The role of agmatine in CNS injury

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The role of agmatine in CNS injury

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Abstract

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There is no optimal therapy for CNS injury. Many therapeutic candidates have been investigated until now. Agmatine is a primary amine formed by the decarboxylation of L-arginine synthesized in mammalian brain. The purpose of this study was to demonstrate the role of agmatine in CNS injury.

First, in brain edema following cerebral ischemia, agmatine significantly reduced brain swelling volume 22 hours after 2 hours middle cerebral artery occlusion (MCAO). Water content in brain tissue was clearly decreased 24 hours after ischemic injury by agmatine treatment. It is confirmed that blood-brain barrier (BBB) disruption was markedly lessened in the striatal, hippocampal, and cerebral cortical area of agmatine treatment group than experimental control, using evans blue extravasation. The expression of matrix metalloproteinase (MMP) -2 and -9, correlated with the disruption of BBB, was reduced by agmatine treatment. The expression of aquaporin (AQP) -1

and -4, correlated with brain edema as water channels, was plainly decreased by agmatine treatment.

Second, in spinal cord injury (SCI), agmatine is validated to improve the physiological condition 4 weeks after SCI, using overturning body test. Collagen scar area, physical barrier to axon regeneration, was surely diminished by agmatine treatment 4 weeks after SCI. Agmatine treatment increased the expression of bone morphogenetic protein (BMP) -7, which is neuroprotective and neuroregenerative, in scar region, proximal region to scar and distal to scar more than experimental control in early period after SCI. Agmatine also decreased the TGFβ-2 positive cells in all regions- distal to scar, proximal to scar, and scar compared to experimental control 1 week and 2 weeks after SCI. TGFβ-2 is correlated with deposition of collagen matrix at the lesion site.

These data suggest that agmatine could attenuate brain edema through reducing disruption of the BBB by suppression of the expression of MMP-2 and -9 and through lessening the expression of AQP-1 and -4 and that agmatine could support CNS regeneration by reducing the collagen scar area, decreasing the expression of TGFβ-2 and increasing the expression of BMP-7 in spinal cord injury. Consequently, this study addressed the neuroprotective and neuroregenerative effect of agmatine in CNS injury.

Key words : Agmatine, Cerebral ischemia, Brain edema, Aquaporins, Matrix metalloproteinases, Blood-brain barrier, Spinal cord injruy, Collagen scar, Transforming growth factor, Bone morphogenetic protein-7

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PART 1. The effect of agmatine for brain edema in cerebral ischemia

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 agmatinase[1]. Recently, agmatine, ADC, and agmatinase were found in mammalian brain[2]. 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 pore[10]. Despite this work, the mode and site(s) of action for agmatine in the brain have not been fully defined. Nitric oxide synthases (NOSs) generate nitric oxide (NO) by sequential oxidation of the guanidino group in arginine, and agmatine is an arginine analogue with a guanidino group. Being structurally similar to arginine, agmatine has been known as a competitive inhibitor of NOS[11,12]. This suggests that agmatine may protect the brain from ischemic injury by interfering with NO signaling[8].

Stroke is one of the leading causes of death in most developed countries with its incidence increasing worldwide[13,14]. The majority of these strokes are ischemic and are caused in most cases by thrombotic or embolic occlusion of a cerebral artery[15]. In stroke, the extracellular matrix that supports microvascular homeostasis and integrity[16,17] are degraded by a variety of proteolytic enzymes, including a family of proteases known as matrix metalloproteinase (MMP), which are divided into five classes[16]. Increasing evidence indicates that MMP-2 and -9 are up-regulated after the onset of focal ischemia with[18,19,20,21,22,23] or without[17,18,24] reperfusion in experimental animals, as well as in human patients[25,26]. The early appearance of activated MMP-2 or -9 is associated with an alteration of blood-brain barrier (BBB) permeability and the formation of vasogenic edema after transient focal cerebral ischemia[19,22,24,27].

Edema is frequently observed in brain ischemia. Brain edema, defined as an abnormal increase in brain water content, which leads to an expansion of brain volume, has a crucial impact on morbidity and mortality after stroke, in that it increases intracranial pressure, favors herniations, and contributes to additional ischemic injuries[28]. Aquaporins (AQPs) are a family of water channel proteins that facilitate the diffusion of water through the plasma

membrane[29]. In the rodent brain, three aquaporins have been clearly identified, AQP-1, AQP-4, and AQP-9[30,31]. AQP-1 has been detected in epithelial cells of the choroid plexus[32], AQP-4 in astrocytes with a polarization on astrocyte endfeet[33], and AQP-9 in astrocytes of the white matter and in catecholaminergic neurons [34,35]. AQP-1 and AQP-4 are permeable only to water and are presumed to be involved in cerebrospinal fluid formation and brain water homeostasis[36]. AQP-9 is an aquaglyceroporin, a subgroup of the aquaporin family, and is permeable to water and also glycerol, monocarboxylates, and urea[37]. These three channels may be implicated in water movements occurring during the formation and resolution of cerebral edema after ischemia.

Based on these evidences, it was hypothesized that agmatine may have neuroprotective effect on brain edema. The purpose of the present study was to investigate the effect of agmatine for brain edema in ischemic brain damage and to evaluate on the expression of AQP-1and -4 and on the expression of MMP-2 and -9.

II. MATERIALS AND METHODS

1. Animals

ICR mice from Sam (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. Stroke model

Male ICR mice weighing 36 ± 2 g were subjected to transient middle cerebral artery occlusion (MCAO, *n* = 50). Animals were anesthetized with chloral hydrate (400 mg/kg) intraperitoneally. Depth of anesthesia was assessed by toe pinch every 15 min. In a separate set of animals (*n* = 5 agamatine-treated, *n* = 5 control), a femoral arterial line was placed and physiological parameters including mean arterial blood pressure, arterial blood gases, and rectal temperature were monitored before, during, and after ischemia. Ischemia was induced using an occluding intraluminal suture as previously described[38,39]. In brief, an uncoated 15-mm segment of 6–0 nylon monofilament suture with the tip rounded by a flame was inserted into the arteriotomy and advanced under direct visualization into the internal carotid artery 11 mm from the bifurcation to occlude the ostium of the middle cerebral artery (MCA). After 2 h, the suture was withdrawn and surgical incisions were closed. Twenty-two hours later, the animals were euthanized with an isoflurane overdose. The brains were removed and 2-mm thick blocks were cut in the coronal plane, stained with triphenyl tetrazolium chloride (TTC, Sigma) to delineate regions of infarction, and embedded in paraffin. After paraffin embedding, 6-㎛ thick sections were stained with H&E and immunostained[38].

Agmatine was dissolved in normal saline (100 mg/kg IP, Sigma) and given after the suture was removed (Agm, *n* = 21). Controls received normal saline in equivalent volumes (EC, *n* = 18).

3. Assessment of brain edema and infarct volume.

Brain swelling and infarct volumes were determined by TTC staining, using a computer-assisted image analysis system (Optimas ver 6.1, Optimas, Bothell, WA, USA), and corrected for the presence of edema using previously

published methods[38,39]. The volume of infarct was expressed as a percentage of the total area of ipsilateral hemisphere.

4. Water content

Mice were killed 24 hours after reperfusion, at the time point of maximal brain edema formation. Brains were removed, hemispheres separated, and weighed to assess the wet weight (WW). Thereafter, the hemispheres were dried for 24 hours at 110 ℃ and the dry weight (DW) was determined[40]. Hemispheric water content (%) was calculated using the following formula: ((WW−DW) / WW) × 100(%)

5. Blood-brain barrier(BBB) disruption

The integrity of the blood–brain barrier was investigated using Evans blue extravasation[41,42,43]. Evans blue at 2 % in saline (100 ㎕) was injected in the tail vein and allowed to circulate for 90 min. The chest wall was then opened under chloral hydrate anesthesia (400 mg/kg, i.p.). Blood sample was obtained from the heart. Animals were perfused transcardially with saline at 100 mmHg pressure until blue color was absent of the effluent. Brains were removed, tissue samples (cortex, hippocampus, and striatum) were dissected out and weighed. They were homogenized in 500 ㎕ 50% trichloroacetic acid (weight/volume), and centrifuged (10,000 rpm, 20 minutes). The supernatant was measured at 445 nm using ELISA reader. Evans blue content of the plasma was similarly determined and the ratio of tissue to plasma Evans blue content was calculated as tissue Evans blue (㎍/g wet weight) / plasma Evans blue (㎍/g).

6. Immunohistochemical staining for AQPs and MMPs

Brains were fixed with 4 % paraformaldehyde, and embedded in paraffin. Brain sections were made by 10 ㎛. Sections were immunostained with antibodies against AQP-1 (Abcam, Cambridgeshire, UK), AQP-4, AQP-9, MMP-2, or MMP-9 (Chemicon, Temecula, CA, USA), 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.

7. Immunoblotting of AQPs and MMPs

Expression of AQP-1, -4, and -9 and MMP-2 and -9 proteins was estimated by immunoblotting in ischemic injured brain. Immunoblotting was performed using anti-AQPs, anti-MMPs, and anti-actin (Santa Cruz, Santa Cruz, CA, USA) antibodies. Equal amounts of protein, 100 ㎍ per condition, were separated on an 8 % polyacrylamide gel and electrotransferred onto Immobilon-NC membrane (Millipore, Bedford, MA, USA). Immunoreactive bands were visualized with the ECL detection system using Kodak X-AR film[8].

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 ± standard error of mean (SEM).

III. RESULTS

1. Assessment of brain edema and infarct volume

To investigate the effect of agmatine in ischemic damage, brain swelling volume was assessed in serial coronal sections of mouse brain. The data are summarized in Figure 1. The average total brain swelling volume (cortical plus subcortical areas) in experimental control group was 117.11 ± 2.37 % after 2 hours middle cerebral artery occlusion (MCAO) and 22 hours reperfusion. In agmatine treatment group, the average total brain swelling volume was 102.73 ± 0.16 % after 2 hours MCAO and 22 hours reperfusion. Agmatine significantly reduced brain swelling volume (14.38 ± 2.21 %, $P < 0.01$, Figure 1).

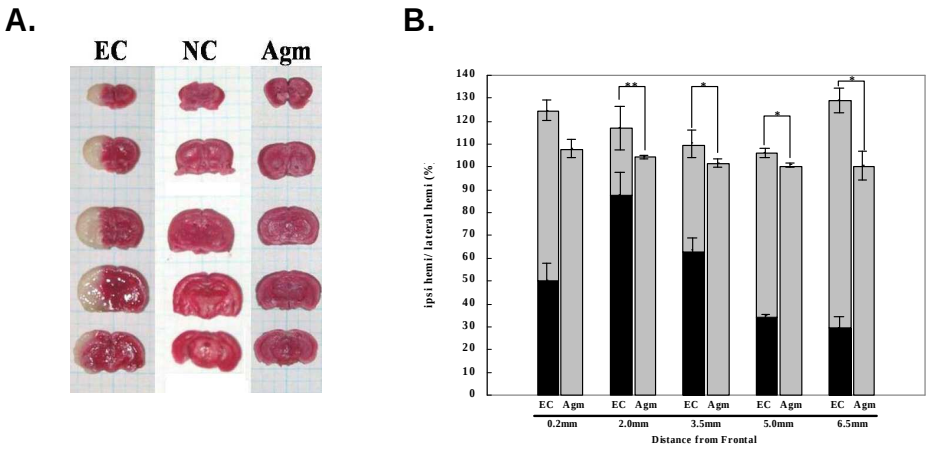

Figure 1. Brain edema and infarct volume on cerebral ischemia. Agmatine reduced infarct volume and brain swelling after ischemic injury. A. Serial coronal sections (1.5 mm of thickness) of mouse brain stained with 2 % TTC solution. B. Graph of brain swelling percent (%) 22 hours after 2 hours MCAO (*, $P < 0.05$; **, $P < 0.01$ vs EC). Data are expressed as the mean ± SEM. Gray bar, Non infarct area; Black bar, Infarct area; EC (n = 7), Experimental control group; NC (n = 4), Normal control group; Agm (n = 9), Agmatine treatment group

2. Water content

The water content in ischemic injured brains at 22 hours reperfusion is shown in Figure 2. In normal control group, water content averaged 79.63 ± 1.10 % in the ipsilateral hemispheres. Ischemia led to a significant increase in water content in the ipsilateral hemispheres (88.23 ± 0.59 %, P < 0.01, Figure 2). However, in agmatine treatment group, water content was significantly decreased in the ipsilateral hemispheres (80.61 ± 1.33 %, P < 0.01, Figure 2).

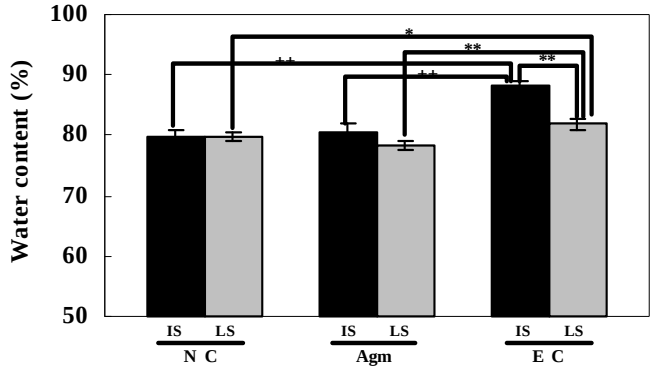

Figure 2. Brain water content on cerebral ischemia. Brain water content as a measure of brain edema of the ischemic hemisphere 22 hours after 2 hours MCAO. Agmatine decreased the water content of ischemic injured brain to normal level (*, P < 0.05; **, P < 0.01 vs LS of EC; ++, P < 0.01 vs IS of EC). Data are expressed as the mean ± SEM. EC (n = 3), Experimental control group; NC (n = 5), Normal control group; Agm (n = 4), Agmatine treatment group; IS, ipsilateral ischemic side; LS, contralateral ischemic side

3. The expression of AQPs

AQP-1 was expressed in only epithelial cells of choroid plexus in normal condition, but AQP-1 was strongly expressed in endothelial cells 22 hours after MCAO without agmatine. Aquaporin-1 positive endothelial cells were shown in cortex (Figure 3A) and striatum (Figure 3B) except penumbra (data not shown). The expression of AQP-1 were reduced in endothelial cells of cortex (Figure 3A), striatum (Figure 3B), and penumbra (data not shown) of agmatine treatment group. Agmatine treatment also decreased the expression of AQP-1 in blood vessels of the choroid plexus (Figure 3C).

The expression of AQP-4 was very feeble compared to that of AQP-1. Agmatine, however, reduced the expression of AQP-4 as ever (Figure 4). The protein expression of AQP-1 and AQP-4 was clearly diminished in agmatine treatment group, but the expression of AQP-9 was not clear (Figure 5).

4. Blood-brain barrier (BBB) disruption

It is needed to confirm whether the brain edema is vasogenic accompanied by BBB disruption or cytotoxic without BBB disruption. At 24 hours after ischemic injury, Evans blue contents in the striatal, hippocampal, and cerebral cortical area of agmatine treatment group (2.74 ± 0.307 in striatum; 3.08 ± 0.134 in hippocampus; 1.14 ± 0.042 in cerebral cortex) were significantly less than that of experimental control group (4.90 ± 0.120 in striatum; 4.24 ± 0.135 in hippocampus; 1.96 ± 0.110 in cerebral cortex, $P < 0.01$, Table 1). However, in the penumbra area, there is no difference between experimental control (2.44 ± 0.157) and agmatine treatment group (2.50 ± 0.145, Table 1).

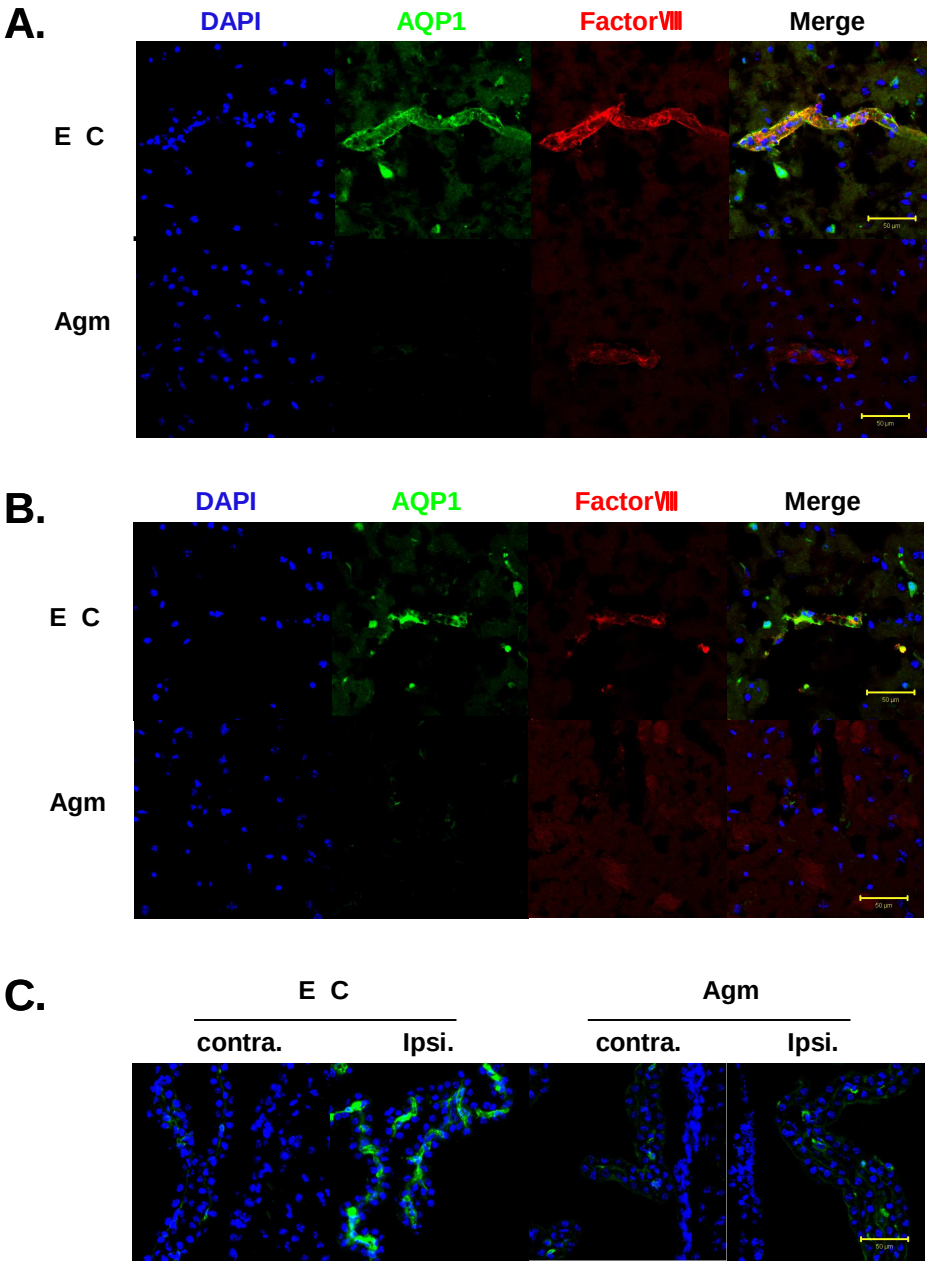
A.
DAPI
AQP1
FactorⅧ
Merge
E C
Agm
B.
DAPI
AQP1
FactorⅧ
Merge
E C
Agm
C.
E C
Agm
contra.
Ipsi.
contra.
Ipsi.

Figure 3. Macrographs of AQP-1 immunofluorescence in the ischemic injured brain 22 hours after 2 hours MCAO with and with out agmatine. Blood vessel marked with factorⅧ (red) was stained with AQP-1 (green) in cortex (A) and striatum (B) of experimental control (EC), but was not merged with AQP-1 (green) in cortex (A) and striatum (B) of agmatine treatment group (Agm). AQP-1 (green) was boldly detected at blood vessel of the choroid plexus of ipsilateral side (Ipsi) in experimental control (EC) but wasn't in agmatne treatment group (Agm) (C). Scale bar is 50 ㎛. EC, Experimental control group; Agm, Agmatine treatment group; Contra, Contralateral side; Ipsi, Ipsilateral side

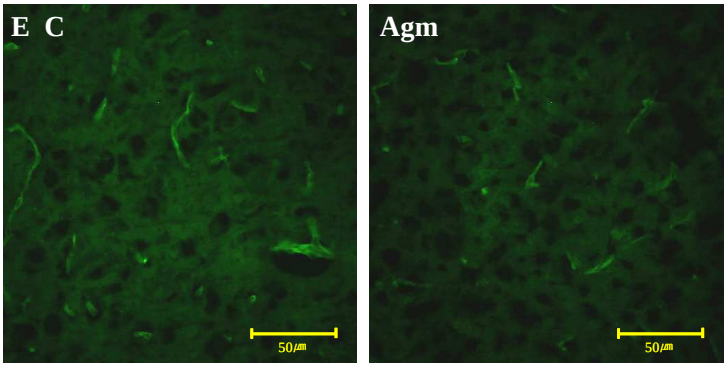

Figure 4. Macrographs of AQP-4 immunofluorescence in the ischemic injured brain 22 hours after 2 hours MCAO with and with out agmatine. Blood vessel was stained with AQP-4 (green) in cortex. AQP-4 (green) was feebly detected compared to AQP-1 in experimental control (EC) and agmatne treatment group (Agm) . Scale bar is 50 ㎛. EC, Experimental control group; Agm, Agmatine treatment group

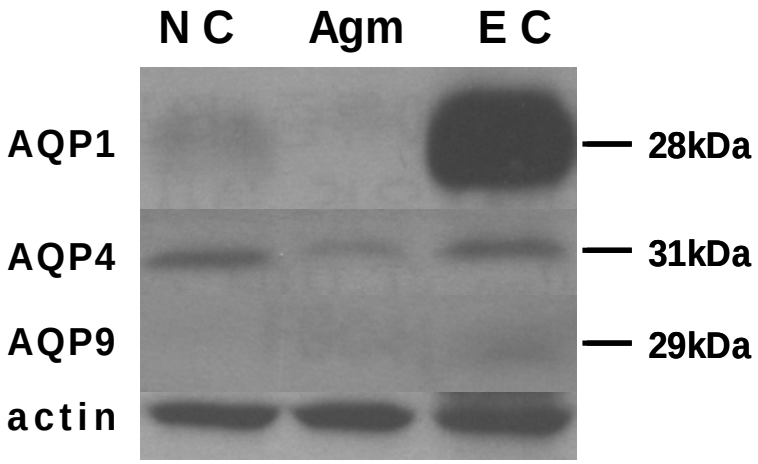

Figure 5. Immunoblotting of AQP-1, -4, and -9. The expression of AQP-1 (AQP1) and -4 (AQP4) were decreased in agmatine treatment group (Agm), but the expression of AQP-9 (AQP9) was not obvious. NC, Normal control group; EC, Experimental control group; Agm, Agmatine treatment group

EB Ratio

(Evans blue(㎍/g wet weight) / plasma Evans blue(㎍/g))

	Penumbra	Hippocampus	Striatum	Cortex
EC	2.44 ± 0.157	4.24 ± 0.135	4.90 ± 0.120	1.96 ± 0.110
Agm	2.50 ± 0.145	3.08 ± 0.134*	2.74 ± 0.307*	1.14 ± 0.042*

Table 1. Blood-brain barrier (BBB) disruption. Agmatine treatment reduced the BBB disruption in hippocampus, striatum, and cortex (*, $P < 0.01$ vs EC). EB, Evans blue; EC (n = 3), Experimental control group; Agm (n = 3), Agmatine treatment group

5. The expression of MMPs

Immunohistochemical staining of MMP-2 and MMP-9 was increased in cerebral cortex and striatum 22 hours after 2 hours MCAO. The number of MMP-2 positive cells was dramatically decreased in cortex and striatum by agmatine treatment. In similar to MMP-2, agmatine treatment reduced the number of MMP-9 positive cells (Figure 6). These decreased expression of MMP-2 and -9 by agmatine treatment was clearly shown in immunoblotting of MMPs (Figure 7).

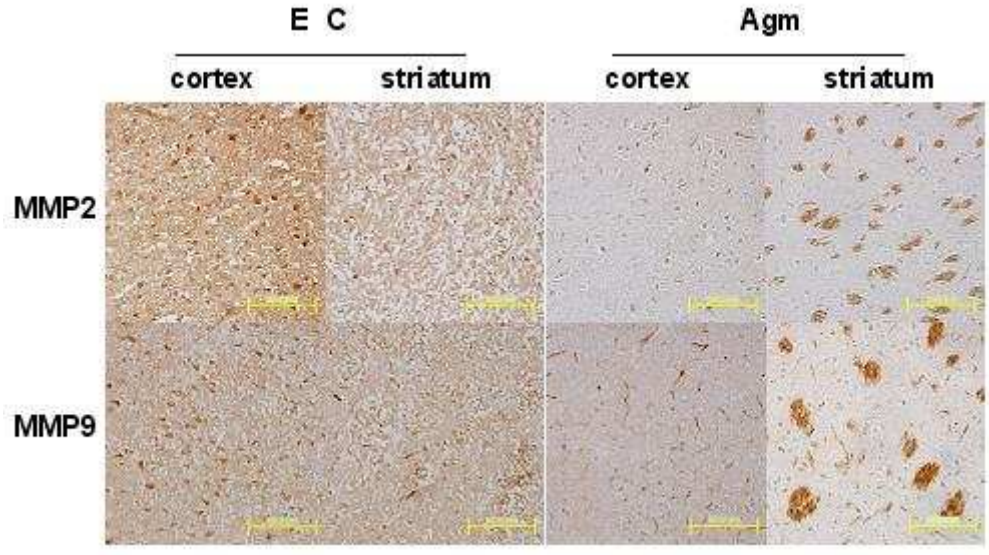

Figure 6. Macrographs of MMPs immunopositive cells in the ischemic injured brain 22 hours after 2 hours MCAO with and with out agmatine. There were few MMP-2 positive cells (brown) in cortex and striatum of agmatine treatment group. MMP-9 positive cells (brown) in agmatine treatment group were decreased compared to experimental control group (EC). Scale bar is 50 ㎛. EC, Experimental control group; Agm, Agmatine treatment group

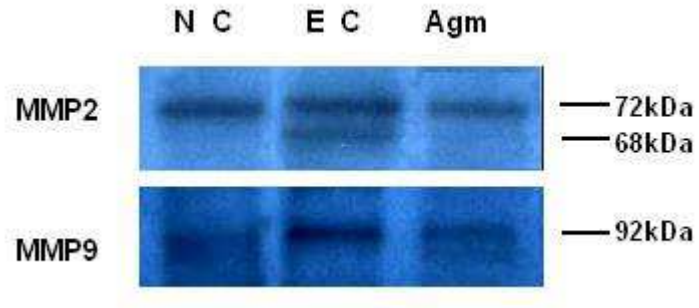

Figure 7. Immunoblotting of MMPs. MMP-2 (MMP2) and -9 (MMP9) were expressed down in agmatine treatment group (Agm) compared to experimental control group (EC). NC, Normal control group; EC, Experimental control group; Agm, Agmatine treatment group

IV. DISCUSSION

It is shown that agmatine reduces brain swelling and brain edema in experimental stroke in this study. This effect of agmaitne on the brain edema is associated with the decrease of matrix metalloproteinase (MMP) -2, MMP-9, aquaporin (AQP) -1 and AQP-4 expressions. It also appears that agmatine reduces MMP-2 and MMP-9 expressions in glial cells to a greater extent than in endothelial cells, and lessens the expression of AQP-1 in endothelial cells in cerebral ischemia.

Recent studies have emphasized the importance of the neurovascular unit, comprised of cerebral endothelial cells, astrocytes and neurons along with the extracellular matrix, in maintaining the integrity of brain tissue in stroke[16]. Perturbation of the extracellular matrix, including basement membrane components (i.e. type IV collagen, heparan sulphate proteoglycan, laminin and fibronectin), disrupts microvascular homeostasis and integrity[16,17]. In stroke, these proteins and polysaccharides that compose part of the extracellular matrix are degraded by a variety of proteolytic enzymes, including a family of proteases known as MMPs, which are divided into five classes[16]. MMP-2 and -9 are reported to be increased after cerebral ischemia in experimental animals[17,18,19,20,21,22,23,24], as well as in human patients[25,26]. The early expression of MMP-2 or -9 is associated with blood-brain barrier (BBB) disruption and the formation of vasogenic edema after transient focal cerebral ischemia[19,22,24,27], or other acute cerebral injuries[44,45]. In addition, pharmacologic inhibition of MMPs was able to ameliorate edema after focal cerebral ischemia[17,22], and MMP-9 deficient knockout mice reduced BBB disruption and edema after transient focal cerebral ischemia[46,47] and traumatic brain injury[48]. The results that MMP-2 and MMP-9 expressions were reduced by agmatine treatment after

stroke in this study support the idea that the neuroprotective effect of agmatine is associated with improved BBB function.

Recent reports suggest a potential extracellular proteolysis pathway to neuronal cell death in which S-nitrosylation by NO activates MMPs, and further oxidation results in a stable posttranslational modification with pathological activity[49]. In this study, agmatine treatment decreased Evans blue extravasation and the expression of MMP-2 and MMP-9. Treatment with the non selective NOS inhibitor in a mouse model of MCAO significantly reduced vascular damage, as indicated by decreasing of Evans blue extravasation and MMP-9 expression[50]. Using trophoblast cells isolated from human placentas, a positive regulatory role of NO on the activity and protein expression of MMP-2 and MMP-9 was demonstrated, as NO donors stimulated, whereas NOS inhibitors reduced, the expression and gelatinolytic activity of MMP-2 and MMP-9[51]. Agmatine was demonstrated that reduces cerebral ischemic injury and may act by inhibiting the detrimental effects of nNOS in animal stroke model[8] and that suppresses inducible nitric oxide generation[52,53,54]. In this study, it is shown that agmatne decreases the expression of MMP-2 and MMP-9 and consequently, it is suggested that BBB disruption and brain edema after stroke could be reduced through the diminution of MMP-2 and MMP-9 expression which may be due to NOS inhibition by agmatine.

There are two types of brain edema, cytotoxic edema and vasogenic edema after brain injury, such as brain trauma and stroke[55]. Cytotoxic edema is leaded without the BBB disruption, but vasogenic edema with the BBB disruption. The disruption of BBB was observed 22 hours after 2 hours MCAO in this study (Table 1). AQP-1 and AQP-4 are two members of AQP family known to be expressed in the central nervous system, and it is possible that these proteins contribute to water transport across the blood-brain barrier[56].

In a study of AQP-1 expression in human brain, a small number of microvessels were positively stained, but there are marked up-regulation in endothelium in astrocytomas and metastatic carcinomas[57]. Function of blood-brain barrier (BBB) is known to be impaired in such brain tumors, leading to formation of edema[58]. Down-regulation of the tight-junction proteins claudin and occludin has also been demonstrated in microvessels in glioblastoma multiforme[59]. Thus, loss of BBB function and the expression of AQP-1 may both be regarded as down-regulation of blood-brain barrier phenotype[56]. The expression of AQP-4 mRNA is strongly induced when BBB is preserved. However, the expression of AQP-4 mRNA is reduced in astrocytes when BBB is disrupted after brain trauma[60]. It explains that the expression of AQP-4 is much less than that of AQP-1 in the brain tissue after MCAO injury in this study. (Figure 5). AQP-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke[61]. Recently, Amiry-Moghaddam et al. (2004) demonstrated, by electron microscopy, sparse AQP-4 staining in the endothelium in mouse hippocampus and cerebellum[62]. This supports the expression of AQP-4 in blood vessel in this study (Figure 4). Herrera et al. (2006) reported that AQP-1 transports NO across cell membranes[63] and Gunnarson et al. (2006) announced that AQP-4 phosphorylation is related with NO in astrocyte[64]. It is possible that agmatine would suppress the expression of AQP-4 in astrocyte by inhibiton of NO synthesis and in endothelial cells by blocking NO transport across cell membranes through decreasing AQP-1 expression, although the mechanism of AQP-1 expression suppressed by agmatine is unexplainable except the possibility of reduced BBB disruption through subduing the expression of MMP-2 and MMP-9.

PART 2. The effect of agmatine for spinal cord injury

I. INTRODUCTION

Spinal cord injury (SCI) usually results in long-lasting deficits, involving loss of motor and sensory function. Following injury to central nervous tissues, spontaneous axonal regeneration of damaged neurons is restrictive. The failure of regeneration is attributed to the nonpermissive environment of the damaged adult mammalian spinal cord, the milieu of which is formed of astrocyte-derived inhibitory molecules in the scar tissue, myelin components of oligodendrocytes interfering with the regeneration of axons and lack of trophic support for axotomized neurons, and the intrinsic neuronal changes, including cell atrophy and death after axotomy[65,66]. Therefore, effective repair strategies for SCI require the creation of a permissive environment within the injured spinal cord that protects damaged neurons from the effects of secondary injury and also facilitates axonal regeneration. following traumatic injury of the central nervous system (CNS) a collagenous wound healing scar develops at the lesion site. In the past the lesion scar was suspected several times to be an impediment for axonal regeneration the lesion scar is comprised of afibrous scar in the lesion core and a glial scar in the surrounding parenchyma[67,68]. Different cell types and extracellular matrix (ECM) components contribute to the lesion scar in SCI. Especially with rupture of the dura, various non-neural meningeal or inflammatory cells invade CNS lesions.

Due to inflammation, cytokines are released in the CNS lesion area, such as transforming growth factor (TGF) and connective tissue growth factor (CTGF)[69,70,71]. TGFβ-1 expression is increased immediately after injury, whereas TGFβ-2 concentration increases more slowly in astrocytes, endothelial cells, and macrophages in proximity to the lesion site. While TGFβ-1 modulates the inflammatory and neuronal response, TGFβ-2 regulates glial/collagenous scarring[72] and induces the production of proteoglycans by astrocytes. CTGF is a downstream mediator of TGFβ in fibroblasts, where it stimulates proliferation and ECM synthesis via an autocrine mechanism[73]. Bone morphogenetic protein-7 (BMP-7) is a member of the BMP subfamily of the TGFβ superfamily[74]. It is reported that BMP-7 mRNA expression increases in glial cells and motor neurons of the spinal cord following injury[74] and that transient occlusion of the middle cerebral artery (MCA) for 60 minutes causes elevated BMP-7 mRNA level at 8 hours after stroke in the cortex and striatum both ipsilateral and contralateral to the ischemic hemisphere[75]. Recent reports indicate that BMP-7 exerts neuroprotective effects in the CNS[76] and selectively stimulates dendrite growth and branching from sympathetic, cerebral cortical, or hippocampal neurons in culture independent of cell survival or axon growth[77,78,79].

Based on these evidences, it was hypothesized that agmatine may have neuroprotective effect on spinal cord injury. The purpose of the present study was to investigate the effect of agmatine for spinal cord injury and to evaluate on the deposition of collagen at lesion site and on the expression of BMP-7 and TGFβ-2.

II. MATERIALS AND METHODS

1. Animals

Studies were conducted on male IcrTacSam:ICR mice, 8 - 10 weeks old, weighing 36 ± 2 g (Sam tako, O San-shi, Korea). All animal procedures were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine.

2. Spinal cord injury model

Mice (*n* = 60) were anesthetized with chloral hydrate (400 mg/kg) intraperitoneally. Body temperature was maintained with a heating pad at 36.5 ± 0.5 ℃. Complete transection of the spinal cord was made at the T9 level as previously described (Lu et al., 2001). The skin was shaved and cleaned using Betadyne solution. A incision was made over the lower thoracic area, and muscle and connective tissue were dissected to expose the T8 - T10 vertabrae. A T9 laminectomy was completed using a microsurgery bone rongeur, taking care not to damage the spinal cord. The spinal cord was completely transected using a surgical blade (NO. 11, made in Sheffield. England). A hooked needle (31 Gauge (0.25 mm) x 8 mm) was used to scrape the transection site to ensure a complete transection. After completion of the transection injuries, mice were sutured and dosed with prophylactic gentamycin sulfate (1 mg/kg, IM) for 1 week. Bladders were manually expressed twice daily[80].

Agmatine was dissolved in normal saline (100 mg/kg, Sigma) and given intraperitoneally 5 minutes after complete transection and daily for 4 weeks (Agm, n = 30). Controls received normal saline as the same manner (EC, n = 30)[8].

3. Neurological test

Open-field locomotor function was evaluated using the Basso-Beattie-Breshnahan (BBB) locomotor rating scale[81], a multiple function test of locomotor outcome that provides an efficient and unambiguous locomotor rating. Briefly, mice were exposed daily for one week to the behavioral testing environment in order to adjust them to open field exploration. All operated mice were tested by two blind testers for 3 minutes weekly from 1 day post-injury to 4week.

4. Overturning body test

Mouse was placed to put its back on bedding and to set its ventral side upward. The time required to overturn its body to normal position was measured to ascertain its physiological condition. All operated mice were tested 4 times by two blind testers at 4 week post-injury.

5. Immunoblotting of BMP-7

Expression of BMP-7 proteins was estimated by immunoblotting in transected spinal cord. Immunoblotting was performed using anti-BMP-7 (Santa Cruz, Santa Cruz, CA, USA) antibodies. Equal amounts of protein, 50 ㎍ per condition, were separated on an 10 % polyacrylamide gel and electrotransferred onto Immobilon-NC membrane (Millipore, Bedford, MA, USA). Immunoreactive bands were visualized with the ECL detection system using Kodak X-AR film[8].

6. Immunoreactivity of BMP-7

Spinal cords were fixed with 4 % paraformaldehyde, and embedded in paraffin. Spinal cord sections were made by 10 ㎛. Sections were immunostained with antibodies against BMP-7 (Santa Cruz, Santa Cruz, CA,

USA), followed by an appropriate FITC-conjugated secondary antibody. Immunostaining controls were prepared by tissue without primary antibodies. All incubation steps were performed in a humidified chamber. Fluorescence-labeled immunostaining was evaluated using a fluorescence microscope (LSM 510 META, Carl Zeiss). The immunoreactivity of BMP-7 was computed by Zeiss LSM Image Browser ver. 4.0 (Carl Zeiss).

7. Analysis of collagen scar area

Collagen scar area was determined by Masson's trichrome staining[82], using a computer-assisted image analysis system (Optimas ver 6.1, Optimas, Bothell, WA, USA). Briefly, spinal cord sections were stained by Masson's trichrome method. Collagen scar dyed with a blue was evaluated using low power field (x 4) light microscopy (VANOX-S, Olympus, Tokyo, Japan). Collagen scar area was automatically calculated by computer-assisted image analysis system.

8. Immunohistochemical staing for TGFβ-2

Spinal cords were fixed with 4 % paraformaldehyde, and embedded in paraffin. Spinal cord sections were made by 10 ㎛. Sections were immunostained with antibodies against TGFβ-2 (Santa Cruz, Santa Cruz, CA, USA), followed by an appropriate biotinylated secondary antibody. Stains were visualized using the ABC kit (Vector, Burlingame, CA, USA)[38], then reacted with diaminobenzidine (DAB, Sigma, St. Louis. MO, USA). Immunostaining controls were prepared by tissue without primary antibodies. All incubation steps were performed in a humidified chamber.

9. Statistical analysis

Statistical tests to determine differences between groups were performed with Mann-Whitney test using SPSS ver. 13.0 (SPSS, Chicago, IL, USA). *P* value < 0.05 was considered significant. Data are expressed as the mean ± standard error of mean (SEM).

III. RESULTS

1. Neurological test

Neurological score was higher in agmatine treatment group than experimental control over the next 2 week post-injury. The sham-operated animals showed little or no hind limb movements from 1day post-injury to 4 week, but the agmatine-treated animals had movements of one joint or two joints from 2 week post-injury. It was not significant (Figure 8).

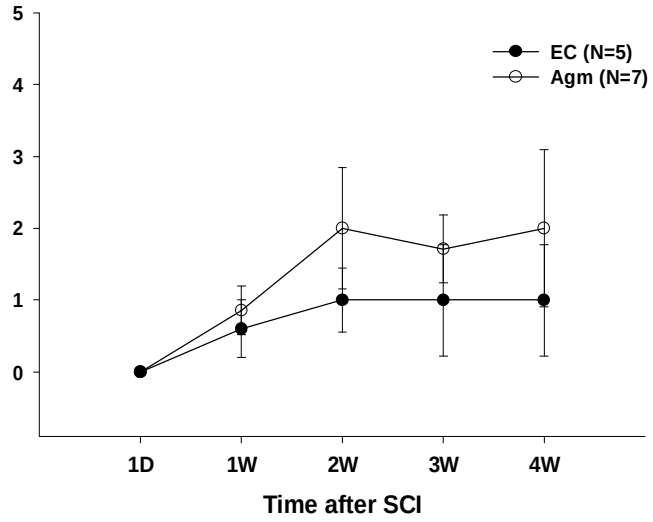

Figure 8. Neurological test after spinal cord injury. Agmatine treated animals had high mean neurological score from 2 weeks after SCI compared to experimental control. Data are expressed as the mean ± SEM. EC, Experimental control group; Agm, Agmatine treatment group

2. Overturning body test

Overturning body test was done 4 weeks after SCI. The time required to overturn body was reduced in agmatine treatment group (4.08 ± 1.35 seconds) compared to experimental controls (8.66 ± 3.17 seconds, $P < 0.05$, Figure 9).

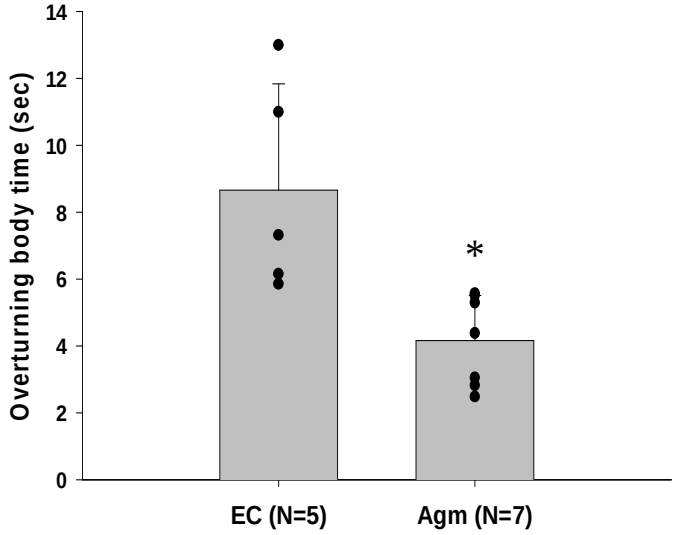

Figure 9. Overturning body test after spinal cord injury. Agmatine treated animals needed short time to overturn body than sham-operated animals (*, $P < 0.05$ vs EC). Data are expressed as the mean ± SEM. EC, Experimental control group; Agm, Agmatine treatment group

3. The expression of BMP-7

Agmatine treatment increased the expression of BMP-7 more than experimental control in scar region, proximal region to scar, and distal region to scar of spinal cord (T8 - T10) through BMP-7 immunoreactivity at 1 week, 2 weeks, and 4 weeks after SCI (Figure 10A). In immunoblotting, BMP-7 expressed higher in agmatine treatment group than experimental control at 1 day, 3 days, 1 week after SCI, but there was no significance at 2 weeks (Figure 10B and C).

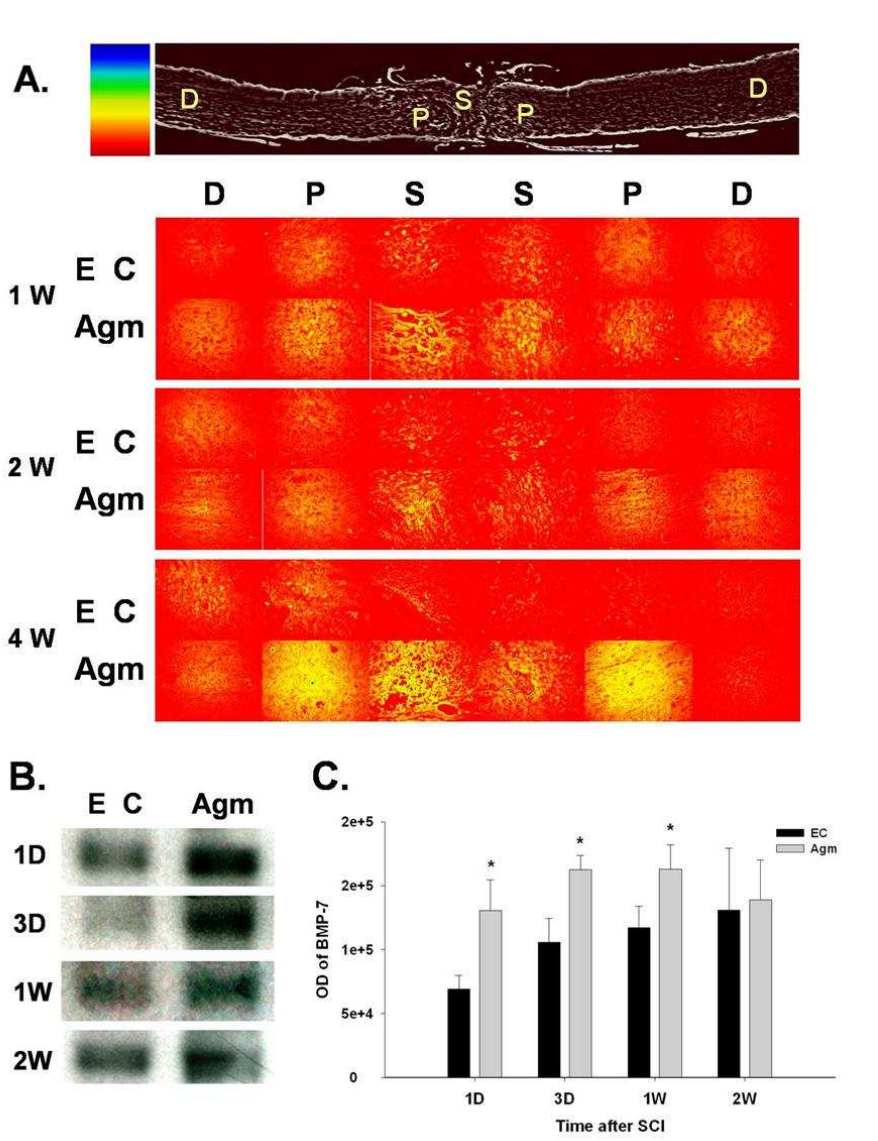

Figure 10. The expression of BMP-7 after spinal cord injury in agmatine treatment group (Agm, n = 4) and experimental control (EC, n = 4) using immunoreactivity (A), immunoblotting (B), and optical densities (OD) of BMP-7 are expressed as the band density (*, $P < 0.05$ vs EC at each time, C). Data are expressed as the mean ± SEM. EC, Experimental control group; Agm, Agmatine treatment group; D, Distal region to scar; P, Proximal region to scar; S, Scar region

4. Analysis of collagen scar area

All operated animals showed collagen scar in transection site at 4 weeks after SCI. Many cavities were shown in experimental control, and the spinal cord was shown to be more compact in agmatine treatment group than experimental control. Collagen scar area was significantly reduced in agmatine treatment group (104593 ± 16001 ㎛^{2}) compared to experimental control (156968 ± 24925 ㎛^{2} , P< 0.05, Figure 11).

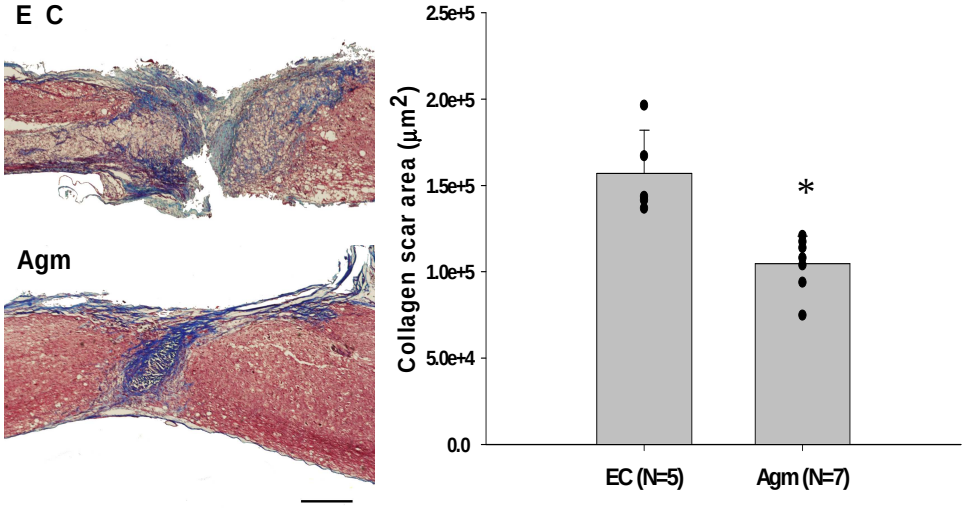

Figure 11. Analysis of collagen scar area after spinal cord injury. Collagen scar area was estimated 4 weeks after SCI in agmatine treatment group (Agm) and experimental control group (EC) using Masson's trichrome stained slides. Collagen was shown as blue. Agmatine treatment reduced collagen scar area (*, P < 0.05 vs EC). Data are expressed as the mean ± SEM. Scale bar is 200 ㎛. EC, Experimental control group; Agm, Agmatine treatment group

5. Immunohistochemistry of TGFβ-2

Many TGFβ-2 positive cells were shown in all regions-distal to scar, proximal to scar, and scar of experimental control 1 week and 2 weeks after SCI, but TGFβ-2 positive cells were few or weakly stained in agmatine

treatment group compared to experimental control. Agmatine treatment, however, increased TGFβ-2 positive cells 4 weeks after SCI more than experimental control. Many TGFβ-2 positive blood vessels, on the other hand, were shown in experimental control 4 weeks after SCI (Figure 12).

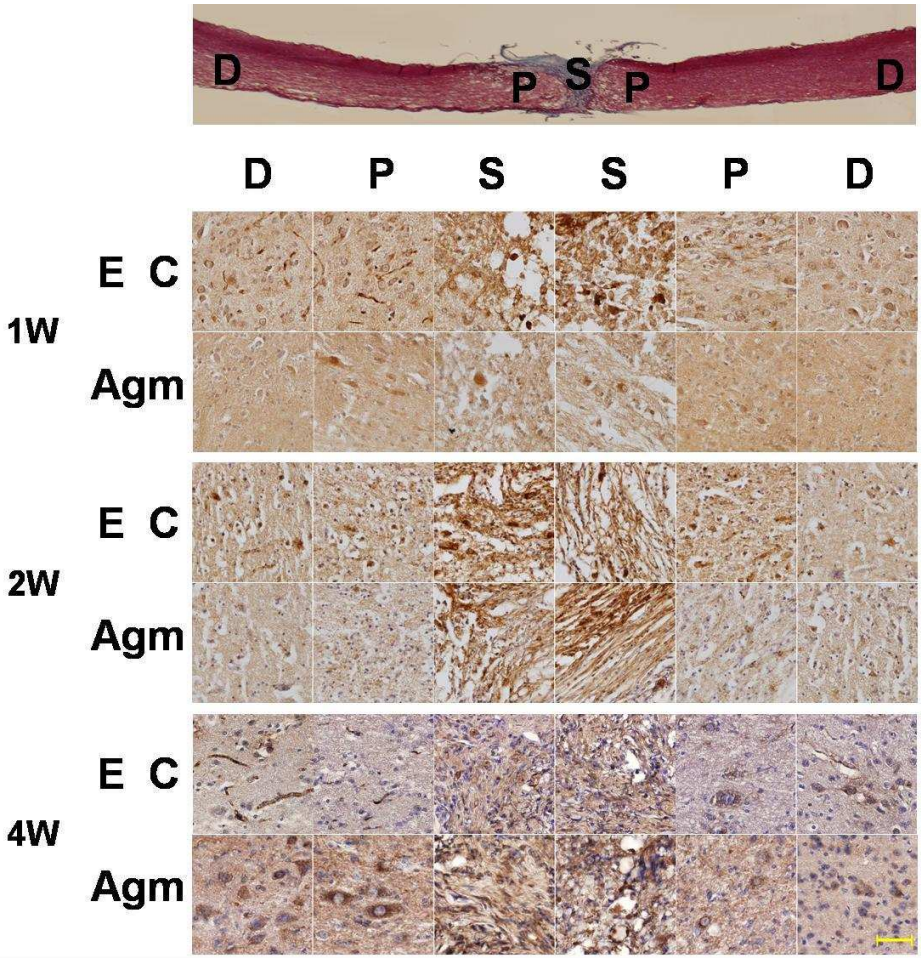

Figure 12. Immunohistochemistry of TGFβ-2 after spinal cord injury. TGFβ-2 positive cells shown to have brown-colored cytosol were increased in all regions of experimental control group (EC) 1 week and 2 weeks after SCI compared to agmatine treatment group (Agm) except 4 weeks after SCI. Scale bar is 25 ㎛. EC, Experimental control group; Agm, Agmatine treatment group; D, Distal region to scar; P, Proximal region to scar; S, Scar region

IV. DISCUSSION

It is shown that agmatine which is neuroprotective material in CNS injury[76,77,78,79] improves physiological condition, increases the expression of BMP-7, reduces collagen scar in lesion, and decreases the expression of TGFβ -2 in this study. Agmatine, however, has no significant restoration of the motor function of hind limb in neurological test using BBB locomotor rating scale (Figure 8). All treated animals with or without agmatine recorded low BBB score, other report using same experimental model also showed BBB score similar to that of this study[83], but in contusion spinal cord injury, BBB score was higher than transected model[9]. These are shown that the regeneration of completely transected spinal cord is very hard and needs long time. Agmatine treatment significantly reduced the time required to overturn body, indicating that agmatine treated animals had improved physiological condition. Overturning body test was designed to confirm the recovery of physiological condition for the first time. Tissue breakdown in the skin, leading to pressure ulcer formation, is a common complication developed in persons with spinal cord injury when prolonged unrelieved pressure has been applied to the body and skin and underlying tissues[84]. Decreasing the required time to overturn the body in agmatine treatment group means that agmatine can make it easy to move body or to change the position in spinal cord injured patients, so it is possible that pressure ulcer formation could be reduced by agmatine.

BMP-7 is a member of the BMP subfamily of the TGFβ superfamily[74]. The expression of BMP-7 mRNA is reported to be increased in CNS injury[74,75]. BMP-7 is announced to improve functional recovery, local cerebral glucose utilization and blood flow after cerebral ischemia[85], and to improve locomotor function after stroke[86]. In this study, agmatine significantly increased the expression of BMP-7 for 1 week after spinal cord injury in

immunoblotting, and intensified the immunoreactivity of BMP-7 from 1 week to 4 weeks after spinal cord injury (Figure 10). These results suggests the possibility to reduce the time to overturn the body through the enhancing of BMP-7 expression by agmatine after spinal cord injury. Recently, Setoguchi et al. (2004) showed that BMPs alter the fate of adult spinal cord-derived neural precursor cells in culture from neurogenesis to astrocytogenesis and in response to spinal cord injury, it is implied that ependymal neural stem cells proliferate to generate migratory cells, which differentiate into astrocytes and participate in glial scar formation[87,88]. They suggested that BMPs contribute to the formation of glial scars[89]. Enzmann et al. (2005), however, reported that neutralizing endogenous BMP in the injured spinal cord significantly increased both the lesion volume and the number of infiltrating macrophages[90]. Therefore, the area of the lesion was analyzed by measurement of the collagen scar area, since collagen matrix is formed at the lesion after transected spinal cord injury. Collagen scar is regarded as physical barrier to axonal regeneration[91,92,93], and considered to contribute at least in part to the frequently observed cavity formation, because of physical force causing contraction of the injured CNS tissue[94]. In this study, Agmatine treatment significantly diminished the collagen scar area (Figure 11). The scar-suppressing treatment results in regeneration of transected corticospinal tract fibers[92]. Suppression of fibrous scarring in spinal cord injury of rat promotes long-distance regeneration of corticospinal tract axons, rescue of motor neurons in somatosensory cortex and significant functional recovery[93]. These reports support that agmatine may have the potential to improve the motor function of spinal cord injured animal through decreasing collagen scar. Last, Lagord et al. published that expression of TGFβ -2 but not TGFβ-1 correlates with the deposition of scar tissue in the lesioned spinal cord, and that TGFβ-1 modulates the inflammatory and neuronal responses, TGFβ-2 regulates glial/collagen scarring[72]. Agmatine reduced the

expression of TGFβ-2 around the lesion site 1 week and 2 weeks after spinal cord injury (Figure 12). Consequently, the deposition of collagen at the lesion site is likely to be diminished through the suppression of TGFβ-2 expression by agmatine. It is worthy to notice that agmatine increased the expression of TGFβ-2 in neuron around the lesion site 4 weeks after spinal cord injury (Figure 12) and TGFβ-2 attenuates the injury-induced death of mature motor neurons[95]. These results might support that agmatine has the capability to save motor neurons by means of upregulation of TGFβ-2 expression.

V. CONCLUSION

The role of agmatine in CNS injury has been demonstrated in cerebral edema after ischemia and in spinal cord injury. The following results of the role of agmatine in CNS injury have been derived:

1. Agmatine significantly reduced brain swelling volume in cerebral edema after ischemia by about 14.38 % compared to experimental control.

2. Water content was significantly decreased in agmatine treatment group by approximately 7.62 % compared to experimental control in cerebral edema.

3. Blood-brain-barrier disruption was markedly lessened 22 hours after MCAO in the striatal, hippocampal, and cerebral cortical area of agmatine treatment group than experimental control.

4. The expression of MMP-2 and of MMP-9 were clearly reduced 22 hours after 2 hours MCAO by agmatine treatment.

5. The expression of AQP-1 and AQP-4 were plainly decreased 22 hours after 2 hours MCAO by agmatine treatment.

6. Agmatine treatment abridged time to overturn the body compared to experimental control 4 weeks after SCI.

7. Agmatine treatment reduced collagen scar area compared to experimental control 4 weeks after SCI.

8. Agmatine treatment increased the expression of BMP-7 around scar more than experimental control in early period of SCI.

9. TGFβ-2 positive cells were few or weekly stained around scar in agmatine treatment group compared to experimental control 1 week and 2 weeks after SCI

Taken together, these data suggest that agmatine could attenuate brain edema through reducing disruption of the blood-brain barrier (BBB) by suppression of the expression of matrix metalloproteinases and lessening the expresssion of aquaporins and propose that agmatine could support CNS regeneration by reducing the collagen scar area, decreasing the expression of TGFβ-2, and increasing the expression of BMP-7 in spinal cord injury.

This study addresses the neuroprotective and neuroregenerative effect of agmatine in CNS injury.

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Abstract (in Korean)

중추신경계 손상시 아그마틴의 역할

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중추신경계 손상 시 신경손상을 줄이는 최적의 치료법은 아직 부족한 실정이며, 많은 치료후보물질들이 연구되고 있다. 아그마틴은 엘-알기닌이 탈탄산화 되어 생성되는 일차 아민으로 포유류의 뇌에 존재함이 알려져 있다. 본 연구의 목적은 중주신경계 손상 시 아그마틴의 역할을 확인하는 것이다.

먼저 허혈 손상 후 발생하는 뇌부종에서 아그마틴은 팽창되는 뇌의 부피를 유의하게 줄여 주는 것으로 관찰되었다. 뇌부종의 주요 원인인 뇌 조직 내 수분의 함유량 역시 아그마틴을 투여한 경우 확연히 감소됨을 확인하였다. 뇌혈관장벽의 붕괴 정도를 알아본 실험에서도 아그마틴은 해마부위와 선조 부위 그리고 대뇌피질부위에서 각각 의미 있게 뇌혈관 장벽의 붕괴를 막았다. 이러한 뇌혈관장벽의 붕괴와 밀접한 연관이 있는 세포외 기질분해 효소인 MMP-2와 MMP-9의 뇌부종 시 발현 정도를 조사한 결과, 아그마틴의 투여가 이 세포외 기질 분해 효소인 MMP-2와 MMP-9의 발현을 감소시키는 것으로 나타났다. 또한 뇌부종의 주요원인 물질인 수분을 이동시키는 수분이동통로인 AQP-1과, AQP-4의 발현도 아그마틴의 투여로 감소됨을 확인하였다.

다음으로 척수 손상 시 아그마틴의 역할에 대해 알아보았다. 아그

마틴을 투여한 경우 실험동물의 생리적 상태가 호전되는 것을 척수 손상 후 4주차에 확인하였다. 또한 신경보호와 신경재생에 효과가 있는 것으로 보고된 뼈 형태형성 단백질인 BMP-7의 발현이 아그마틴을 투여함으로써 척수 손상 후 초기 회복단계에서 그 발현이 실험대조군에 비해 상대적으로 유의하게 증가됨을 손상부위와 손상근위부 그리고 손상원위부에서 관찰하였다. 그리고 신경재생에 물리적 장벽이 되는 결합조직상흔의 형성정도를 측정한 결과 아그마틴의 투여가 결합조직상흔의 크기를 줄이는 것을 확인하였으며, 이러한 결합조직 상흔의 형성에 관련된 물질로 알려진 형태변형 성장 인자인 TGFβ-2의 발현이 아그마틴에 의해 감소되었음을 확인하였다. 이상의 결과들로 비춰볼 때, 아그마틴은 세포외 기질분해 효소인 MMP-2와 MMP-9의 발현을 감소시켜 뇌혈관 장벽의 붕괴를 줄이고, 뇌부종을 일으키는 수분의 이동통로인 AQP-1과 AQP-4의 발현을 억제하여 조직 내로의 수분의 유입을 막아 허혈 손상 후 일어나는 뇌부종을 억제할 수 있는 것으로 판단되며, 척수손상 시, 회복기에 생리적 상태를 호전시키고, 뼈 형태형성 단백질인 BMP-7의 발현을 손상 후 초기에 증가시킴과 더불어 형태변형 성장 인자인 TGFβ-2의 발현을 억제하여 결합조직 상흔의 크기를 줄임으로써 중추신경손상에서 신경의 재생을 촉진할 수 있을 것이라고 판단된다. 따라서 본 실험결과로부터 아그마틴은 손상기전이 다른 허혈 손상과 외상에 대해 손상을 억제하고 재생을 촉진하는 신경보호 기능을 담당함을 알 수 있었다.

핵심 되는 말 : 아그마틴, 뇌 허혈 손상, 뇌부종, 수분이동통로 (AQP), 세포외 기질분해효소 (MMP), 뇌혈관 장벽, 척수손상, 결합조직 상흔, 형태변형 성장 인자 (TGF), 뼈 형태형성 단백질 (BMP)