

Effect of agmatine in Mitogen-activated
protein kinases (MAPKs) activation
after traumatic brain injury

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after traumatic brain injury

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Abstract

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Agmatine is a primary amine formed by the decarboxylation of L-arginine and is an endogenous clonidine-displacing substance synthesized in mammalian brain. Many studies suggest that agmatine reduces various brain injury. We investigate the effect of agmatine in mitogen-activated protein kinase (MAPKs) activation after traumatic brain injury (TBI). Agmatine was treated at 30 minutes after trauma. Through the treadmill test, motor function was clearly increased in agmatine treatment group at 2 weeks after TBI. Agmatine reduced necrotic brain area, the number of eosinophilic neurons and TUNEL positive cells. Especially, Agmatine increased p-ERK expression and decreased p-JNK and p-p38. The expression of inflammatory cytokines were decreased in agmatine treatment group than experimental control group. These results were associated with an induction of nuclear factor-kB (Nf-kB) nuclear translocation. Western blot analysis showed that agmatine induces nuclear translocation of Nf-kB. Aquaporins (AQPs), correlated with brain edema as water channels. The expression of AQPs were decreased by agmatine treatment. Bone

morphogenetic protein (BMP) -7 is neuroprotective and neuroregenerative. Agmatine treatment increased the expression of BMP-7 more than experimental control after TBI.

In these results, neuroprotective effect of agmatine on TBI is associated with activated MAPKs expression through Nf-kB translocation into the nucleus.

Key words : Agmatine, Traumatic brain injury, Mitogen-activated protein kinases, Inflammatory cytokine, Aquaporins, Bone morphogenetic protein-7

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I. INTRODUCTION

Agmatine, formed by the decarboxylation of L-arginine by arginine decarboxylase (ADC), was first discovered in 1910. It is hydrolyzed to putrescine and urea by agmatinase¹. Recently, agmatine, ADC, and agmatinase were found in mammalian brain². Agmatine is an endogenous clonidine-displacing substance, an agonist for the 2-adrenergic and imidazoline receptors, and an antagonist at N-methyl-D-aspartate (NMDA) receptors^{2,3,4}. It has been shown that agmatine may be neuroprotective in trauma and ischemia

models^{1,5,6,7,8,9}. Agmatine was shown to protect neurons against glutamate toxicity and this effect was mediated through NMDA receptor blockade, with agmatine interacting at a site located within the NMDA channel pore¹⁰.

Traumatic brain injury (TBI) is one of three major causes of death in Korea, along with cancer and cardiovascular disease. Dynamic mechanical deformation of neuronal tissue produces a microstructural response at the cellular level triggering a complex array of signaling events. These events, such as activation of second messengers, phosphorylation of proteins, and changes in the synthesis of transcriptional factors^{11,12}, can lead to delayed damage and cell death in the initially affected cells as well as neighboring cells within hours and days post-injury¹³. However, the mechanisms by which these initial disturbances caused by membrane damage lead to cell death are poorly understood. Recent studies on the molecular mechanisms mediating apoptosis have focused on activation of mitogen-activated protein kinase (MAPKs) following traumatic injury in both in vivo and in vitro models¹⁴⁻²².

Inflammatory response and edema following TBI has been proposed as an important factor in the development of secondary tissue damage. The proinflammatory cytokines interleukin-1 and TNF, are induced early after brain injury and have been implicated in the delayed damage²³. Brain edema is a pathophysiological condition of increased water content due to variety of coexisting brain injuries, including ischemia, trauma, tumor and infection, and has been classified into several subtypes by the pathogenesis of the edema development, including cytotoxic, vasogenic and interstitial brain edema²⁴.

Mitogen-activated protein kinases(MAPKs) cascades are a family of protein kinases activated by a wide spectrum of extracellular stimuli. There are at least three subtypes of MAPKs cascades, including extracellular signal regulated kinase(ERK)1/2, c-jun NH2 terminal kinase(JNK), and p38 cascades, and they regulate various cellular processes such as cell growth, differentiation, inflammation and apoptosis. MAPKs is crucial for many physiological or pathological responses²⁵.

MAPK participates in the TRAF pathway of IL-1 intracellular signal transduction^{26,27}. In addition, may play an important role in ischemia or trauma-induced neuronal damage in the hippocampus²⁸.

Based on these evidences, we hypothesized that agmatine may have neuroprotective effect through MAPKs activation after TBI.

II. MATERIALS AND METHODS

1. Animals

Sprague-Dawley rats from Sam tako(Osan, Korea) were used for this study. All animal procedures were carried out according to a protocol approved by the Yonsei University Animal Care and Use Committee in accordance with NIH guidelines.

2. Traumatic brain injury model

Male adult Sprague-Dawley rats weighting 300 ± 20 g were anesthetized with mixture of ketamine (75 mg/kg), sedazect (0.75 mg/kg), and rompun (4 mg/kg). The scalps were incised and a craniotomy was made over the right forelimb motor cortex (the rats were put into a stereotactic frame and a craniotomy was drilled +2 ~ -2 mm anterior and posterior from bregma and + 1 ~ + 5 mm lateral from the midline). The bone was thinned with the drill bit over the forelimb motor cortex, and then the bone was removed by a forceps. Special care was taken to keep the dura intact to prevent bleeding. Cortical lesion was induced by attachment of metal probe (diameter 3mm) cooled by liquid nitrogen onto the brain surface 5 times for 30 seconds. The physiological parameters before cold traumatic injury were monitored and maintained throughout the experiment.

Agmatine was dissolved in normal saline (100 mg/kg IP, Sigma) and given 30min after TBI. Controls received normal saline in equivalent volumes.

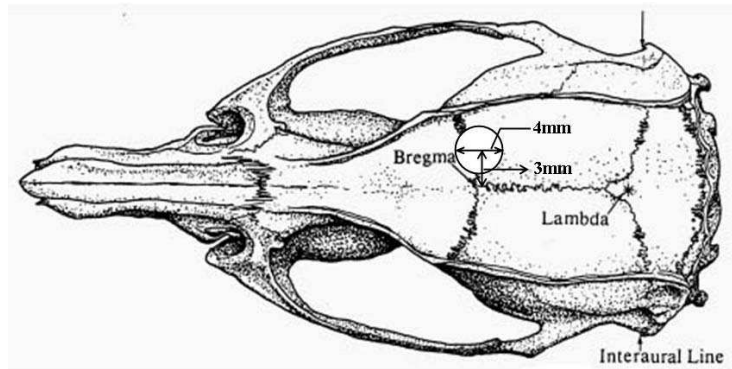


Figure 1. Dorsal view of the rat skull. Figure shows the position of traumatic cold lesion. Figure from "The rat brain in stereotaxic coordinates fourth edition, George Paxinos & Charles Watson, Academic Press 1998"

3. Assessment of necrotic volume.

Necrotic volume was determined by H-E staining, using a computer-assisted image analysis system (Image J 1.36b, NIH, USA). The volume of necrosis was expressed as a percentage of the necrotic area of ipsilateral hemisphere.

4. Terminal Deoxynucleotidyl transferase-Mediated dUTP Nick End Labeling (TUNEL) staining.

For TUNEL staining, we used the *In Situ* Cell Death kit (Roche Diagnostics). Brain sections were deparaffin, rehydrate and washed with PBS. Then add Proteinase K and incubate for 30min at 37°C. Sections were then washed twice with PBS, stained with the TUNEL reaction mixture for 60 min at 37°C, washed twice with PBS. DNA fragmentation was observed under a fluorescence microscope (LSM 510 META, Carl Zeiss).

5. Immunohistochemical staining

Brains were fixed with 4 % paraformaldehyde, and embedded in paraffin. Brain sections were made by 10 μ m. Sections were immunostained with primary antibodies, followed by an appropriate biotinylated secondary antibody. Stains were visualized using the ABC kit (Vector, Burlingame, CA, USA) (Lee et al., 2002), then reacted with diaminobenzidine (DAB, Sigma, St. Louis, MO, USA). When double-labeled fluorescent immunohistochemistry was used, stain were visualized using fluorescein-conjugated secondary antibody. Double-labeled immunostaining was evaluated using a fluorescence microscope (LSM 510 META, Carl Zeiss). Immunostaining controls were prepared by tissue without primary antibodies. All incubation steps were performed in a humidified chamber.

6. Immunoblotting

Proteins were isolated from rat brain and lysed in solublizing buffer (10mM HEPES pH7.4, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 10% NP-40, and 1mM PMSF, 1g/ml aprotinin, leupeptin). Equal amounts of protein were subjected to electrophoresis on 10~12.5% SDS-polyacrylamide gels. Separated proteins were then electro-transferred to PVDF membrane (Millipore, Bredford, MA, USA). The membranes were blocked for 1h at room temperature in 5% skim milk in TBS. The membranes were incubated overnight with antibodies. After washing 5 times with TBS-T for every 5min, blots were incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies for 3h at RT. Finally blots were rinsed and proteins were visualized using an ECL protein detection kit (ECL plus, Amersham international plc, Little Chalfont, UK) according to the manufacturer's instructions.

7. Behavior test

To examine the change of behavioral function recovery, all animals were behaviorally tested at 2 weeks after TBI. Behavioral test was performed by using the forepaw adjusting steps. We assessed contralateral forepaw adjusting steps on a treadmill, which moved at a rate of 90 cm/12 sec. This test is consisted of 3 trials each animal forepaw and used to measure motor coordination.

8. Statistical analysis

Statistical tests to determine differences between groups were performed with student's *t* test using SAS ver 8.01 (SAS Institute Inc., NC). *P* value < 0.05 was considered significant. Data are expressed as the mean \pm standard error of mean (SEM).

III. RESULTS

1. Behavior test.

To measure the motor coordination, we used treadmill test. 2 weeks after TBI, counted the number of forepaw step to examine the change of behavioral function recovery. In normal condition, the number of forepaw step was 16~18. In agmatine treatment group, the number of step was increased (7 ± 2 steps) compared to experimental controls (2 ± 1 steps).

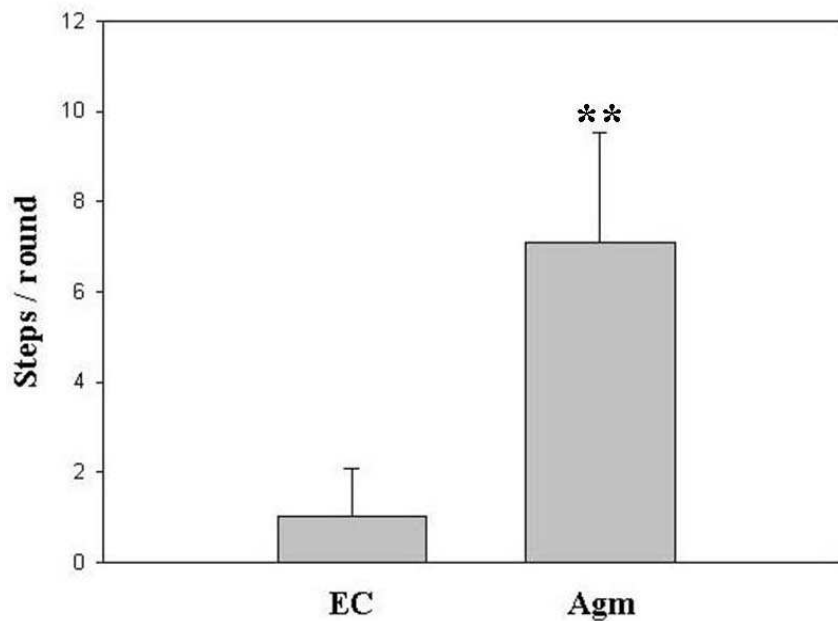
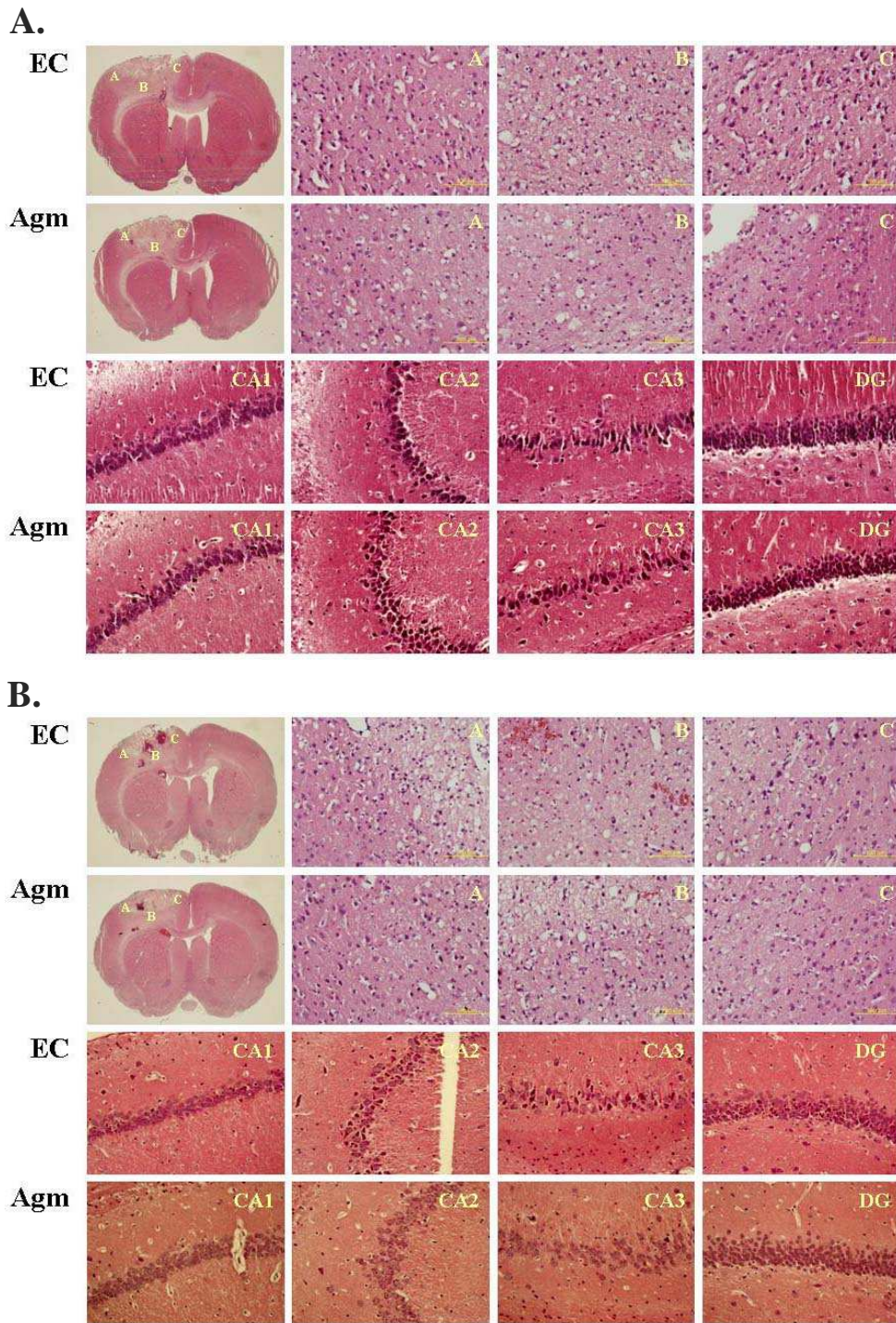
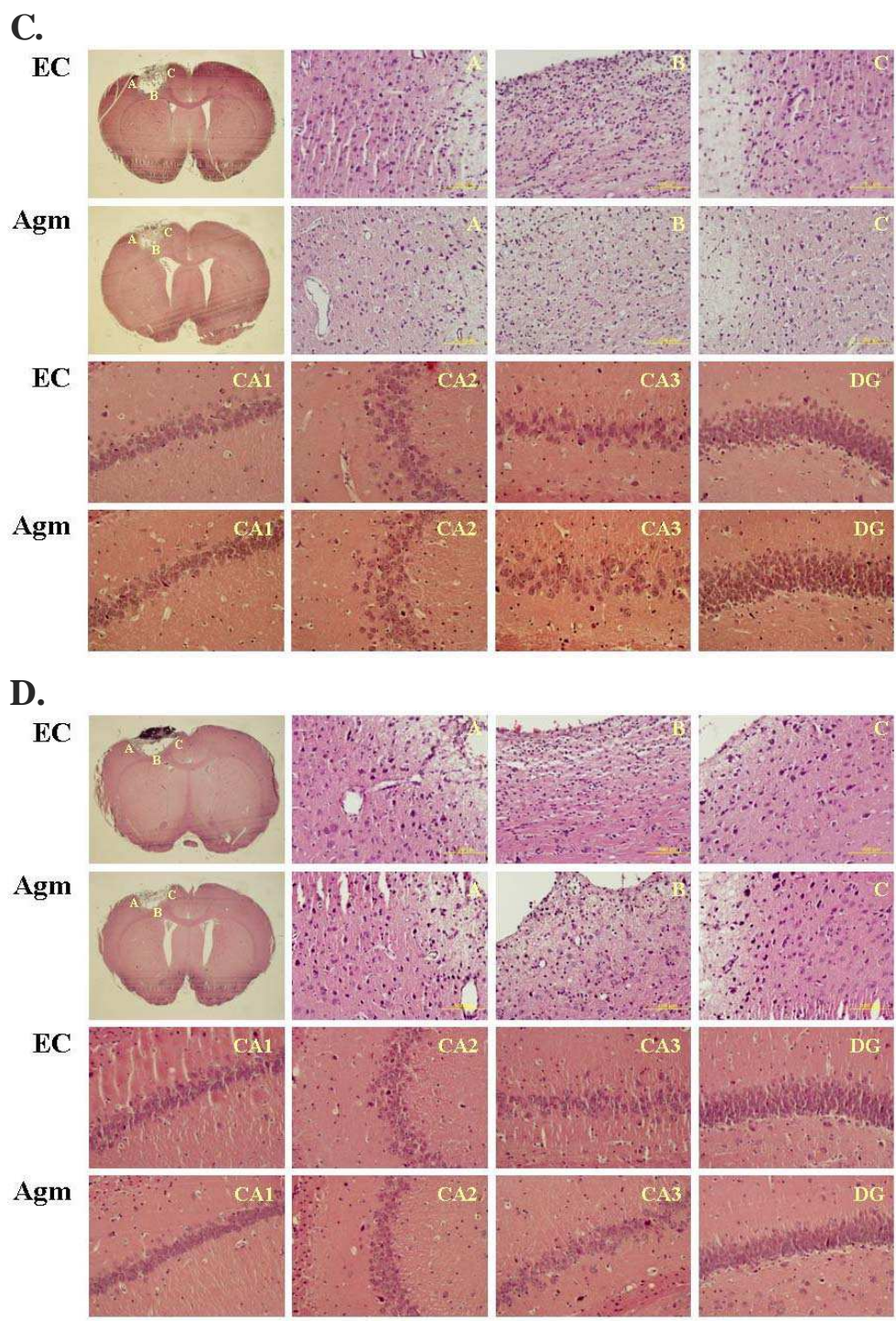


Figure 2. Treadmill test 2 weeks after TBI. The motor function was clearly increased in agmatine treatment group than in EC at 2weeks after TBI. (**, $P < 0.01$ vs EC). Data are expressed as the mean \pm SEM. EC, Experimental control group; Agm, Agmatine treatment group.

2. Histological analysis for neuroprotective effect of agmatine after TBI.

To analysis the morphological change such as pyknosis and eosinophilic cell after TBI, we used H-E staining in serial coronal sections of the brain. Necrotic brain area and the number of eosinophilic neurons was reduced in agmatine treatment group than EC in all time point. 1day after TBI, the number of eosinophilic neurons were reduced in cerebral cortex and hippocampus by agmatine treatment (Figure 3A). Two days after TBI, eosinophilic neurons were significantly reduced agmatine treatment group hippocampus (Figure 3B). The necrotic volume of brain was reduced in agmatine treatment group at 1 and 2 week after TBI compared to that of EC group (Figure 3C, 3D). The volume of necrosis was expressed as a percentage of the ipsilateral hemisphere of necrotic area (Figure 3E).





E.

Necrosis area

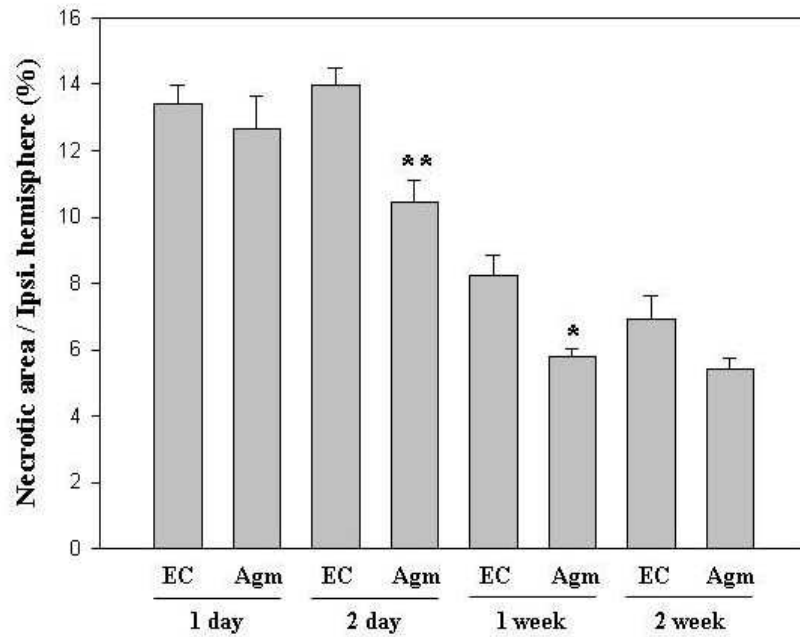


Figure 3. Trauma-induced the number of eosinophilic neurons was markedly reduced and improved neurological deficits in agmatine treatment group. Figure shows that 1day (A), 2 day (B), 1 week (C), and 2 weeks (D) after TBI. In all time point necrosis area was also reduced in agmatine treatment group (E). Scale bar is 100 μ m. (*, P < 0.05, **, P < 0.01 vs EC) EC, Experimental control group; Agm, Agmatine treatment group; CA1, CA2, CA3, DG, Dentate gyrus of hippocampus

3. TUNEL staining after TBI.

To determine the protective effect of agmatine against to the damaged brain, we used TUNEL staining.

In cerebral cortex (Figure 4A) and hippocampus (Figure 4B), the number of TUNEL positive cells (green) was decreased in agmatine treatment group from 2 days to 2 weeks after TBI compared to experimental control.

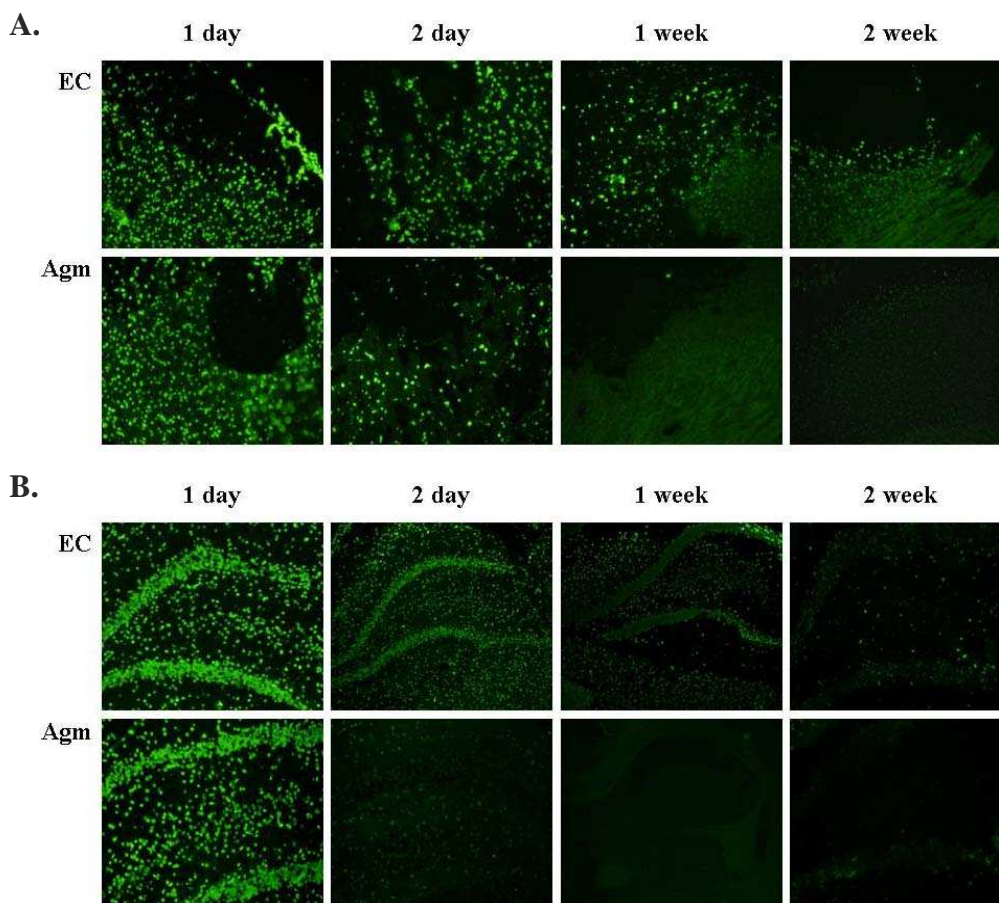


Figure 4. TUNEL staining after TBI with and without agmatine. TUNEL positive cells (green) were decreased in Agm than EC from 2 day to 2 week after TBI in both cerebral cortex (A) and hippocampus (B). EC, Experimental control group; Agm, Agmatine treatment group.

4. Immunohistochemistry of mitogen-activated protein kinases (MAPKs).

To investigate the effect of agmatine in MAPKs expression, we performed immunohistochemistry using anti- p-ERK, p-JNK and p-p38 antibodies.

The number of p-ERK positive cells was increased at 1 and 2days after TBI in agmatine treatment group than experimental control (Figure 5A). Agmatine treatment reduced the number of p-JNK and p-p38 positive cells in cerebral cortex (Figure 5B, 5C).

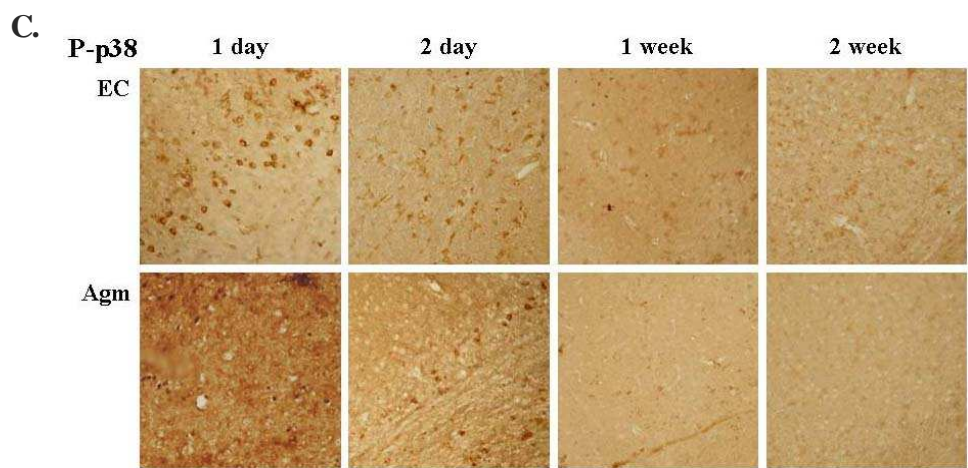
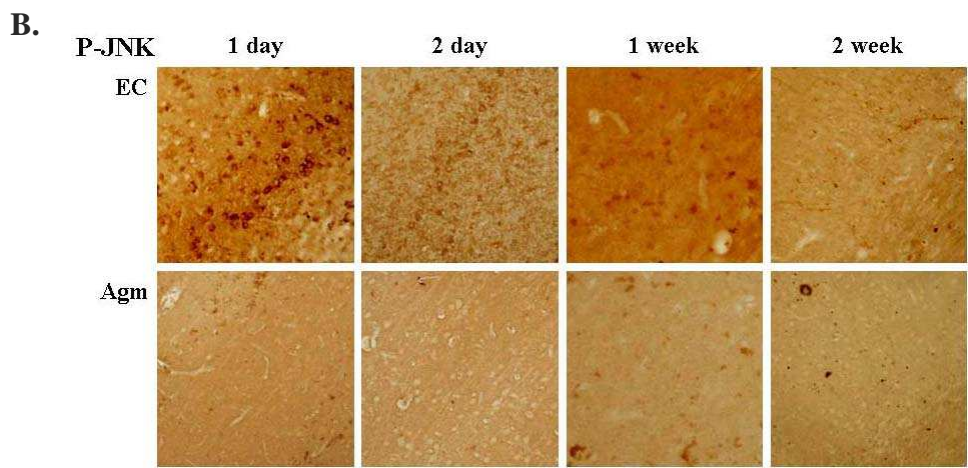
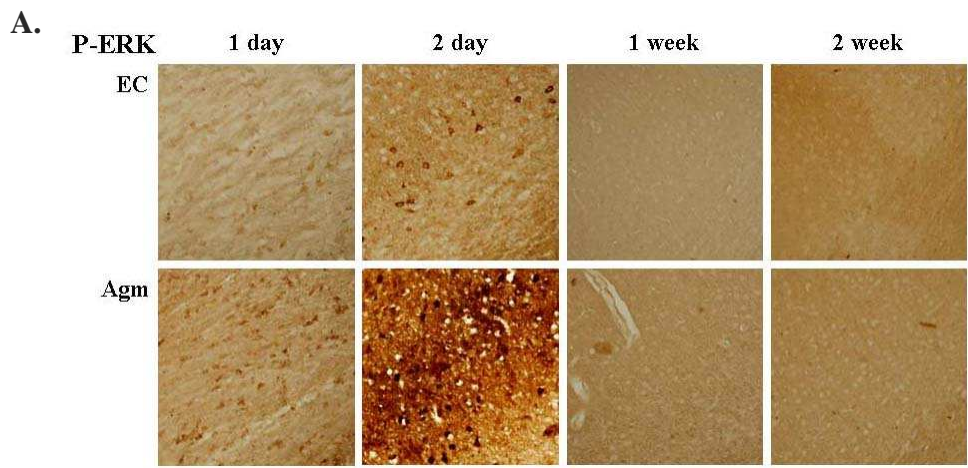


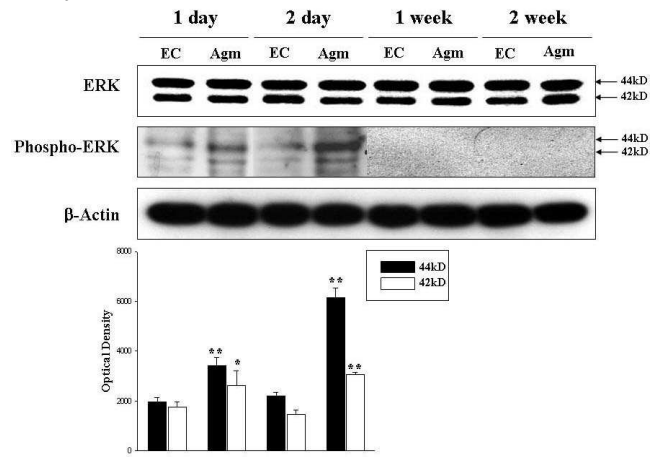
Figure 5. Immunohistochemistry of MAPKs after TBI. p-ERK, p-JNK and p-p38 positive cells shown to have brown-colored. The number of p-ERK positive cells were increased in Agm than EC (A). But p-JNK (B) and p-p38 (C) were decreased in Agm. EC, Experimental control group; Agm, Agmatine treatment group.

5. Immunoblotting of Mitogen-activated protein kinases (MAPKs).

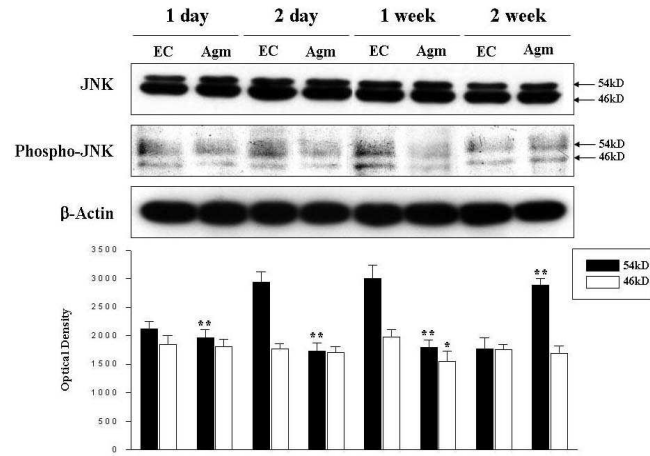
In cerebral cortex, p-ERK was increased at 1 and 2days after TBI in agmatine treatment group. p-ERK was not detected 1 and 2weeks after TBI in all condition (Figure 6A). The expression of p-JNK was decreased after TBI in agmatine treatment group (Figure 6B). p-p38 MAPK was decreased all time point in agmatine treatment group (Figure 6C).

In hippocampus, p-ERK was also increased at 1 and 2days after TBI in agmatine treatment group. p-ERK was not detected 1 and 2weeks after TBI in all condition (Figure 6D). The expression of p-JNK was decreased from 1 day to 1 week after TBI in agmatine treatment group. But 2 weeks after TBI, p-JNK was increased in agmatine treatment group (Figure 6E). The expression of p-p38 was not detected in hippocampus.

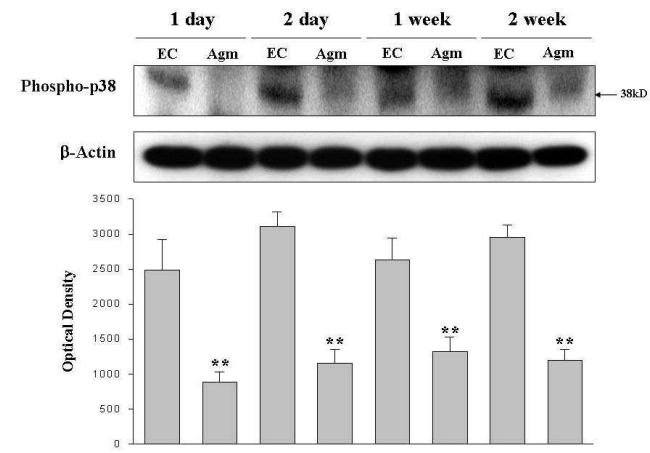
A.



B.



C.



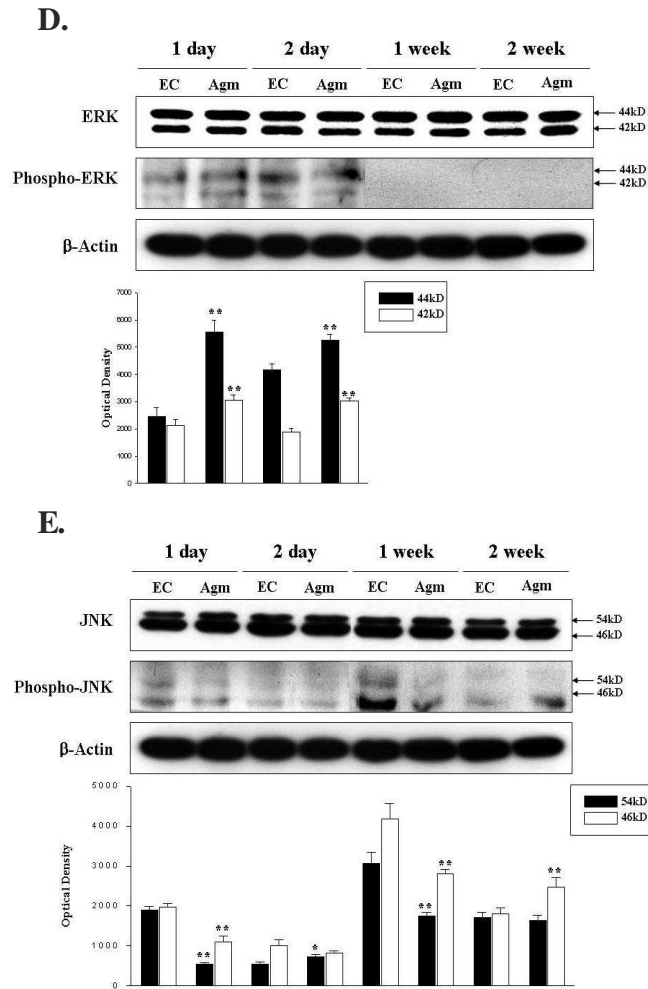


Figure 6. The expression of MAPKs after TBI. In cerebral cortex, expression of p-ERK (A) but not p-JNK (B) and p-p38 (C), was increased by agmatine treatment. In hippocampus, expression of p-ERK (D) was increased and p-JNK (E) was decreased in agmatine treatment group. p-p38 was not detected. (*, $P < 0.05$, **, $P < 0.01$ vs EC) EC, Experimental control group; Agm, Agmatine treatment group.

6. The expression of inflammatory cytokines.

The acute inflammatory response following TBI has been shown to play an important role in the development of secondary tissue damage. The proinflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α), are induced early after brain injury and have been implicated in the delayed damage. So we determine the anti-inflammatory effect of agmatine after TBI by western blot analysis.

In cerebral cortex, TNF- α (Figure 7A) and IL-1 β (Figure 7B) was decreased in all time point agmatine treatment group.

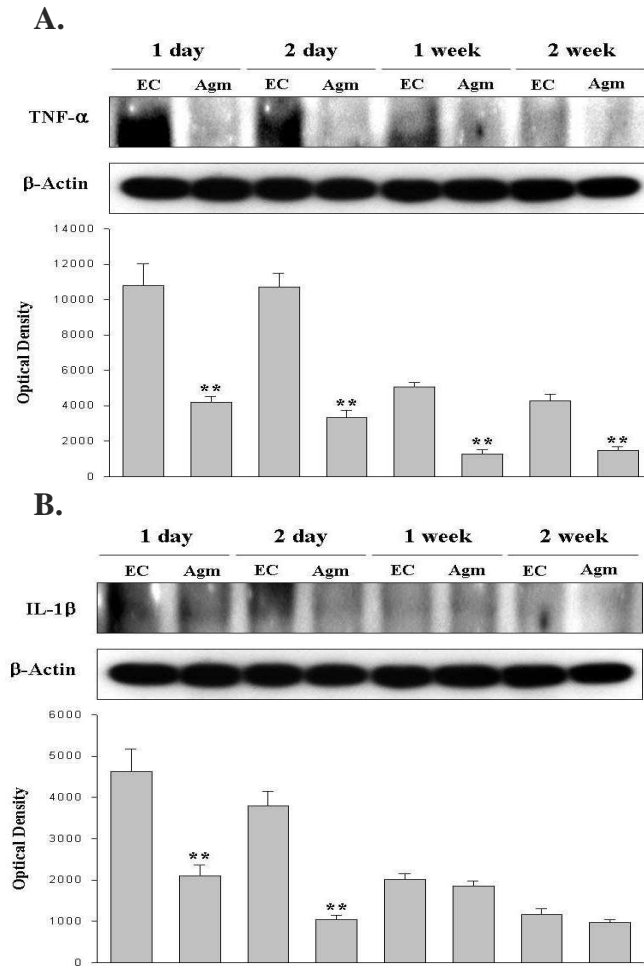


Figure 7. We examined the expression of inflammatory cytokine using immunoblotting in cerebral cortex (A, B). In all time point, TNF- α and IL-1 β were decreased in agmatine treatment group compared to experimental control after TBI. (*, P < 0.05, **, P < 0.01 vs EC) EC, Experimental control group; Agm, Agmatine treatment group.

7. Effect of agmatine on the Nf-kB nuclear translocation after TBI.

To determine the regulation of Nf-kB p65 translocation to the nuclei by agmatine, we performed western blot analysis of the cytosolic (Figure 8A) and the nuclear (Figure 8B) fractions. Agmatine induced Nf-kB p65 nuclear translocation at 1 and 2 day after TBI. Nf-kB p65 was not detected at 1 and 2 week after TBI. This data suggest that Nf-kB p65 nuclear translocation by agmatine may be involved with increased expression of p-ERK at 1 and 2 day after TBI.

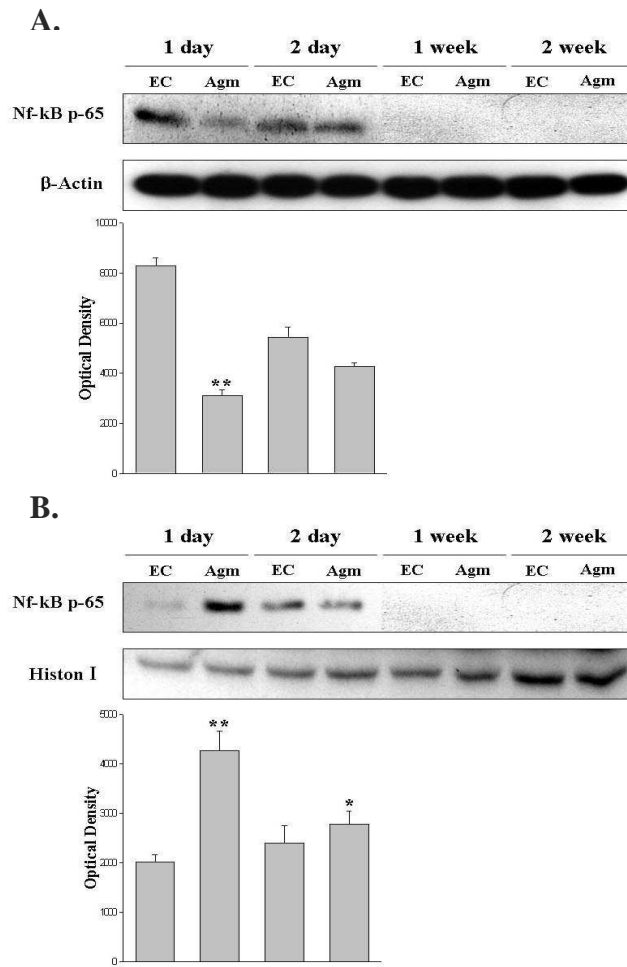


Figure 8. Western blot analysis of Nf-kB nuclear translocation by agmatine. Cytosolic (A) and nuclear (B) Nf-kB p65 were visualized. (*, $P < 0.05$, **, $P < 0.01$ vs EC) EC, Experimental control group; Agm, Agmatine treatment group.

8. Immunofluorescence of AQPs expression after traumatic brain injury.

AQP-1 was boldly detected at the choroid plexus of experimental control but wasn't in agmatine treatment group (Figure 9A). The expression of AQP-4 was colocalized with GFAP and increased in experimental control than agmatine treatment group (Figure 9B). The expression of AQP-9 was also increased in experimental control than agmatine treatment group (Figure 9C).

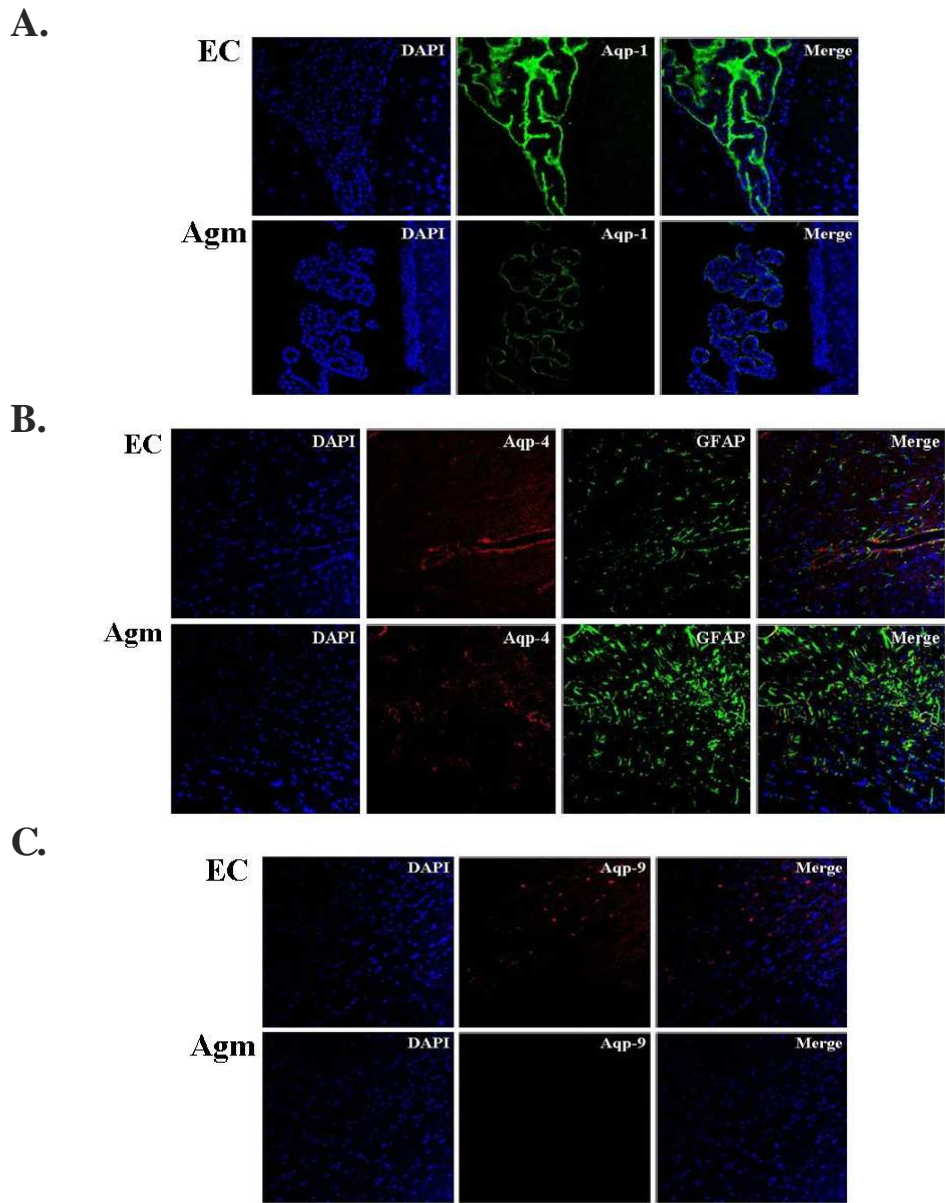


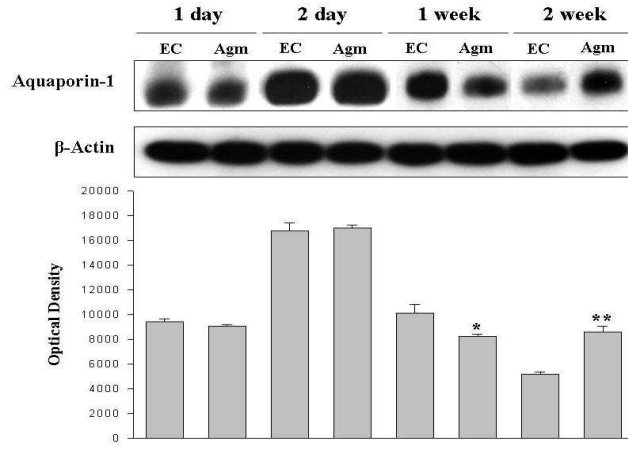
Figure 9. Macrographs of aquaporin 1 immunofluorescence in the choroid plexus after TBI with and without agmatine. AQP-1 (green) was boldly detected at the choroid plexus of experimental control (EC) but wasn't in agmatine treatment group (Agm) (A). The expression of AQP-4 (red) was colocalized with GFAP (green) and increased in EC than Agm (B). The expression of AQP-9 (red) was increased in EC than Agm (C). EC, Experimental control group; Agm, Agmatine treatment group.

9. Suppression of aquaporins (AQPs) expression in cytotoxic and vasogenic brain edema after traumatic brain injury by agmatine administration.

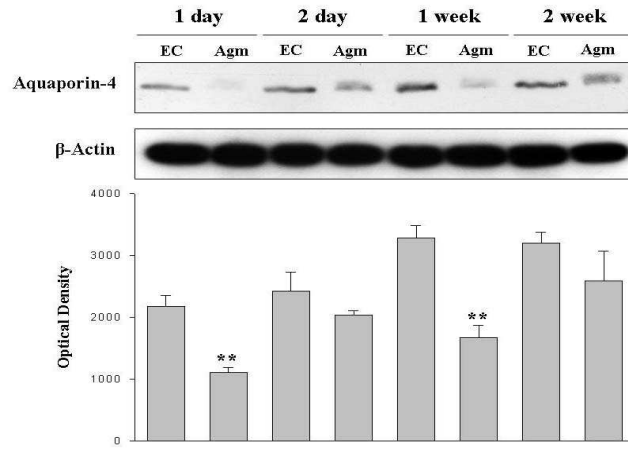
In cerebral cortex, AQP-1 was strongly expressed at 2days after TBI. The expression of AQP-1 was decreased after TBI except 2weeks in agmatine treatment group (Figure 10A). The expression of AQP-4 was decreased all time point in agmatine treatment group (Figure 10B). The expression of AQP-9 was not detected at 1day after TBI. AQP-9 was decreased in agmatine treatment group (Figure 10C).

In hippocampus, AQP-1 was strongly expressed at 2days after TBI. The expression of AQP-1 was decreased after TBI in agmatine treatment group (Figure 10D). The expression of AQP-4 was decreased from 1day to 1week after TBI in agmatine treatment group. But 2weeks after TBI, AQP-4 was increased in agmatine treatment group (Figure 10E). The expression of AQP-9 was not detected at 1day after TBI. AQP-9 was increased at 2days after TBI in agmatine treatment group. But the expression of AQP-9 was suppressed in agmatine treatment group than experimental control at 1 and 2weeks after TBI (Figure 10F).

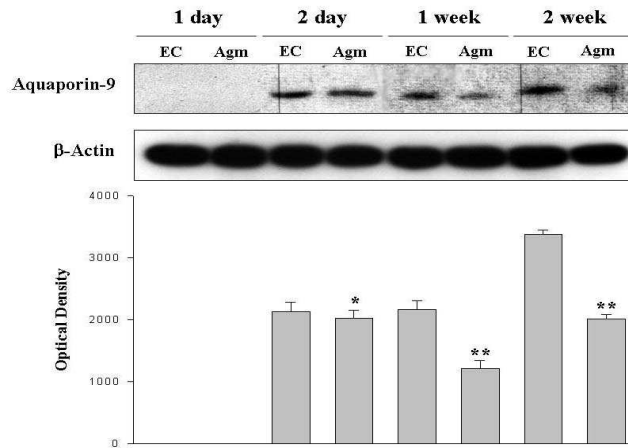
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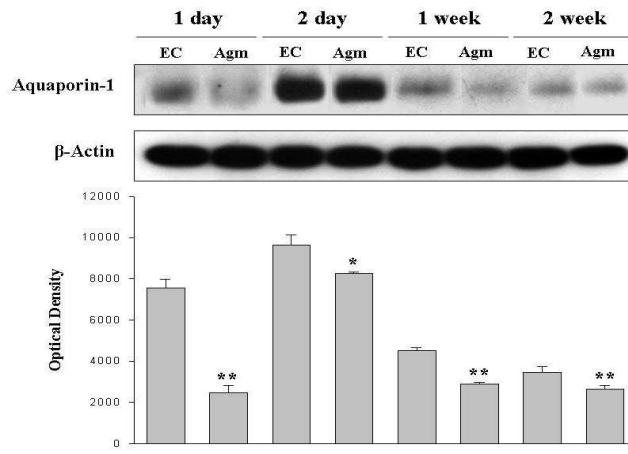
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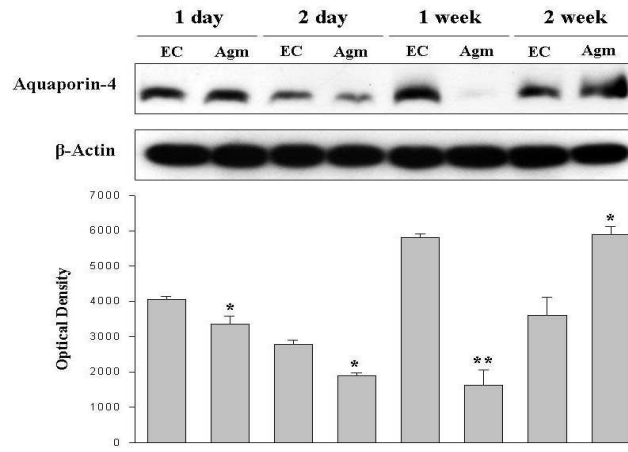
C.



D.



E.



F.

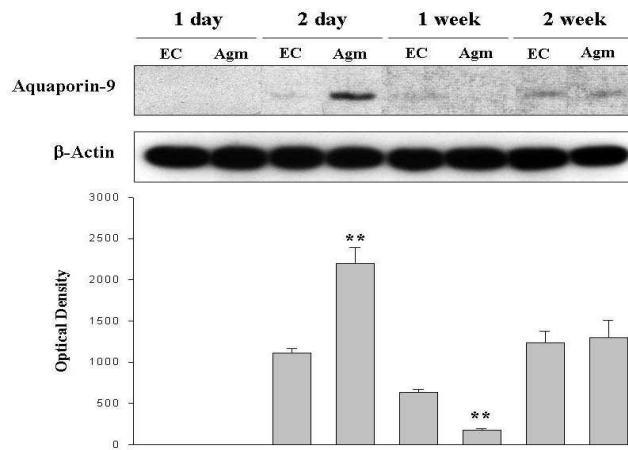


Figure 10. We examined the expression of AQP-1,-4 and -9 using immunoblotting in cerebral cortex (A, B, C) and hippocampus (D, E, F). In all time point, aquaporin-1 was decreased in agmatine treatment group compared to experimental control after TBI. Aquaporin -4, -9 were similar expressed in agmatine treatment group and experimental control at 1, 2 day after TBI. But 1 week after TBI, the expressions of aquaporin -4,-9 were decreased in agmatine treatment group. (*, $P < 0.05$, **, $P < 0.01$ vs EC) EC, Experimental control group; Agm, Agmatine treatment group.

10. The expression of bone morphogenetic protein (BMP)-7

BMP-7 is induces differentiation in astrocyte lineage cells and induces dendritic growth. In the expression of BMP-7 promotes survival of neurons and glial cells after TBI⁵⁴. So we performed immunoblotting to examined the expression of BMP-7 after TBI by agmatine.

In cerebral cortex, the expression of BMP-7 was similar (Figure 11A).

In hippocampus, agmatine increased the expression of BMP-7 from 1 day to 1 week after TBI in agmatine treatment group (Figure 11B).

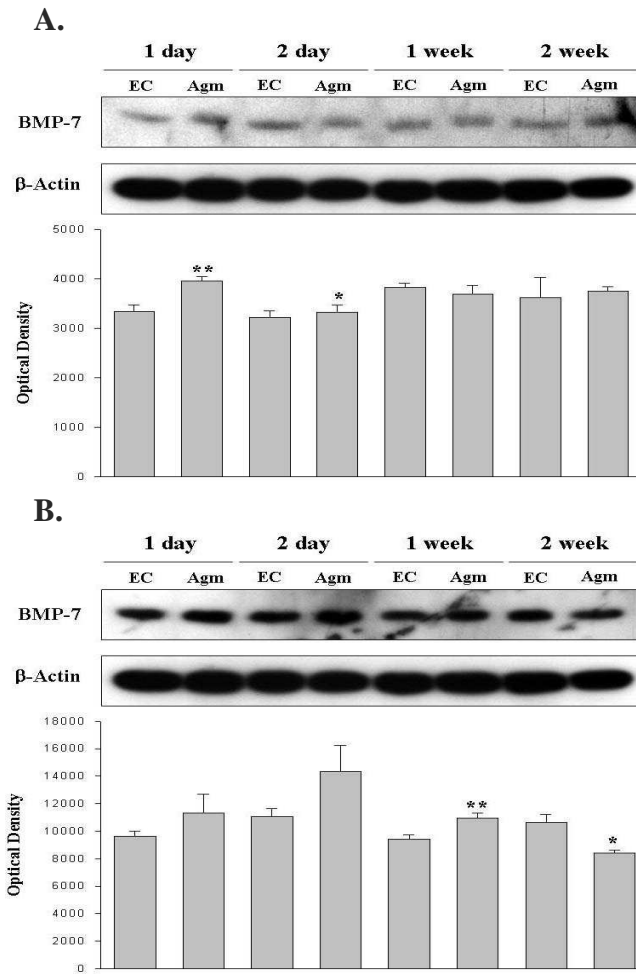


Figure 11. Increased expression of BMP-7 from 1day to 1week after TBI in agmatine treatment group. Figure shows that BMP-7 was similarly expressed in cerebral cortex (A) but in hippocampus, BMP-7 was increased from 1day to 1week after TBI in agmatine treatment group hippocampus. (*, $P < 0.05$, **, $P < 0.01$ vs EC) EC, Experimental control group; Agm, Agmatine treatment group.

IV. DISCUSSION

Following previously reports, agmatine decreased infarct sizes in mouse ischemia model, and promotes survival in neurons exposed OGD⁸. Agmatine has been shown to reduce excitotoxicity in vitro by blocking NMDA receptor activation^{1,10} and to protect neurons from injury² by inhibiting of NOS⁸. To investigate the neuroprotective effect of agmatine on brain traumatic injury, cold injury has been used in this study. Traumatic brain injury is a complex process which include primary, secondary or additional injury, and regeneration⁵⁸. Secondary injury mechanisms include complex biochemical and physiological processes, which are initiated by the primary insult and manifest over a period of hours to days⁵⁹. The initial disturbances caused by membrane damage lead to cell death, but the molecular mechanisms mediating apoptosis are poorly understood. In this study, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling), a method commonly used to detect apoptotic cell death was performed to determining the numbers of dying cells. Because it is possible that cells undergoing necrotic cell death could also be detected apoptosis. Therefore, TUNEL positive means a 'dying' cell. In this study TUNEL positive cells were decreased in agmatine treatment group than experimental control from 2days to 2weeks after TBI. It is suggested that agmatine protect neurons from cold injury.

Recent studies have shown that MAPKs is implicated in the progression of acute brain damage after ischemia or trauma. For example, after serum withdrawal and oxidative stress in cerebellar neuronal cultures, the ERK pathway mediates protective effects and the p38/JNK pathways mediate deleterious effects²⁹. ERK has been shown to be involved in preconditioning responses that protect against secondary insults³⁰, and has been associated with neuroprotection in global cerebral ischemia¹⁶. For the most part, these protective effects of ERK appear to be related to

the transcriptional upregulation of neuroprotective antioxidant systems³⁰. In this study, it is shown that the expression of p-ERK was increased and p-JNK was decreased at 1, 2 days after TBI in agmatine treatment group. Also, p-p38 is decreased in all time point in agmatine treatment group. It may suggest that increased p-ERK and decreased p-JNK expression mediate protective effect by agmatine.

TBI models have shown increased mRNA expression of IL-1 β and TNF- α in hippocampus early after head injury²³. Local induction of TNF- α and IL-6 mRNA expression as well as intrathecal release of these cytokines have been demonstrated in various head trauma models such as experimental cortical contusion^{31,32,33}, surgical brain injury^{34,35,36}, fluid percussion trauma^{37,38,39}, and experimental axonal injury⁴⁰. Furthermore, evidence from *in vivo* and *in vitro* studies shows that administration of recombinant TNF can induce intracranial inflammation and a breakdown of the blood-brain barrier (BBB), a pathophysiological hallmark of neurotrauma^{41,42,43,44}. Production and release of cytokines depend on inducible gene expression mediated by activation of cell signalling. The primary inflammatory stimulus may act through membrane receptors such as TNF- α receptor (TNFR)1 and TNFR2, causing the activation of four major intracellular signalling cascades: the three distinct MAPKs pathways (p38, JNK and ERK) and the pathway leading to activation of nuclear factor kB (NF-kB)⁶⁵. MAPKs are strongly activated by stress stimuli, bacterial lipopolysaccharide (LPS) and cytokines, and have been suggested to contribute to cell death and survive.

The NF-kB regulates various genes involved in immunoresponses, cell proliferation and apoptosis. NF-kB triggers a number of anti-apoptotic genes which interrupt the apoptotic cascade at multiple levels^{66,67}, and a pivotal role of NF-kB in the regulation of cell survival and death has therefore been suggested. Although findings reported are controversial, strong evidence

supports the notion that NF- κ B functions as an anti-apoptotic transcription factor in various cell populations including neurons^{60,61,62}. In this study, it is shown that the expression of TNF- α and IL-1 β were decreased in agmatine treatment group after TBI. This result suggested agmatine has anti-inflammatory effect in cold TBI.

Brain edema is concomitant with brain dysfunction and is explained by several pathophysiological mechanisms. Because the brain is enclosed in a rigid skull, brain swelling produces displacement of water from low-pressure compartments, including CSF and venous blood (~10 mmHg), into peripheral blood. Severe brain edema increases intracranial pressure and causes a decrease of cerebral blood perfusion, resulting in secondary brain injury⁶³. In all brain edema types, excess fluid leaves the brain parenchyma along three different routes: across the blood-brain barrier into the bloodstream, across the ependyma into the ventricles, and across the glia limiting membrane into the CSF in the subarachnoid space. However, the mechanisms of edema fluid clearance are less well understood than the mechanisms of edema fluid formation.

Aquaporins (AQPs) are a family of water channel proteins that facilitate the diffusion of water through the plasma membrane⁴⁵. In the rodent brain, three aquaporins have been clearly identified, AQP-1, AQP-4, and AQP-9^{46,47}. AQP-1 has been detected in epithelial cells of the choroid plexus⁴⁸, AQP-4 in astrocytes with a polarization on astrocyte endfeet⁴⁹, and AQP-9 in astrocytes of the white matter and in catecholaminergic neurons^{50,51}. AQP-1 and AQP-4 are permeable only to water and are presumed to be involved in cerebrospinal fluid formation and brain water homeostasis⁵². AQP-9 is an aquaglyceroporin, a subgroup of the aquaporin family, and is permeable to water and also glycerol, monocarboxylates, and urea⁵³. These three channels may be implicated in water movements occurring during the formation and resolution of cerebral edema. In this study, it is shown that the expression of

AQP-1,4 and -9 were decreased after TBI in agmatine treatment group. It is suggested that agmatine attenuate brain edema through lessening the expression of aquaporins after TBI.

In this study, agmatine protect cold injured brain by regulating the MAPKs signaling and the expression of TNF- α and IL-1 β . It is also shown that agmatine decrease brain edema through regulating the aquaporin expression. Furthermore agmatine enhanced neuronal survival itself in H-E or TUNEL stained brain tissues from this study. Based on these results, it was hypothesized that agmatine may have a modulatory effect on neural homeostasis or cell survival in TBI. Bone Morphogenetic Proteins (BMPs) are growth factors which are thought to be involved in multiple aspects of cell signaling and homeostasis, including germ cell formation, stem cell maintenance and cell differentiation from brain injury⁶⁴. BMP-7 is a member of the BMP subfamily of the TGF- β superfamily⁵⁴. The expression of BMP-7 mRNA is reported to be increased in CNS injury^{54,55}. BMP-7 is announced to improve functional recovery, local cerebral glucose utilization and blood flow after cerebral ischemia⁵⁶, and to improve locomotor function after stroke⁵⁷. In this study, agmatine increased the expression of BMP-7 from 1day to 1week after TBI in agmatine treatment group. These results suggests the possibility to increase the number of forepaw step through the enhancing of BMP-7 expression by agmatine after TBI.

This study showed that agmatine increased the expression of p-ERK and BMP-7, and decreased the expression of p-JNK, p-p38, AQPs, TNF- α and IL-1 β . These effects of agmatine on TBI were associated with the increase of activated MAPKs expression through Nf-kB translocation into nucleus at 1 and 2 days after TBI.

V. CONCLUSION

We hypothesized that agmatine may not only have neuroprotective effects through MAPKs activation and induction of anti-inflammation signaling, but also enhance cell survival through regulating of BMP7 expression after TBI.

1. The motor function was significantly increased in agmatine treatment group than in EC at 2 weeks after TBI.
2. Agmatine significantly reduced necrotic brain volume and number of eosinophilic neurons after TBI compared to experimental control.
3. The number of TUNEL positive cells was decreased in agmatine treatment group than experimental control group.
4. The expression of p-ERK was increased, whereas p-JNK and p-p38 were decreased by agmatine.
5. The expression of inflammatory cytokines were clearly reduced after TBI by agmatine.
6. Agmatine increased Nf-kB p65 nuclear translocation after TBI.
7. The expression of AQP-1,-4 and -9 was decreased after TBI by agmatine treatment.
8. The expression of BMP-7 was increased in agmatine treatment group than in experimental control after TBI.

These data suggest that agmatine reduced necrotic volume and number of eosinophilic neurons. And agmatine regulate MAPKs expression via Nf-kB nuclear translocation and have anti-inflammatory effect. Moreover, agmatine could attenuate brain edema through lessening the expression of aquaporins and propose that agmatine could support CNS regeneration by increasing the expression of BMP-7 after TBI. This study addresses the neuroprotective and neuroregenerative effect of agmatine after TBI.

REFERENCES

1. Yang XC, Reis DJ. Agmatine selectively blocks the N-methyl-D-aspartate subclass of glutamate receptor channels in rat hippocampal neurons. *J Pharmacol Exp Ther* 1999; 288: 544-9.
2. Li G, Regunathan S, Barrow, CJ, Eshraghi J, Cooper R, Reis DJ. Agmatine: an endogenous clonidine-displacing substance in the brain. *Science* 1994; 263(5149): 966-9.
3. Piletz JE, Chikkala DN, Ernsberger P. Comparison of the properties of agmatine and endogenous clonidine-displacing substance at imidazoline and alpha-2 adrenergic receptors. *J Pharmacol Exp Ther* 1995; 272(2): 581-7.
4. Reynolds IJ. Arcaine uncovers dual interactions of polyamines with the N-methyl-D-aspartate receptor. *J Pharmacol Exp Ther* 1990; 255: 1001-9.
5. Feng Y, Piletz JE, Leblanc MH. Agmatine suppresses nitric oxide production and attenuates hypoxic-ischemic brain injury in neonatal rats. *Pediatr Res* 2002; 52(4): 606-11.
6. Gilad GM, Gilad VH. Accelerated functional recovery and neuroprotection by agmatine after spinal cord ischemia in rats. *Neurosci Lett* 2000; 296(2-3): 97-100.
7. Gilad GM, Salame K, Rabey JM, Gilad VH. Agmatine treatment is neuroprotective in rodent brain injury models. *Life Sci* 1996; 58: 41-6.
8. 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(1): 122-30.
9. Yu CG, Marcillo Ae, Bairbanks CA, Wilcox GL, Yeziarski RP. Agmatine improves locomotor function and reduces tissue damage following spinal cord injury. *NeuroReport* 2000; 11(14): 3203-7

10. Olmos G., DeGregorio-Rocasolano N, Paz Regalado M, Gasull T, Assumpcio Boronat M, Trullas R, Villarroel A, Lerma J, Garcia-Sevilla JA. Protection by imidazol(ine) drugs and agmatine of glutamate-induced neurotoxicity in cultured cerebellar granule cells through blockade of NMDA receptor. *Br J Pharmacol* 1999; 127(6): 1317-26.
11. Guyton, K. Z., Liu, Y., Gorospe, M., Xu, Q., and Holbrook, N. J. Activation of mitogen-activated protein kinase by H₂O₂. Role in cell survival following oxidant injury. *J. Biol. Chem.* 1996; 271: 4138-42
12. Stanciu, M., Wang, Y., Kentor, R., Burke, N., Watkins, S., Kress, G., Reynolds, I., Klann, E., Angiolieri, M. R., Johnson, J. W., et al. Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. *J. Biol. Chem.* 2000; 275: 12200-6
13. McIntosh, T. K., Juhler, M., and Wieloch, T. Novel pharmacological strategies in the treatment of experimental traumatic brain injury. *J. Neurotrauma* 1998; 15: 731-76
14. Alessandrini, A., Namura, S., Moskowitz, M. A., and Bonventre, J. V. MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia. *Proc. Natl. Acad. Sci.* 1999; 96: 12866-9
15. Herdegen, T., Claret, F. X., Kallunki, T., Martin-Villalba, A., Winter, C., Hunter, T., and Karin, M. Lasting N-terminal phosphorylation of c-Jun and activation of c-Jun Nterminal kinases after neuronal injury. *J. Neurosci.* 1988; 18: 5124-35
16. Hu, B. R., Liu, C. L., and Park, D. J. Alteration of MAP kinase pathways after transient forebrain ischemia. *J. Cereb. Blood Flow Metab.* 2000; 20: 1089-95
17. Mori, T., Wang, X., Jung, J. C., Sumii, T., Singhal, A. B., Fini, M. E., Dixon, C. E., Alessandrini, A., and Lo, E. H. Mitogen-activated protein kinase inhibition in traumatic brain injury: in vitro and in vivo effects. *J. Cereb. Blood Flow Metab.* 2002; 22: 444-52
18. Otani, N., Nawashiro, H., Fukui, S., Nomura, N., Yano, A., Miyazawa, T., and Shima, K. Differential activation of mitogen-activated protein kinase pathways after traumatic brain injury in the rat hippocampus. *J. Cereb. Blood Flow*

Metab. 2002; 22: 3213-34

19. Sugino, T., Nozaki, K., Takagi, Y., Hattori, I., Hashimoto, N., Moriguchi, T., and Nishida, E. Activation of mitogen-activated protein kinases after transient forebrain ischemia in gerbil hippocampus. *J. Neurosci.* 2000; 20: 4506-14
20. Walton, K. M., DiRocco, R., Bartlett, B. A., Koury, E., Marcy, V. R., Jarvis, B., Schaefer, E. M., and Bhat, R. V. Activation of p38 MAPK in microglia after ischemia. *J. Neurochem.* 1998; 70: 1764-7
21. Wang, X., Mori, T., Jung, J. C., Fini, M. E., and Lo, E. H. Secretion of matrix metalloproteinase-2 and -9 after mechanical trauma injury in rat cortical cultures and involvement of MAP kinase. *J. Neurotrauma* 2002; 19: 615-25
22. Ozawa, H., Shioda, S., Dohi, K., Matsumoto, H., Mizushima, H., Zhou, C. J., Funahashi, H., Nakai, Y., Nakajo, S., and Matsumoto, K. Delayed neuronal cell death in the rat hippocampus is mediated by the mitogen-activated protein kinase signal transduction pathway. *Neurosci. Lett.* 1999; 262: 57-60
23. Tehrani R, Andell-Jonsson S, Beni SM, Yatsiv I, Shohami E, Bartfai T, Lundkvist J, Iverfeldt K, Improved recovery and delayed cytokine induction after closed head injury in mice with central overexpression of the secreted isoform of the interleukin-1 receptor antagonist. *J Neurotrauma.* 2002; 19(8):939-51
24. Murakami K, Kondo T, Yang G, Chen SF, Morita-Fujimura Y, Chan PH, Cold injury in mice: a model to study mechanisms of brain edema and neuronal apoptosis. *Prog Neurobiol.* 1999; 57: 289-99.
25. S. Davis, P. Vanhoutte, C. Pages, J. Caboche, S. Laroche, The MAPK/ERK cascade targets Elk-1 and camp response element binding protein to control long-term potentiation-dependent gene expression in the dentate gyrus in vivo. *J. Neurosci.* 2000; 20: 4563-72.
26. J.M. Daun, M.J. Fenton, Interleukin-1/Toll receptor family members: receptor structure and signal transduction pathways, *J. Interferon Cytokine Res.* 2000; 20: 843-55.
27. J. Ninomiya-Tsuji, K. Kishimoto, A. Hiyama, J. Inoue, Z. Cao, K. Matsumoto, The kinase TAK-1 can activate the NIK-IB as well as the MAP kinase cascade

in the IL-1 signaling pathway, *Nature* 1999; 398: 252-6.

28. N. Otani, H. Nawashiro, S. Fukui, N. Nomura, A. Yano, T. Miyazawa, K. Shima, Differential activation of mitogen-activated protein kinase pathways after traumatic brain injury in the rat hippocampus, *J. Cereb. Blood Flow Metab.* 2002; 22: 327-44.
29. Xia Z, Dickens M, Rainegaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK/p38 MAP kinase on apoptosis. *Science* 1995; 270: 326-31
30. Gonzalez-Zulueta M, Feldman AB, Klesse LJ, Kalb RG, Dillman JF, Parada LF, Dawson TM, Dawson VL. Requirement for nitric oxide activation of p21(ras)/extracellular regulated kinase in neuronal ischemic preconditioning. *Proc Natl Acad Sci* 2000; 97: 436-41
31. Shohami E, Novikov M, Bass R, Yamin A, Gallily R. Closed head injury triggers early production of TNF α and IL-6 by brain tissue. *J Cereb Blood Flow Metab* 1994; 14: 615-9
32. Shohami E, Bass R, Wallach D, Yamin A, Gallily R. Inhibition of tumor necrosis factor alpha (TNF α) activity in rat brain is associated with cerebroprotection after closed head injury. *J Cereb Blood Flow Metab* 1996; 16: 378-84
33. Holmin S, Schalling M, Höjeberg B, Sandberg Nordqvist AC, Skeftruna AK, Mathiesen T. Delayed cytokine expression in rat brain following experimental contusion. *J Neurosurg* 1997; 86: 493-504.
34. Woodroffe MN, Sarna GS, Wadhwa M, Hayes GM, Loughlin AJ, Tinker A, Cuzner ML. Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: evidence of a role for microglia in cytokine production. *J Neuroimmunol* 1991; 33: 227-36.
35. Yan HQ, Alcaros Banos M, Herregodts P, Hooghe R, Hooghe-Peters EL. Expression of interleukin (IL)-1 α , IL-6 and their respective receptors in the normal rat brain and after injury. *Eur J Immunol* 1992; 22: 2963-71
36. Tchelingierian JL, Quinonero J, Booss J, Jacque C. Localization of TNF α

and IL-1 immunoreactivities in striatal neurons after surgical injury to the hippocampus. *Neuron* 1993; 10: 213–24

37. Taupin V, Toulmond S, Serrano A, Benavides J, Zavala F. Increase in IL-6, IL-1 and TNF levels in rat brain following traumatic lesion. *J Neuroimmunol* 1993; 42: 177–86
38. Fan L, Young PR, Barone FC, Feuerstein GZ, Smith DH, McIntosh TK. Experimental brain injury induces differential expression of tumor necrosis factor- α mRNA in the CNS. *Mol Brain Res* 1996; 36: 287–91
39. Knoblauch SM, Fan L, Faden AI. Early neuronal expression of tumor necrosis factor- α after experimental brain injury contributes to neurological impairment. *J Neuroimmunol* 1999; 95: 115–25
40. Hans VHJ, Kossmann T, Lenzlinger PM, Probstmeier R, Imhof HG, Trentz O, Morganti-Kossmann MC. Experimental axonal injury triggers interleukin-6 mRNA, protein synthesis and release into cerebrospinal fluid. *J Cereb Blood Flow Metab* 1999; 19: 184–94
41. Ramilo O, Saez-Llorens X, Mertsola J, Jafari H, Olsen KD, Hansen EJ. Tumor necrosis factor alpha/cachectin and interleukin 1 beta initiate meningeal inflammation. *J Exp Med* 1990; 172: 497–507
42. Kim KS, Wass CA, Cross AS, Opal SM. Modulation of blood-brain barrier permeability by tumor necrosis factor and antibody to tumor necrosis factor in the rat. *Lymphokine Cytokine Res* 1992; 11: 293–8
43. Megyeri P, Abraham CS, Temesvari P, Kovacs J, Vas T, Speer CP. Recombinant human tumor necrosis factor alpha constricts pial arterioles and increases blood-brain barrier permeability in newborn piglets. *Neurosci Lett* 1992; 148: 137–40
44. de Vries HE, Blom-Roosemalen MCM, van Oosten M, de Boer AG, van Berkel TJC, Breimer DD, Kuiper J. The influence of cytokines on the integrity of the blood-brain barrier in vitro. *J Neuroimmunol* 1996; 64: 37–43
45. Agre P, King LS, Yasui M, Guggino WB, Ottersen OP, Fujiyoshi Y, et al. Aquaporin water channels—from atomic structure to clinical

- medicine. *J Physiol (Lond)*. 2002; 542: 3–16.
46. Badaut J, Lasbennes F, Magistretti PJ, Regli L. Aquaporins in brain: distribution, physiology, and pathophysiology. *J Cereb Blood Flow Metab* 2002; 22: 367-78.
 47. Agre P, Nielsen S, Ottersen OP. Towards a molecular understanding of water homeostasis in the brain. *Neuroscience* 2004; 129(4): 849-50.
 48. Nielsen S, Smith BL, Christensen EI, Agre P. Distribution of the aquaporin CHIP in secretory and resorptive epithelia and capillary endothelia. *Proc Natl Acad Sci USA* 1993; 90: 7275–79.
 49. Nielsen S, Nagelhus EA, Amiry-Moghaddam M, Bourque C, Agre P, Ottersen OP. Specialized membrane domains for water transport in glial cells: high-resolution immunogold cytochemistry of aquaporin-4 in rat brain. *J Neurosci* 1997; 17: 171–80.
 50. Badaut J, Hirt L, Granziera C, Bogousslavsky J, Magistretti PJ, Regli L. Astrocyte-specific expression of aquaporin-9 in mouse brain is increased after transient focal cerebral ischemia. *J Cereb Blood Flow Metab* 2001; 21: 477–82.
 51. Badaut J, Petit JM, Brunet JF, Magistretti PJ, Charriaut-Marlangue C, Regli L. Distribution of Aquaporin 9 in the adult rat brain: preferential expression in catecholaminergic neurons and in glial cells. *Neuroscience* 2004; 128(1): 27-38.
 52. Amiry-Moghaddam M, Ottersen OP. The molecular basis of water transport in the brain. *Nat Rev Neurosci* 2003; 4: 991–1001.
 53. Badaut J, Regli L. Distribution and possible roles of aquaporin 9 in the brain. *Neuroscience* 2004; 129: 971–981.
 54. Setoguchi T, Yone K, Matsuoka E, Takenouchi H, Nakashima K, Sakou T, Komiya S, Izumo S. Traumatic injury-induced BMP7 expression in the adult rat spinal cord. *Brain Res* 2001; 921: 219–25.

55. Chang CF, Lin SZ, Chiang YH, Morales M, Chou J, Lein P, et al. Intravenous administration of bone morphogenetic protein-7 after ischemia improves motor function in stroke rats. *stroke* 2003; 34: 558-64.
56. Liu Y, Belayev L, Zhao W, Busto R, Saul I, Alonso O, Ginsberg MD. The effect of bone morphogenetic protein-7 (BMP-7) on functional recovery, local cerebral glucose utilization and blood flow after transient focal cerebral ischemia in rats. *Brain Res* 2001; 905(1-2): 81-90.
57. Harvey BK, Hoffer BJ, Wang Y. Stroke and TGF-beta proteins: glial cell line-derived neurotrophic factor and bone morphogenetic protein. *Pharmacol Ther.* 2005; 105(2): 113-25.
58. Reilly PL. Brain injury: the pathophysiology of the first hours. "Talk and Die revisited." *J Clin Neurosci* 2001; 8: 398-403.
59. McIntosh TK, Smith DH, Meaney DF, Kotapka MJ, Gennarelli TA, Graham DI. Neuropathological sequelae of traumatic brain injury: relationship to neurochemical and biomechanical mechanisms. *Lab Invest* 1996; 74: 315-42.
60. Kaltschmidt B, Uherek M, Volk B, Kaltschmidt C. Inhibition of NF-kB potentiates amyloid beta-mediated neuronal apoptosis. *Proc Natl Acad Sci* 1999; 96: 9409-14.
61. Glazner GW, Camandola S, Mattson MP. Nuclear factor-kB mediates the cell survival-promoting action of activity-dependent neurotrophic factor peptide-9. *J Neurochem* 2000; 75: 101-8.
62. Mattson MP, Culmsee C, Yu ZF, Camandola S. Role of nuclear factor kB in neuronal survival and plasticity. *J Neurochem* 2000; 74: 443-456.
63. KENSUKE MURAKAMI, TAKEO KONDO, GUOYUAN YANG, SYLVIA F. CHEN, YUIKO MORITA-FUJIMURA, PAK H. CHAN. *Progress in Neurobiology.* 1999; 57: 289-99
64. Urist MR, Lindholm TS, Mirra JM, Grant TT, Finerman GA. Growth of

osteoid osteoma transplanted into athymic nude mice. *Clin Orthop Relat Res.* 1979; 141: 275-80.

65. Saklatvala J, Dean J, Clark A. Control of the expression of inflammatory response genes. *Biochem Soc Symp.* 2003; 70: 95-106.
66. Mattson MP, Culmsee C, Yu Z, Camandola S. Roles of nuclear factor kappaB in neuronal survival and plasticity. *J Neurochem.* 2000 ; 74(2): 443-56.
67. Karin M, Lin A. NF-kappaB at the crossroads of life and death. *Nat Immunol.* 2002; 3(3): 221-27.

Abstract (in Korean)

외상성 뇌손상후 아그마틴에 의한
Mitogen-activated protein kinase(MAPKs) 활성화

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이 용 우

아그마틴은 엘-알기닌의 탈탄소반응에 의해 형성된 일차아민으로서 동물의 뇌에서 합성되어지는 내재성 clonidine 대체 물질이다. 본 연구에서는 외상성 뇌손상후 아그마틴에 의한 Mitogen-activated protein kinase(MAPKs) 활성화에 대해 알아보았다. 외상 2주후, 트레이드밀을 이용한 행동학적 검사결과에서 아그마틴을 투여한 동물군이 실험대조군에 비해서 기능적 회복이 일어남을 알 수 있었다. 아그마틴은 뇌에서 괴사가 일어난 부분과 eosinophilic 신경세포의 수, 그리고 TUNEL-positive 세포의 수를 감소시켰다. 아그마틴에 의해 p-ERK의 발현이 증가하는 반면 p-JNK 와 p-p38의 발현은 감소하였다. 염증성 cytokine의 발현도 실험대조군에 비해서 아그마틴을 투여한 동물군에서 감소되었다. 이러한 결과들은 Nf-kB의 핵으로의 전이가 아그마틴에 의해 증가되는 것과 관련됨이 관찰되었다. 뇌부종의 주요원인 물질이며 수분이동통로인 AQP의 발현도 아그마틴의 투여로 감소됨을 확인하였다. 또한 신경보호와 신경재생에 효과가 있는 것으로 보고된 뼈 형태형성 단백질인 BMP-7의 발현이 아그마틴을 투여함으로써 그 발현이 실험대조군에 비해 증가됨을 관찰하였다.

이러한 결과로부터 외상성 뇌 손상에서 아그마틴에 의한 신경보호

효과는 Nf-kB의 핵으로의 전이가 아그마틴에 의해 증가됨으로써 MAPKs를 활성화 시키는 것과 관련됨을 알 수 있었다.

핵심 되는 말 : 아그마틴, 외상성 뇌손상, MAPKs, 염증성 cytokine, 수분 이동통로 (AQP), 뼈 형태형성 단백질 (BMP)