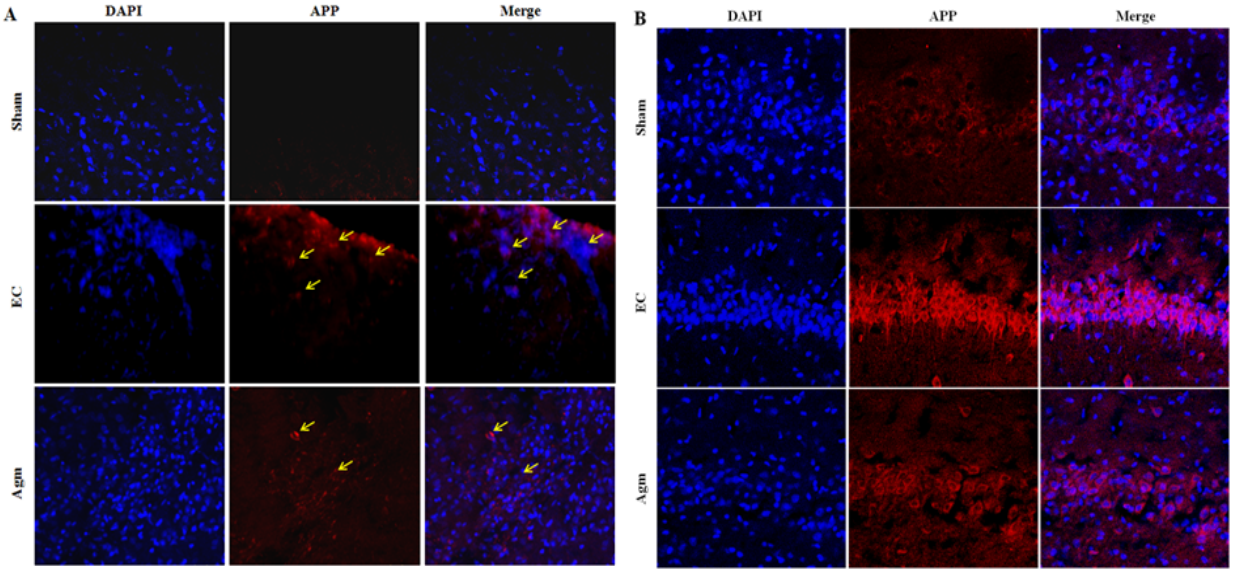

Figure 5. The effect of agmatine treatment on amyloid precursor protein expression in intracerebrally streptozotocin injected rats. (A) The immunohistochemistry of APP in cortex.

(B) The immunohistochemistry of APP in hippocampus. As shown in data, sham showed few expression of APP in cortex and hippocampus. However, EC has the strongest expression of APP among three groups. Compared to EC, agmatine treated group has less APP expression. Therefore, these results inhibit the induction of APP by STZ. Sham : Sham control, EC : experimental control (STZ i.c. injection), Agm : agmatine treated group (Agmatine i.p. injection, daily for 2 weeks from surgery)

On 21st day following streptozotocin injection, amyloid beta staining was conducted to confirm the formation of amyloid beta and its expression in each group in cortex and hippocampus. Figure 6 (A) shows the amyloid beta expression of whole brain on 21st day of surgery. As shown in Figure 6 (A), intracerebral administration of streptozotocin produced amyloid beta obviously. In agamtine treated group, the amyloid beta was less positive in neurons of cortex, compared with EC group. In contrast,

EC revealed the higher expression of amyloid beta, compared with both agmatine treated and sham group.

Similarly, agmatine treated group showed the lower expression of amyloid beta, compared with EC group in hippocampus. These data suggest that agmatine inhibits amyloid beta formation in the brain exposed to streptozotocin.

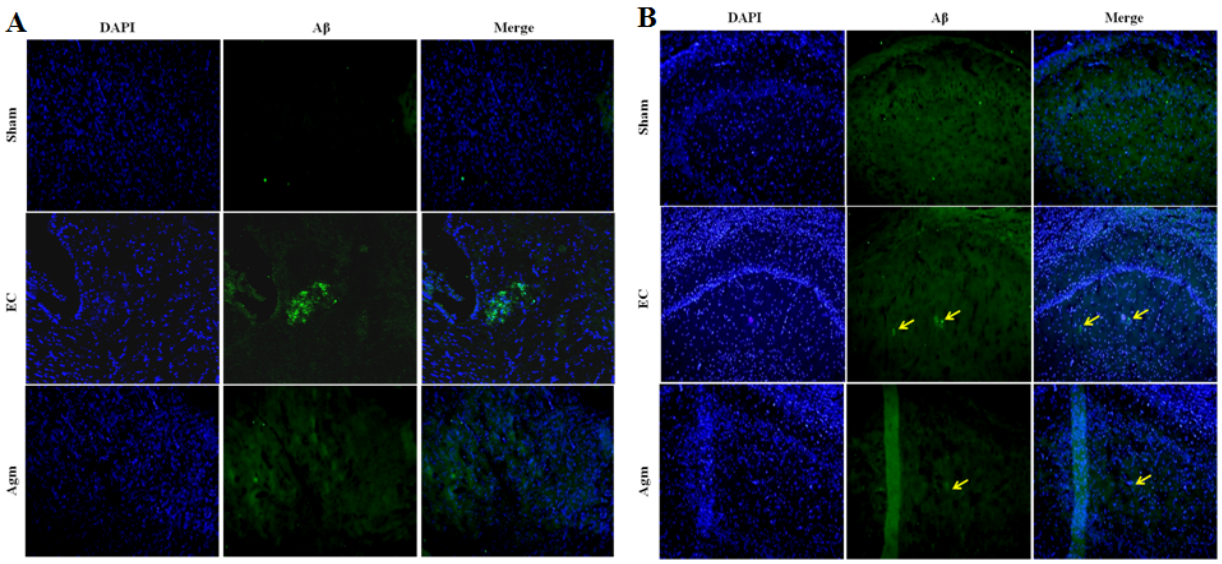

Figure 6. The effect of agmatine treatment on amyloid beta aggregation or formation in intracerebrally streptozotocin injected rats. (A) The immunohistochemistry of Aβ in cortex. (B) The immunohistochemistry of Aβ in hippocampus. In Aβ expression, sham showed few expression of Aβ in cortex and hippocampus. But, EC has the highest expression of Aβ among three groups. Compared to EC, agmatine treated groups have less Aβ expression. These results suggest agmatine attenuates Aβ formation. Sham : Sham control, EC : experimental control (STZ i.c. injection), Agm : agmatine treated group (Agmatine i.p. injection, daily for 2 weeks from surgery)

Caspase 3 immunopositivity was examined to identify the occurrence of neuronal apoptosis in cortex and hippocampus. Figure 7 (A) and (B) showed the robust caspase 3 immunopositivity was observed in EC group. However, agmatine treated group revealed lower expression of caspase 3, compared with EC group in both cortex and hippocampus. Thus these data imply that agmatine protects neuron aginst apoptotic death by streptozotocin.

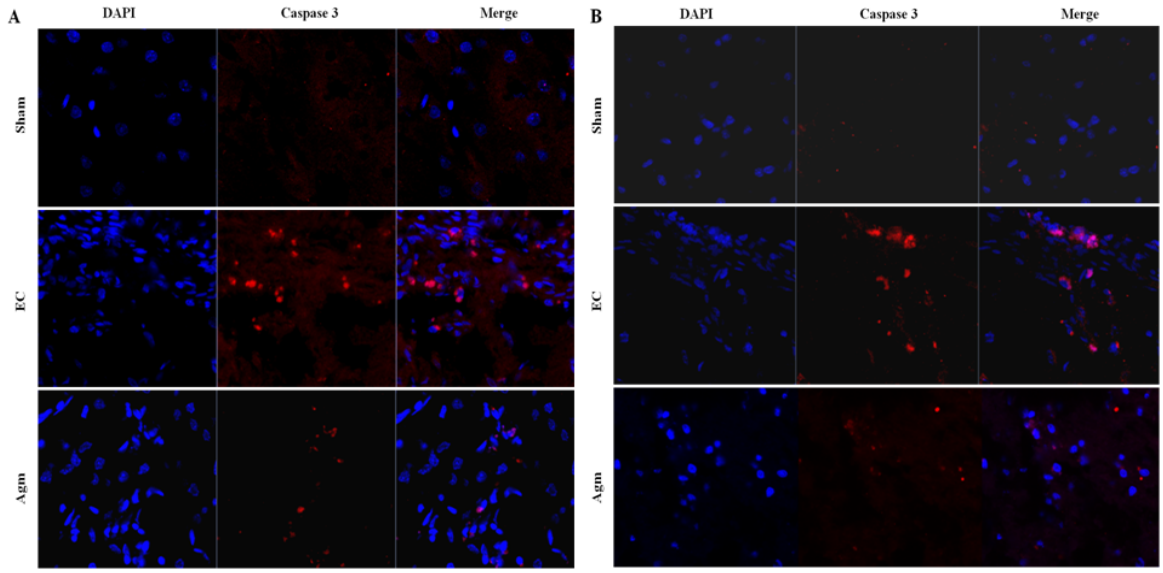

Figure 7. Agmatine treatment attenuates Caspase 3 activation in intracerebrally streptozotocin injected rats. (A) The immunohistochemistry of caspase 3 in cortex. (B) The immunohistochemistry caspase 3 in hippocampus. In these data, sham has no change of caspase 3 in cortex and hippocampus. However, EC has the predominant expression of caspase 3 in both regions, especially, injured parts. Compared with EC, agmatine treated group has lower caspase 3 expression. Thus, the present data suggest that inhibits the activation of apoptotic executioner by STZ. Sham : Sham control, EC : experimental control (STZ i.c. injection), Agm : agmatine treated group (Agmatine i.p. injection, daily for 2 weeks from surgery)

The culture of primary hippocampal neuronal was conducted to study amyloid beta toxicity. On DIV 10 day, neuronal cells were treated with amyloid beta 25-35 fragment to induce toxicity. Experimental groups were divided into five (NC, EC, Agm 50, Agm 100, MK 801), where MK801 is the positive control in this study. This agent has already been reported to be antiapoptotic and NMDA receptor antagonistic. Figure 8 demonstrates both overall morphology and apoptotic caveat. In EC group, neuronal cell revealed markedly shrinkage but, agmatine and MK 801 treated groups showed less shrinkage of neuronal cell, compared with EC group. Two concentrations of agmatine (50μM and 100μM) showed no significant protective effect in treated groups.

Hoechst PI and caspase 3 staining were used to confirm cell death in each group. As shown in data, EC group revealed the obvious cell death. In contrast to, agmatine treated group and MK 801 group showed less of Hoechst PI and caspase 3.

Therefore, cell culture data suggest that agmatine exerts its protective effect against amyloid beta toxicity through a NMDA receptor antagonist.

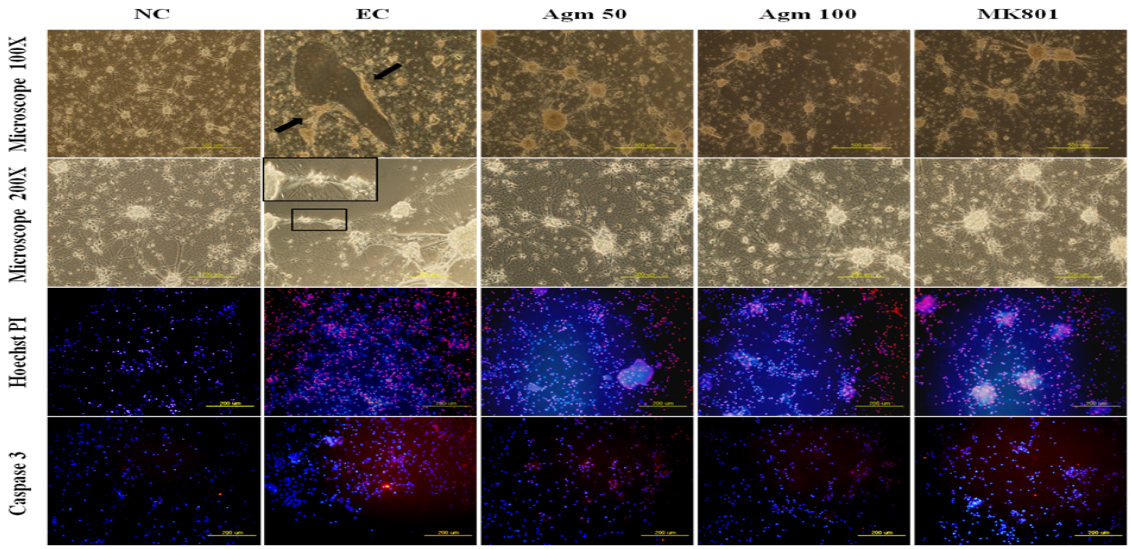

Figure 8. Agmatine treatment inhibits cell death induced by amyloid beta fragment *in vitro*.

In microscopic study, the morphology of primary hippocampal neuron expressed shrinkage in each group by Aβ. In Hoechst/PI staining, agmatine treated group showed less stained cells, compared with EC. Also, caspase 3 immunopositive cells were reduced in agmatine treated group. NC : Normal control, EC : experimental control (Aβ25-35), Agm 50 : Aβ25-35 with agmatine 50μM, Agm 100 : Aβ25-35 with agmatine 100μM, MK801 : positive control (Aβ25-35 with MK801 10μM)

2. The neuroprotective effects of agmatine in rats exposed to streptozotocin via intracerebroventricular route

Before the administration STZ of intracerebroventricular injection, rat brain was confirmed firstly by evans blue at a same does as following mentioned coordinates.

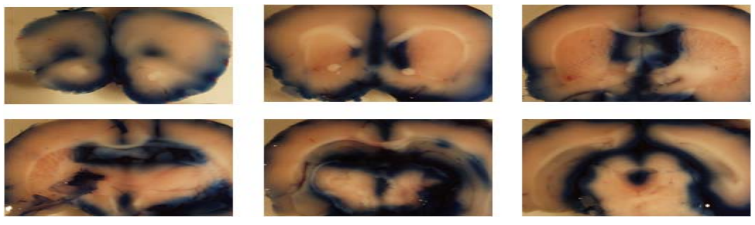

Figure 9. The intracerebroventricular (I.C.V) administration of evans blue.

Figure shows the evans blue expression after injecting in manner of intracerebroventricular (i.c.v) method. The figures were arranged sequentially.

Cognitive impairment is the representative characteristic of AD. Cognition and memory ability were tested with Morris water maze and Radial maze, which are commonly emploted to evaluate reference and working memory.

As shown in figure 10, agmatine treatment group conducted better than EC in both behavioral assessments, these data imply that agmatine has potential to improve learning and memory ability.

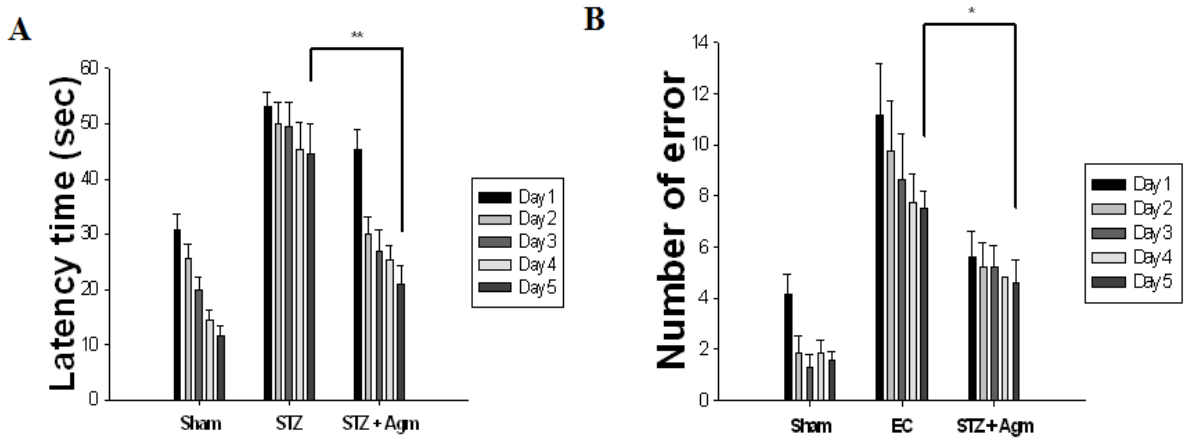

Figure 10. Agmatine treatment improves learning and memory. (A) The latency time of Morris water maze (MWM) among three groups. (B) The numbers of errors of radial arm maze among three groups. Both graphs express the average of daily latency time (5 trials) in each group 3 weeks after surgery (STZ i.c.v injection).

In morris water maze, the latency time of Agm treated group decreased compared to EC. The longest latency time is EC group. In radial maze, the numbers of errors decreased in Agm treated group, compared to EC group. These results show agmatine supports learning and memory ability. Each value represents the mean ±S.E. *P<0.05, **P<0.01 compared with 5 days.

On the 21st day after surgery, rats were sacrificed to prepare brain slices. Brains were embedded with paraffin were cut at the thickness of 5μm and stained with Hematoxylin & Eosin to investigate neuronal morphology. In hippocampus, layers were divided into CA1, CA2, and CA3 regions and showed the large number of degenerated neuronal cells in EC group compared with agmatine treated group.

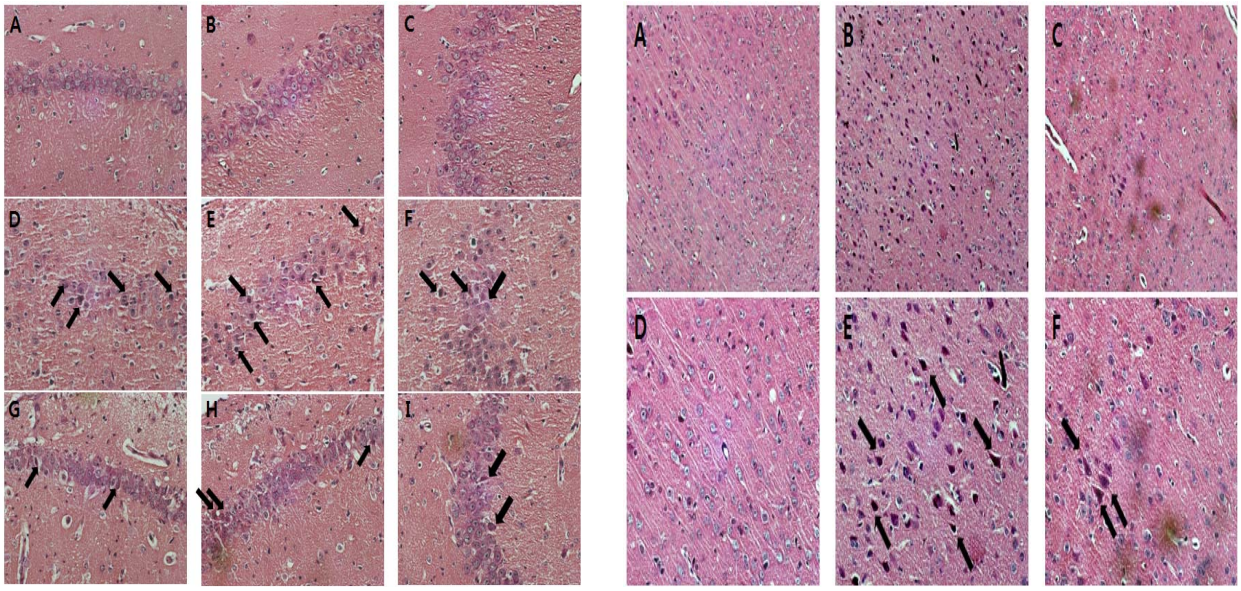

Figure 11. Agmatine reduces histopathological changes in hippocampus CA1. All slides were stained by hematoxyin and eosin. 3 weeks after surgery, agmatine treated groups decreased cell death by STZ. Left (Hippocampus): Sham (A,B,C), EC (D,E,F), Agm (G,H,I). and CA1 (A,D,G), CA2 (B,E,H), CA3 (C,F,I). 40X, Right (Cortex): Sham (A), EC (B), Agm (C) 20X and sham (D), EC (E), Agm (F) 40X in cortex. Black arrows express cell death.

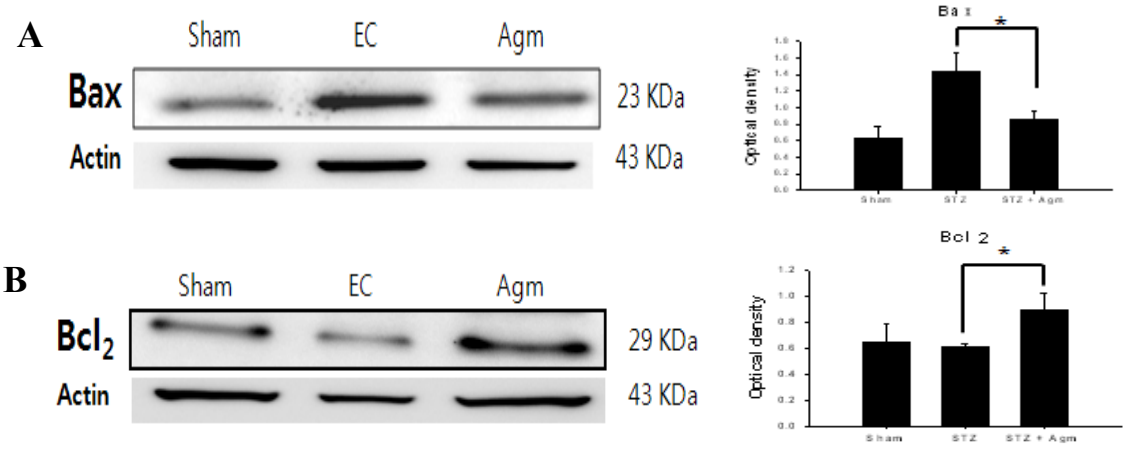

Figure 12. Agmatine decline pro-apoptotic Bax protein and increase anti-apoptotic Bcl2 protein in hippocampus exposed to streptozotocin (A) Bax protein level, (B) Bcl2 protein level. The quantitative graphs show relative expression of these proteins. Each value represents the mean ±S.E. *P<0.05, **P<0.01.

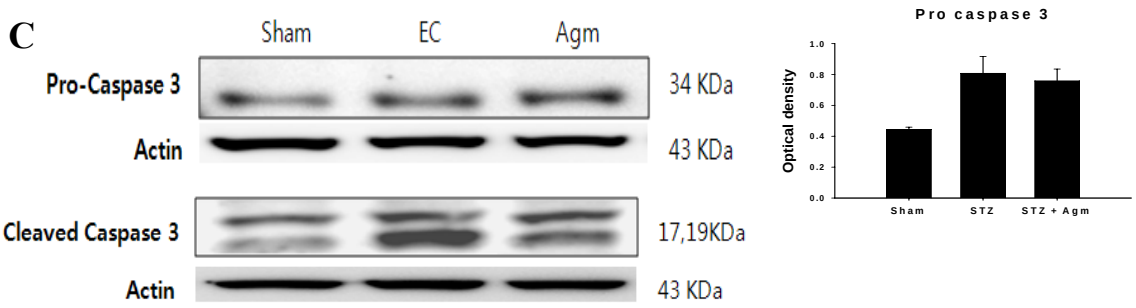

Figure 13. Agmatine suppresses the activation of caspase 3 in hippocampus exposed to streptozotocin. (A) pro-caspase protein level, (B) cleaved caspase 3 protein level. The quantitative graphs show relative expression of these proteins. Each value represents the mean ±S.E. *P<0.05, **P<0.01

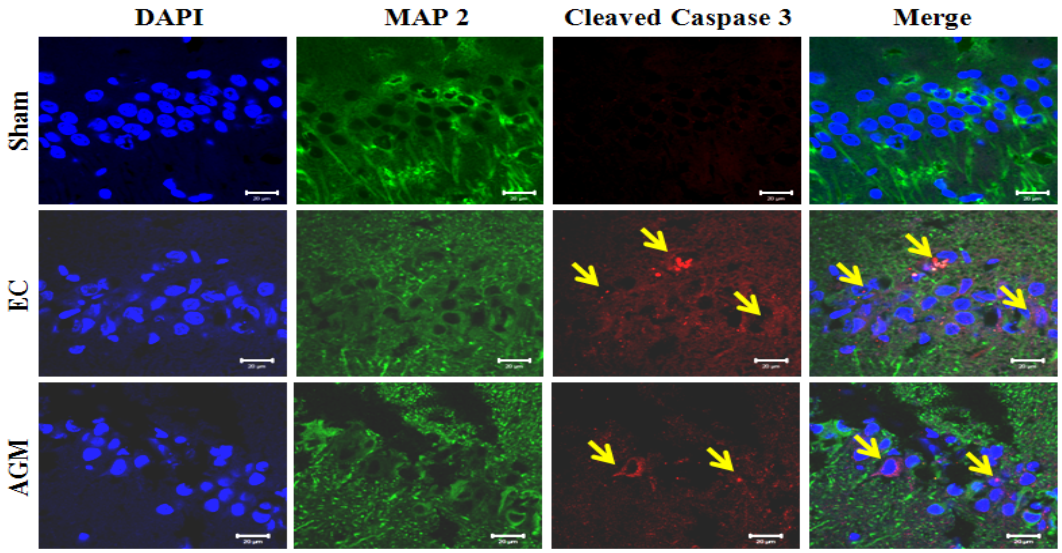

Figure 14. Agmatine reduces cleaved caspase 3 expression in hippocampal CA1 layer. Cleaved caspase 3 positivity represents neuronal apoptosis. This positivity was the highest in EC, compared with other groups. Agmatine decreased this positivity, suggesting that apoptotic event is suppressed by agmatine.

To evaluate anti-apoptotic effect of agmatine in hippocampus exposed to streptozotocin, bax and bcl2 were first investigated. Western blotting data show that agmatine increases bcl2 and decreases bax in EC group, compared with sham group. Second, caspase 3 was examined. Agmatine reduced protein levels of pro- and active- form of caspase 3 in hippocampus exposed to STZ. Therefore, this datum

indicates that agmatine prevents neuronal apoptosis through the inhibition of apoptotic executioner protein.

To investigate the effects of agmatine on cell survival signaling pathway in hippocampus, PI3K, AKT, and P-AKT was examined with western blotting. PI3K protein level was elevated in agmatine treated group, compared with EC group. In contrast, the pro survival protein, AKT expression is no significant agmong three groups, whereas P-AKT increased in agmatine treated group, compared to EC group. These data suggest agmatine activates cell survival related proteins.

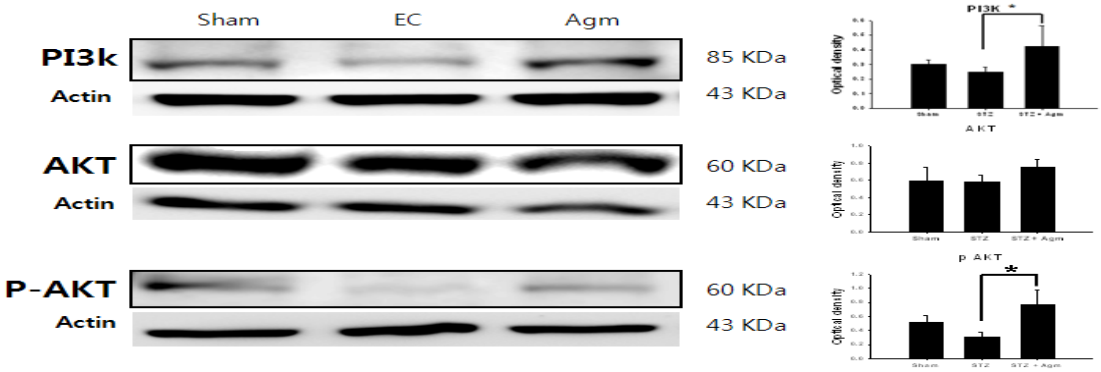

Figure 15. Agmatine increases PI3K, AKT, and P-AKT expression. The representative blots show PI3K, AKT and P-AKT protein level in hippocampus. The quantitation of each band is demonstrated as the bar graph. Each value represents the mean ±S.E. *P<0.05, **P<0.01

To explore the role of agmatine as an antioxidant, Nrf2 and γ-GCS were examined with western blotting. As shown in data, the antioxidant transcription factor, Nrf2 was raised in agmatine group, compared with EC group. Nrf2 is known to regulate the expression of γ-GCS.

Correspondingly, γ-GCS protein was prominently elevated in agmatine treated group. Thus, these data suggest that agmatine stimulates Nrf2 to induce γ-GCS.

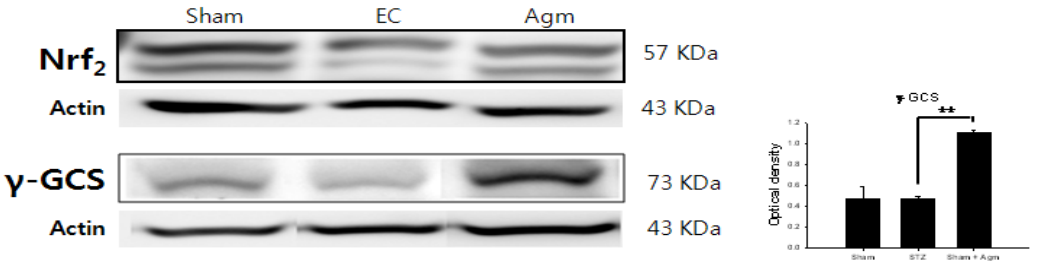

Figure 16. Agmatine increses protein levels of Nrf2 and γ-GCS. The western blot and the quantitative graph demonstrate the significant increase in Nrf2 and γ-GCS proteins in agmatine

treated group compared with the other groups. Each value represents the mean ±S.E. *P<0.05, **P<0.01

To check the role of agmatine as an antioxidant, 8-OHdG profile was examined in hippocampus. Agmatine clearly reduced 8-OHdG positivity in hippocampus exposed to STZ.

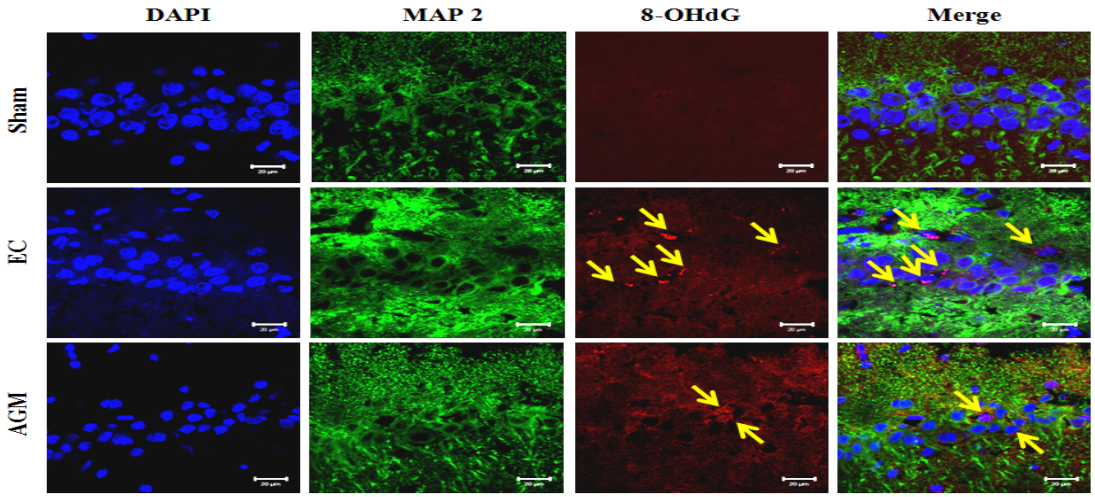

Figure 17. Agmatine significantly reduces 8-OHdG positivity. Immunohistochemistry shows that agmatine treated considerably has low reactivity of 8-OHdG, compared to EC group in hippocampus CA1. In contrast, EC group revealed high reactivity of 8-OHdG.

Collectively, these data suggest that agmatine activates both pro-survival and anti-oxidant proteins to resist oxidative damage.

Ⅳ. DISCUSSION

Alzheimer's disease (AD) is one of the most frequent degenerative diseases in the elderly. AD is clinically diagnosed by progressive memory loss and cognitive impairment. [35]

It is characterized by predominant death of neuron and synapses, especially in cortex and hippocampus, the extra cellular accumulation of Aβ plaque, and the presence of hyperphosphorylated tau protein causing intracellular neurofibrillary tangles (NFT). [36-40]

Agmatine has reported that it exerts beneficial effect in various stress model and protects hippocampal neuron from glucocorticoid induced toxicity via possible blockade, N-methyl-Daspartate receptor channels.[41] In addition, agmatine has potential of anti-apoptotic and antioxidant in the *in vitro* model.[42]

In this study, we focused on the effect of agmatine against AD like pathology in two points of views. In the first group of experiment, the effect of agmatine was investigated to suppress amyloid beta (Aβ) toxicity as a NMDA receptor antagonist. In the second group of experiment, the effect of agmaitne was examined to detoxify oxidative stress involved damage.

In experiment 1 : we investigated the effect of agmatine aginst Aβ plaque toxicity both in the *in vivo* and *in vitro*.

In the *in vivo* study, rats were intracerebrally injected with streptozotocin (STZ) to induce Alzheimer like pathologies. The administration of STZ in central nervous system generates the similar pathology of AD[43] and the application of intracerebral injection of STZ in rat produced Aβ within one month.[44] Aβ (a peptide mostly 40 or 42 aminoacid in length) is derived from the proteolysis of amyloid precursor protein (APP). According to the 'amyloid cascade' hypothesis, the fibrillization of Aβ result in the formation of amyloid plaque that is the cardinal hallmark of AD.[45] Thus, the increase level of APP is closely related to the higher possibility of Aβ formation. In this study, we found that agmatine treated group demonstrated significantly low expression of either APP or Aβ and less cell death in cortex and hippocampus. Furthemore, the expression of caspase 3 expression was less in agmatine treated group, compared with EC group. These results suggest that agmatine protects neuronal death against Aβ toxicity.

In morris water maze test, we found that the latency time was reduced in agmatine treated group, compared with EC group. This means chronic agmatine treatment not only plays an important role in improving learning and memory ability but also exhibits hippocampal neuronal protection.

To investigate how agmatine blocks neuronal death by Aβ toxicity, we explored its role as the NMDA receptor antagonist in primary hippocampal neuron culture treated with Aβ 25-35 fragment.

The reason of Aβ 25-35 fragment is that this short peptide has shown to be more toxic than parent peptide. [46,47]

In pathologic status of AD, the hyper activation of glutamate receptor and continuous Ca^{2+} influx by Aβ plque result in neuronal damage and cognitive dysfunction.[48]

To overcome these pathologies, NMDA antagonists are clinically tested as one of the therapeutics. For instance, memantine provides symptomatic benefits by offering temporary cognitive improvement and retardation of decline.[49] Similarly, agmatine binds to imidazoline and α2-adrenergic receptor to trigger a noncompetitive voltage and concentration dependent blockage of the N-,ethyl-D-asparate (NMDA) receptor inophore.[13,50] Thus, it is conceivable that agmtine antagonizes NMDA receptor channels, resulting in the inhibition of cell death. In the present study, Hoechst-PI staining was obviously reduced in groups treated with agmaitne (50μM and 100 μM), compared with EC group. As a positive control, MK801 was used to confirm the effects of agmatine in hippocampal neuronal cultures.[51-53] In parallel, agmatine demonstrated the significant antiapoptotic effect via the blockage of NMDA receptor.

In summary, the present data experiment 1 suggest that agmatine protects hippocampal neurons, thereby maintain or enhance of cognition and learning and memory ability. These effects of agmatine are believed to be linked with its NMDA receptor antagonistic property.[54]

In experiment 2, it was investigated whether agmatine neutralizes oxidative stress and prevents cell death. Many studies show that in AD, there is increased oxidative stress relevant to the retardation of cognitive ability.[56-58] The intracerebroventricular administration of STZ produced memory impairment and oxidative stress.[59]

To evaluate cognitive ability, morris water maze and radial arm maze were conducted. In morris water maze, the latency time was declined in agmatne treated group, compared with EC group. Similarly, radial arm maze test showed the less number of error in agmatine treated group than EC group significantly. In both behavioral assessments, these data mean agmaitne improved cognitive ability. Based on behavioral results, study has tested how agmatine ameliorates cognitive function.

The morphologic data (H&E staining) indicate that agmatine saved neurons against STZ in both hippocampus and cortex.

Western blotting data indicate that agmatine rases Bcl2 and reduce Bax, As well, both western blotting and immunostaining data show that agmatine inhibis the activation of capase 3 in STZ treated group. Therefore, these data suggest that agmatine demonstrates neuroprotective effect through the inhibition of pto-apoptotic and the activation of anti-apoptotic mediators. To demonstrate how agmaitne effect on cell death protection in AD like pathology, study was focused on oxidative stress caused by streptozotocin.

Then, it was further investigated how agmatine supports cell survival. Western blotting and biochemical data indicate that agmatine elevates P-AKT, Nrf2 and γ-GCS and increase GSH. AKT/Nrf2/ γ-GCS/GSH is known to be major cell survival and antioxidant pathway in most cell types. It is relevant to the availability of intracellular GSH. The reason is that mitochondria produce the large amount of ROS and mitochondrial ROS is detoxified by imported cytosolic GSH. Therefore, the present data suggest that the enhanced cell survival represent the activation of pro-survival factors including AKT, Nrf2, and γ-GCS accompanied by the production of key antioxidant GSH in agmatine treated group.

Taken together, all of the current findings suggest that agmatine stimulates cell survival route to boost cognitive function. In conclusion, this study proposes a new avenue to develop the therapeutic agents including agmatine to treat neurodegenerative disease like AD.

Ⅴ. CONCLUSION

In AD like pathology, agmatine endogenously improves cognitive ability via blocking amyloid beta (Aβ) toxicity and oxidative stress. The different administration manner, intracerebral (i.c) and intracerebroventricular (i.c.v) injection of streptozotocin were subjected to examine different approach.

Intracerebral (i.c) administration of streptozotocin study suggests agmatine play a role as a NMDA antagsnist leading to protect of cell death and cognitive function. *In vivo* study suggest that agmatine reduced the formation of APP and Aβ. Moreover, by caspase 3 staining in hippocampus and cortex, agmaitne may have the potential of decline apoptotic cell death induced by Aβ.

1. To confirm how agmatine prevent apoptotic cell death, *in vitro* study was subjected.
   Agmaitne treatment showed the protective effect as a NMDA receptor antagonist through caspase 3 and Hoechst/PI staining. Inhibition of hippocampal neuronal cell death by agmatine treatment leads to cognitive ability improvement.

2. Intracerebroventricular (i.c.v) administration of steptozotocin study showed that agmatine has potential of an antioxidant role and inhibit cell death, which result in improving learning and memory. Agmtine treated group showed that inhibition of pro-apoptotic protein and amplification of anti-apoptotic protein both western blot and immuno-staining results in hippocampus. The protein level of antioxidants demonstrates that agmatine has potential to be an antioxidant, which may prevent neuronal cell death from oxidative stress. Avoiding hippocampal neuronal cells result in enhanced learning and memory ability.

In conclusion, agmaitne suppressed amyloid beta related neurotoxicity in rats intracerebrally injected with STZ through NMDA receptor antagonistic function. As well, it detoxified oxidative stress mediating neurotoxicity in rat intracerebroventricularly treated with STZ.

These NMDA receptor antagonism and anti-oxidant function of agmatine are considered to boost cognitive impairment

Therefore, the current data provide the possibility that agmatine can be employed to enhance cognitive deficits in the patients of several neurodegenerative diseases.

REFERENCES

1. Hyman BT, Damasio H, Damasio AR, Van Hoesen GW. Alzheimer's disease. Annu Rev Public Health 1989;10:115–40.
2. Van Hoesen GW, Augustinack JC, Dierking J, Redman SJ, Thangavel R. The parahippocampal gyrus in Alzheimer's disease. Clinical and preclinical neuroanatomical correlates. Ann NY Acad Sci 2000;911:254–74.
3. Furukawa K, Abe Y and Akaike N. Amyloid beta protein-induced irreversible current in rat cortical neurones. Neuroreport 1994;5:2016–18.
4. Arispe N, Rojas E and Pollard HB. Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminum. Proc Natl Acad Sci USA 1993;90:567–71.
5. Mattson MP, Barger SW, Cheng B, et al. beta-Amyloid precursor protein metabolites and loss of neuronal Ca^{2+} homeostasis in Alzheimer's disease. Trends Neurosci 1993;16:409–14.
6. 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.
7. Alberdi E, Sanchez-Gomez MV, Cavaliere F, Perez-Samartin A, Zugaza JL, Trullas R, Domercq M, Matute C. Amyloid beta oligomers induce Ca^{2+} dysregulation and neuronal death through activation of ionotropic glutamate receptors. Cell Calcium 2010;47:264–72.
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.
9. 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.
10. 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.
11. Reis DJ, Regunathan S. Agmatine: a novel neurotransmitter? Adv Pharmacol 1998;42:645–9.
12. 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.
13. Reis DJ, Regunathan S. Is agmatine a novel neurotransmitter in brain? Trends Pharmacol Sci 2000;21:187–93.
14. Halaris A, Piletz J. Agmatine: metabolic pathway and spectrum of activity in brain. CNS Drugs 2007;21:885–900.

15. 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.
16. 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.
17. Liu P, Collie ND. Behavioral effects of agmatine in naive rats are task- and delay dependent. Neuroscience 2009;163:82–96.
18. Lu W, Dong H, Gong Z, Su R, Li J. Agmatine inhibits morphine-induced memory impairment in the mouse step-down inhibitory avoidance task. Pharmacol Biochem Behave 2010;97:256–61.
19. McKay BE, Ladol WE, Martin LJ, Galic MA, Fournier NM. Learning and memory in agmatine-treated rats. Pharmacol Biochem Behave 2002;72:551–7.
20. 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.
21. Hoyer, S. Glucose metabolism and insulin receptor signal transduction in Alzheimer disease. Eur J Pharmacol 2004;490:115–25.
22. 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.
23. Grunblatt E, Salkovic-Petrisic M, Osmanovis J, Riedere P, Hoyer S. Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. J Neurochem 2007;101:757-70
24. Weinstock M, Shoham S. Rat models of dementia based on reduction in regional glucose metabolism, cerebral blood flow and cytochrome oxidative activity. J Neural Transm 2004;111:347-66
25. Jellinger KA. Cell death mechanisms in Parkinson's disease. J Neural Transm 2000;107:1–29.
26. Kim TW, Pettingell WH, Jung YK, Kovacs DM, Tanzi RE. Alternative cleavage of Alzheimer-associated presenilins during apoptosis by a caspase-3 family protease. Science 277:373–6.
27. Butterfield DA. Proteomics: a new approach to investigate oxidative stress in Alzheimer's disease brain. Brain Res 2004;1000:1-7
28. Gary EG, Hsueh-Meei H. Oxidative stress in Alzheimer's disease. Neurobiol Aging 2005;26:575-8
29. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 3rd ed. Oxford:

Clarendon Press. 1999.

30. Polidori MC, Mecocci P. Plasma susceptibility to free radical-induced antioxidant consumption and lipid peroxidation is increased in very old subjects with Alzheimer disease. J Alzheimer's Dis 2002;4(6):517-22.
31. Ahmad M, Saleem S, Ahmad AS, Yousuf S, Ansari MA, Khan MB. Ginkgo biloba affords dose-dependent protection against 6-hydroxydopamine-induced parkinsonism in rats: neurobehavioural, neurochemical and immunohistochemical evidences. J Neurochem 2005;93:94-104.
32. Ansari MA, Ahmad AS, Ahmad M, Salim S, Yousuf S, Ishrat T. Selenium protects cerebral ischemia in rat brain mitochondria. Biol Trace Elem Res 2004;101:73.86.
33. Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 4th ed. San Diego, CA: Academic Press, 1998.
34. Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 1984;11:47–60.
35. Flynn BL. Pharmacologic management of Alzheimer disease. Part I: Hormonal and emerging investigational drug therapies. Ann Pharmacother 1999;33:178–87.
36. Querfurth HW, LaFerla FM. Alzheimer's disease. N Engl J Med 2010;362:329–44.
37. Goedert M, Spillantini MG. A century of Alzheimer's disease. Science 2006;314:777–81.
38. Moreira PI, Duarte AI, Santos MS, Rego AC, Oliveira CR. An integrative view of the role of oxidative stress, mitochondria and insulin in Alzheimer's disease. J Alzheimers Dis 2009;16:741–61.
39. Moreira PI, Honda K, Zhu X, Nunomura A, Casadesus G, Smith MA, Perry G. Brain and brawn: parallels in oxidative strength. Neurology 2006;66:S97–S101.
40. Moreira PI, Santos MS, Seica R, Oliveira CR. Brain mitochondrial dysfunction as a link between Alzheimer's disease and diabetes. J Neurol Sci 2007;257:206–14.
41. 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
42. Iizuka Y, Hong S, Kim CY, Kim SK, Seong GJ. Agmatine pretreatment protects retinal ganglion cells (RGC5 cell line) from oxidative stress in vitro. Biocell 2008;32:245-50
43. Selkoe DJ, Alzheimer's disease results from the cerebral accumulation and cytotoxicity of amyloid beta-protein. J Alzheimers Dis 2001;3:75–80.
44. Salkovic-Petrisic M, Hoyer S. Central insulin resistance as a trigger for sporadic Alzheimer-like pathology: an experimental approach. J Neural Transm Suppl 2007;72:217–33.

45. 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.
46. Selkoe DJ. Alzheimer's disease: a central role for amyloid. J Neuropathol Exp Neurol 1994;53:438–47.
47. Varadarajan S, Yatin S, Aksenova M, Butterfield DA. Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity. J Struct Biol 2000;130:184–208.
48. 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.
49. Danysz W, Parsons CG. The NMDA receptor antagonist memantine as a symptomatological and neuroprotective treatment for Alzheimer's disease: preclinical evidence. Int J Geriatr Psychiatry. 2003;18:S23–32.
50. Salloway S, Mintzer J, Weiner MF, Cummings JL. Disease-modifying therapies in Alzheimer's disease. Alzheimers Dement 2008;4:65–79.
51. Ogden F, DeCoster MA, Bazan NG. Recombinant plasma-type platelet-activating factor acetylhydrolase attenuates NMDA-induced hippocampal neuronal apoptosis. J Neurosci Res 1998;53:677–84.
52. Lee EH, Hsu WL, Ma YL, Lee PJ, Chao CC. Enrichment enhances the expression of sgk, a glucocorticoid-induced gene, and facilitates spatial learning through glutamate AMPA receptor mediation. Eur J Neurosci 2003;18:2842–52.
53. Lu J, Goula D, Sousa N, Almeida OF. Ionotropic and metabotropic glutamate receptor mediation of glucocorticoid-induced apoptosis in hippocampal cells and the neuroprotective role of synaptic N-methyl-D-aspartate receptors. Neuroscience 2003;121:123–31.
54. Mikati MA, Abi-Habib RJ, El Sabban ME, Dbaibo GS, Kurdi RM, Kobeissi M, Farhat F, Asaad W. Hippocampal programmed cell death after status epilepticus: evidence for NMDA-receptor and ceramide-mediated mechanisms. Epilepsia 2003;44:282–91.
55. Mayer B, Schmidt K, Humbert P, Böhme E, Biosynthesis of endothelium-derived relaxing factor: a cytosolic enzyme in porcine aortic endothelial cells Ca^{2+} dependently converts L-arginine into an activator of soluble guanylyl cyclase. Biochem Biophys Res Commun 1989;164:678–85.
56. Launer LJ, Kalmijn S. Anti-oxidants and cognitive function: a review of clinical and epidemiologic studies. J Neural Transm Suppl 1998;53:1-8
57. Pratico D, Delanty N. oxidative injury in diseases of the central nervous system: focus on

Alzheimer's disease. Am J Med 2000;109:577-85.

58. Hartman D. Free radical theory of aging: Alzheimer's disease pathogenesis. Age 1995;18:97-119
59. 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

ABSTRACT (IN KOREAN)

스트렙토조토신이 주입된 백서에서 규명된 아그마틴의 신경보호작용

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허보은

알츠하이머 질환은 노인들에서 점진적인 인지능력의 장애와 기억력 손실이 관찰되는 것이 특징인 대표적인 신경퇴행성 질환이다. 중요하게, 몇몇의 연구에서 NMDA 수용체의 길항제와 항산화 물질들은 이러한 알츠하이머 병적 양상을 막아줄 수 있는 것으로 증명해왔다. 아그마틴은 양이온다중성이며 내제적인 아민으로 아르기닌 탈탄산효소에 의해 생성된다. 아그마틴은 다양한 뇌질환 동물모델에서 신경보호기능을 담당하고 있음이 보고되어 왔으며, 최근 일부 연구들에서는 NMDA 수용체 길항제 또는 항산화 인자로서의 기능을 담당한다고 보고되어왔다. 본 연구는 스트렙토조토신에 노출된 백서들의 뇌에서 아그마틴의 효과들을 연구하였다. 특히, 스트렙토조토신에 노출된 백서에서 아그마틴이 어떻게 아밀로이드 베타와 산화성 스트레스 중개된 독성을 완화시키는지를 관찰하고자 하였다.

첫번째로, 정위방법으로 대뇌에 스트렙토조토신을 주입하고 아그마틴을 복강 내 주사로 14일 동안 처치하였다. 아그마틴은 모리스 수중미로와 방사형 팔방미로와 같은 행동검사에서, 스트렙토조토신으로 처치된 쥐들이 인지기능과 기억력 손실을 회복시키는 것으로 증명되었다.

또한, 아그마틴은 아밀로이드 전구 단백질과 아밀로이드 베타 단백질의 유도를 억제시키고 caspase 3의 면역염색양상을 경감시켰다.

아그마틴의 보고효과를 근본적으로 조사하기 위해서, 일차 해마 신경세포에 아밀로이드 베타, MK801 (NMDA 수용체 길항체) 또는 이 약물들을 혼합하여 처리하였다.

아그마틴은 아밀로이드 베타로 유도된 일차 해마 신경세포상에서, 세포 생존률의 지표인 포마잔의 환원을 억제시키고, 세포 괴사의 지표인 Hoechest/PI 반응을

억제시켰다. 이러한 효과는 아그마틴이 MK801과 유사했다. 그러므로, 이러한 결과는 아그마틴이 NMDA 수용체의 길항제로써 긍정적인 효과를 가하는 것으로 예상된다.

두번째로, 백서들은 정위방법으로 양측 뇌실에 스트렙토조토신을 주입하였고, 아그마틴을 복강내 주입으로 14일 동안 진행하였다.

아그마틴은 스트렙토조토신을 대뇌에 주입한 것과 비슷한 양상으로, 인지와 기억의 장애를 증진시켰고, caspase 3 를 억제시켰으며, 이는 행동검사들과 면역염색결과에서도 증명되었다.

아그마틴의 신경보호효과를 규명하기 위해, Bax, Bc12, PI3K, AKT, Nrf2 그리고 r-GCS에 대한 웨스턴 블롯을 시행하였다. 아그마틴 처치후 bax의 발현이 경감되었고, 열거한 상위 단백질의 발현이 증가되었다.

아그마틴 처치 후 Nrf2와 r-GCS 단백질의 발현이 증가된 것은 주목할 결과로, 이 단백질들은 산화성 손상을 중화시키는데 필수적인 역할을 하는 것으로 알려져 있다.

이와 유사하게, 아그마틴 처치 후 스트렙토조토신으로 유도된 해마 신경세포에서 8-oxo 면역 형광 발현이 감소되었으며, 이러한 결과는 세포사이에서 산화성 스트레스를 해독하는 잠재력으로 보여진다.

이러한 결과들은 아그마틴이 인지기능을 담당하는 신경세포의 생존을 높이고, 이들 세포에서의 항산화제 발현을 유도하는 것을 보여주었다.

결론적으로, 아그마틴은 알츠하이머 질환, 파킨슨 질환, 헌팅턴 질환 그리고 루게릭 질환과 같은 신경 퇴행성 질환에서 세포 생존과 항산화 역할을 통해 인지기능 장애를 완화시키는 것으로 예측된다.

핵심되는 말: 아그마틴, 알츠하이머 질환, 인지기능, NMDA 수용체 길항제, 세포괴사, 산화성 스트레스, 세포 생존