The neuroprotective effects of agmatine on rat global cerebral ischemia

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The neuroprotective effects of agmatine on rat global cerebral ischemia

Directed by Professor Jong Eun Lee

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문진희의 석사학위논문을 인준함

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It is a fresh memory to me when I first got into the complicated laboratory. The narrow paths, so many rooms like a maze, different people made me thrilled and excited. Spring in 2003, I met these so nice people who I've never seen before with having funny, sometime very hard and great time with them for scientific and beautiful life. The fruit of this work over last two years is my thesis in my life through the joy and sorrow, trial and error.

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ABSTRACT

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In ischemic strokes, apoptosis is caused by excitotoxicity, ionic imbalance, oxidative/nitrosative stress and apoptotic-like pathways. One of free radicals which are elevated after the ischemic insult is *nitric oxide* (NO). NO generated primarily by neuronal and inducible NO synthases (NOS) promotes neuronal damage following ischemia. Evidence obtained in recent years has demonstrated that endoplasmic reticulum (ER)-mediated cell death plays an important role in cerebral ischemia.

Agmatine is an endogenous substance synthesized from L-arginine by arginine decarboxylase (ADC) and is present in the mammalian brains. Previous studies have shown that agmatine may be neuroprotective in *in vitro* and *in vivo* ischemia models.

Also, we have previously studied and reported the effects of agmatine on the middle cerebral artery occlusion (MCAO) model in mice and shown that agmatine contributes to neuroprotection from ischemic injury.

Transient global ischemia for 20 minutes was induced by 4 vessel occlusion (4-VO) using the method described originally by Pulsinelli et al. (1979). Animals were anesthetized with isoflurane and agmatine was administered by IP injection in dose of 100 mg/kg at the same time of reperfusion. The rats were sampled at 6, 24, 48, and 72h after reperfusion to determine the effect of agmatine on NOS expression. We also checked the relation of agmatine and calcium influx with ER by using immunohistochemistry. We detected the expression of NOS by using immunoblotting. Agmatine treatment prevented the delayed neuronal cell death in the hippocampal CA1 neurons in global cerebral ischemia. It also blocked NOS expression and calcium influx in the rat brain. On the other hand, the expression of NOS was different according to the area of the rat brain. Agmatine induced the level of NF-kB, Hsp70 and Grp 78, and so on. Also, agmatine reduced the level of MMP-9 and MMP-2 slightly. Agmatine has shown different neuroprotective effects according to various brain regions.

These results show that agmatine inhibits the production of nitric oxide by decreasing the expression of three isoforms of NOS differently on global forebrain ischemia. It regulates anti-apoptotic

and pro-apoptotic proteins through different routes to protect neurons from transient global ischemia. Moreover, neuroprotective effects of agmatine were concerned with ER stress-mediated condition.

Key words : agmatine, global ischemia, nitric oxide synthase, neuronal cell death, ER stress

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I. Introduction

Ischemic stroke is the third leading cause of death in industrially advanced countries and a major cause of long-lasting disability.[1] Even though a large number of compounds have been proven to reduce ischemic injury, clinical trials have been unsuccessful because of toxic side effects. Thus, the development of new drugs and discovery of novel mechanisms for treating cerebral ischemia are needed[2]. In ischemic strokes, apoptosis is caused by excitotoxicity, ionic imbalance, oxidative/nitrosative stress and apoptotic-like pathways[3]. The increase of calcium ion mediated by glutamate receptors causes ionic imbalance, the production of reactive oxygen families. The reactive oxygen families directly impair lipids, proteins, nucleic acids and secrete apoptotic proteins[1,4,5].

A main event during ischemia is the generation of free radicals; due to their high reactivity, they provoke damage to lipids, DNA and proteins and produce neuronal death[6]. They also contribute to the breakdown of the blood-brain barrier and brain edema[6]. One of these radicals which is elevated after the ischemic insult is *nitric oxide* (NO)[5,7]. As it will be discussed below, NO generated primarily by neuronal and inducible NO synthases (NOS) promotes neuronal damage followed by ischemia[5,6,7]. In addition, the conversion of xanthine dehydrogenase to xanthine oxidase promotes the cellular formation of toxic oxygen free radicals such as the superoxide anion which further breaks down membrane, cytoskeletal, and nuclear structures[1,6]. An important source of oxidative stress-mediated brain damage is the oxidant reactions due to the formation of peroxynitrite, a powerful oxidant that results from the interaction between NO and superoxide[8]. This anion has been shown to cause cell damage by several mechanisms that include lipid peroxidation, tyrosine nitration, sulfhydril oxidation and nitrosylation, and DNA breakage, etc[8,9]. Ischemia-induced nitric oxide overproduction is in part caused by glutamatergic-mediated increased in intracellular calcium concentration, resulting in a calmodulin-dependent upregulation of nitric oxide synthase[6,7]. Nitric oxide is enzymatically synthesized from L-arginine and is massively increased by ischemia[10]. Three nitric oxide synthases (NOS) have been reported (eNOS, nNOS and iNOS), so named because of their originally defined endothelial

(eNOS) and neuronal (nNOS) localization, or ability to be upregluated when induced (iNOS)[6]. Nitric oxide has also been shown to inhibit mitochondrial respiration *via* competition with oxygen for cytochrome oxidase and play a role in the initiation of apoptosis[11,12]. Although little has been reported on the effects to bring nitric oxide inhibitors to clinical investigation, there is no doubt that nitric oxide plays a pivotal role in mediating oxidative stress[13].

Endoplamic reticulum (ER) is the site of synthesis and folding of secretory proteins. Perturbations of ER homeostasis affect protein folding and cause ER stress[14]. ER can sense the stress and respond to it through translational attenuation, upregulation of the genes for ER chaperones and related proteins, and degradation of unfolded proteins by a quality-control system[15]. Evidence obtained in recent years has demonstrated that endoplasmic reticulum (ER)-mediated cell death plays an important role in cerebral ischemia[16]. Therefore, targeting the ER may provide a therapeutic approach for blocking the pathological process induced by cerebral ischemia[17]. However, no pharmacological approach for treating ischemia-induced ER dysfunction has been reported so far.

Agmatine, [$(NH_2(CH_2)_4NH_2C(NH=)NH$] is an endogenous substance synthesized from L-arginine by arginine decarboxylase (ADC)[18] and is present in the mammalian brains[18,19,20]. Previous studies have shown that agmatine may be neuroprotective in *in vitro*[21]and *in vivo* ischemia models[22]. It is not a precursor for

NOSs, but is rather a weak competitive inhibitor of various NOS isozymes[23,24]. NOSs generate nitric oxide (NO) by sequential oxidation of the guanidinium group in L-arginine, and agmatine is an L-arginine analogue with a guanidinium group[25,26]. This suggests that agmatine may protect the brain from ischemic injury by interfering with NO signaling. Also, we have previously studied and reported the effects of agmatine on the MCAO (middle cerebral artery occlusion) model in mice and shown that agmatine contributes to neuroprotection from ischemic injury[27]. NO production synthesized by NOS was suppressed by agmatine in hypoxic-ischemic brain injury model and some reports have indicated the interaction of agmatine and many apoptotic proteins as well as NO[28,29].

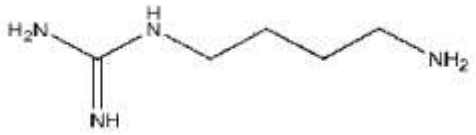


Figure 1. Structure of agmatine

A typical feature of global ischemia is that the ischemia is of brief or intermediate duration, thus allowing recirculation and long-term recovery[8,30,31]. Global ischemia, as occurs in cardiac arrest, can be sustained only for periods of up to 12 minute[32]. Transient global cerebral ischemia leads to cell death of hippocampal CA1 pyramidal neurons starting 2-3 days after reperfusion[33,34]. This phenomenon is commonly referred to as

selective neuronal vulnerability of delayed neuronal cell death. Several mechanisms such as glutamate excitotoxicity, calcium overload, free radical injury, and disturbance in protein synthesis have been implicated in ischemia-mediated neuronal cell death and transcriptional activation of various genes has been demonstrated in postischemic neurons[35,36,37].

In this study, we investigated the effects of agmatine and its NO regulation according to the occlusion and reperfusion time on the neuronal death of transient global cerebral ischemia in rats using immunohistochemical and Western blot analysis. In addition, we have demonstrated the relationship of NO and calcium influx with agmatine treatment elucidating the function of endoplasmic reticulum (ER).

II. Materials and Methods

1. Animals

Male Sprague Dawley rats 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 the NIH guidelines. Male Sprague Dawley rats weighing 300-350g were used. Anesthesia was induced with 3% isoflurane, 70% N_2O, 30% O_2 and maintained with 2% isoflurane. The left femoral artery was cannulated to monitor arterial blood pressure, blood gases and blood glucose concentration. Rectal temperature was monitored continuously and maintained at 37±0.5℃ using a heating blanket (Homeothermic Blanket Control Unit, Harvard apparatus; Edenbridge, UK).

2. Global cerebral ischemia

Before the experiment, food was withheld overnight, but water was freely available. The animals were anesthetized with 3% isoflurane and maintained during surgery at a level of 2% isoflurane in 70% N_2O and 30% O_2 with spontaneous breathing. Twenty minutes of transient global ischemia was induced by 4 vessel occlusion (4-VO) using the method described originally by Pulsinelli et al. (1979)[38]. In brief, after the animals were positioned

in stereotaxic ear bars (Kopf Tujunga, CA) with the head tilted down at ~30 to the horizontal, an incision of 1 cm in length was made behind the occipital bone directly overlying the first two cervical vertebrae. The paraspinal muscles were separated from the midline, and the right and left alar foramina of the first cervical vertebrae were exposed with the use of an operating microscope. A 0.5-mm diameter electrocautery needle was inserted through each alar foramen and both vertebral arteries electrocauterized and permanently occluded. Next, both common carotid arteries were isolated via a ventral, midline cervical incision. An atraumatic arterial clasp was loosely placed around each common carotid artery without interrupting the carotid blood flow and the incision was closed with a single suture. On the following day, 20 min of 4-VO ischemia was induced by tightening the clap around the common carotid arteries. Carotid claps were then removed. Sham-operated animals that underwent surgery were used for non-ischemic control.

The rectal temperature was controlled at 37±0.5℃ during surgery with a feedback-regulated heating blanket. The femoral artery was exposed and catheterized with a PE-50 catheter to allow continuous recording of arterial blood pressure and withdrawal of blood samples for blood gas analysis. After the arterial blood pressure was recovered, the arterial blood was collected for blood gas analysis.

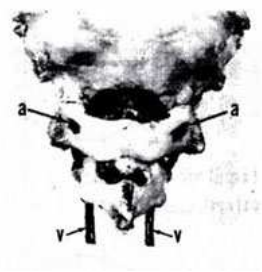


Figure2 Dorsal view of rat skull plus first and second cervical vertebrae. a. alar foramina of first cervical vertebrae, v. vertebral arteries as they pass rostrally through vertebral canal and beneath alar foramina.

3. Agmatine administration

Agmatine was purchased from Sigma (St. Louis, MO, USA), and dissolved in normal saline and given as 100 mg/kg injections by the intraperitoneal route, at the same time of reperfusion[27]. Control was received normal saline in equivalent volumes.

4. Histological assessment of ischemic injury

For histological and immunohistochemical study, 6, 24, 48 and 72 hours (each n = 5) after reperfusion, the animal were perfused transcardially with 200 ml of saline and then 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH7.4, and killed by decapitation. Brains were removed and postfixed in the same paraformaldehyde solution for days and then embedded in paraffin, and 5 μm sections through the dosal hippocampus (anteroposterior coordinate, bregma - 3.0mm) were cut on a microtome. For histological assessment of damage in the hippocampus, the paraffin embedded brain sections were stained with hematoxylin and eosin

(H&E). Neuronal counts in a predesignated region of CA1 were obtained from four to six animals per condition.

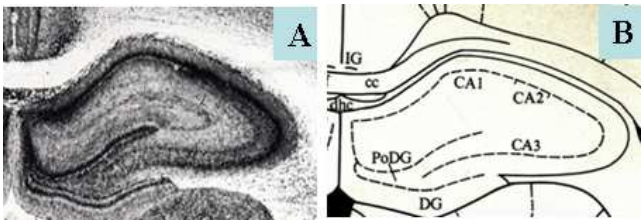


Figure 3. The CA1, CA2 region of hippocampus

5. Western blot analysis

For western blot analysis, animals were killed and decapitated 6, 24, 48 and 72 hours (each n = 4) after exposing to global ischemia. Brains were perfused through the heart arota with cold PBS to rinse out blood, and protein was extracted, as previously described[37]. The region of hippocampus and cerebral cortex in rat brain was dissected for extraction of cellular proteins, treated with 10× lysis buffer containing 1× PBS, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, proteinase inhibitor-PMSF, Aprotinin and Sodium orthovandate) and isolated protein used homogenizer (Dramel, Racine WI, USA). The homogenate was centrifuged at 13000rpm for 17 min at 4℃. The determination of protein concentration in the supernatant was performed by the BCA method (PIERCE, Rockford, USA).

Table 1. Antibodies used for immunohistochemistry and Western blot

Protein	Format	Concentration Immuno/WB	Manufacturer
nNOS	Rabbit polyclonal	1:200/1:500	Upstate[a]
iNOS	Rabbit polyclonal	1:5000/1:5000	Chemicon[b]
eNOS	Rabbit polyclonal	1:500/1:1000	Transduction Lab[c]
Nitrotyrosine	Rabbit purified IgG	1:200/ -	Upstate
NF-kB	Rabbit polyclonal	1:500/1:1000	Santa Cruz[d]
MMP-2	Rabbit polyclonal	- /1:2000	Chemicon
MMP-9	Rabbit polyclonal	- /1:2000	Chemicon
Hsp70	Mouse monoclonal	1:500/1:1000	Stressgen[e]
actin	Mouse monoclonal	- /1:1000	Santa Cruz
Grp78	Rabbit polyclonal	1:500/1:1000	Santa Cruz
GADD153	Mouse monoclonal	1:500/1:2000	Santa Cruz
Bcl-2	Rabbit polyclonal	- /1:1000	Santa Cruz

Immuno, Immunohistochemistry; WB, Western blot.

[a]Lake Placid, NY.

[b]Temecula, CA.

[c]Hercules, CA.

[d]Santa Cruz, CA.

[e]Victoria, Canada.

To confirm the expression of proteins, Western blots were performed as previously descibed[37]. Western blot analysis was performed using primary antibodies (Table 1). Equal amounts of protein, 40 g per condition, were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and

electrotransferred onto Immobilon-NC membrane (Milipore, MA), which were probed with primary antibody at 4℃ overnight and secondary antibody at room temperature for 1 hour. To reduce nonspecific antibody binding, the membranes were blocked with 5% nonfat dry milk for 1 hour at room temperature and then incubated for 24 hours at 4℃ with primary antibodies. After three washes with TBS containing 0.05% Tween 20, immunoreactive signals were visualized by chemiluminescence, the ECL detection system (Amersham Life Science, Buckinghamshire, UK) using Kodak X-AR film. Immunoblot signals were quantified using a computer program (GelScopeTM, Imageline INC, CA, USA).

6. Immunohistochemistry

Brain sections were fixed with 4% paraformaldehyde and rehydrated. Sections were immunostained with primary antibodies followed by an appropriate biotinylated secondary antibody. Stains were visualized using the ABC kit (Vector Laboratories Inc, CA) (Lee et al., 2001)[37], then reacted with diaminobenzidine as substrate (DAB, St. Louis, Sigma) and counterstained with hematoxylin. Results were recorded with a DP-70 digital camera (Olympus, Melville, NY). Controls included omission or preabsorption of the primary antibody or omission of the secondary antibody.

7. Detection of nitric oxide (NO) production

To assess an amount of NO production in tissue samples, the stable end-products of NO metabolism, peroxynitrite was measured using anti-nitrotyrosine (Upstate, MA). Brain sections were fixed with 4% paraformaldehyde, rehydrated and immunostained with anti-nitrotyrosine (1:200, Upstate, MA) followed by an appropriate biotinylated secondary antibody. Then, reacted with diaminobenzidine as substrate (DAB, St. Louis, Sigma).

8. Transmission electron microscopy (TEM)

For electron microscopy, animals were killed and decapitated 24 hours (each n = 3) after exposing to global ischemia. Brains were perfused through the heart arota with 2.5% glutaraldehyde and 2% paraformaldehyde. The brain sections were immersed in a solution containing 3% glutaraldehyde in 0.1M cacodylate buffer for 1 h, rinsed in cacodylate buffer and post-fixed for 1 h in a 1% OsO4 solution in the same buffer. The fixed tissue blocks were then dehydrated through gradient concentrations of ethanol, transferred to propylene oxide, and the specimens were embedded in Epon 812/Araldite. One-micrometer sections were cut with glass knives and stained with toluidine blue (0.1% aqueous solution). Thin sections for ultrastructural evaluation were cut on an LKB ultramicrotome with a diamond knife, contrasted with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop 102.

9. Cell counting and Statistical analysis

Cell counting was performed in 2 coronal brains slices (4 hippocampi, approximately at 1.0 and 2.0 mm posterior from the bregma) for each animal, and performed using the light microscope equipped with a 10× objective by independent observers in a blind manner, and the results were averaged.

All data were presented as mean ± SEM. Statistical comparison between different treatments was done by Student's ***t***-test. Differences with ***P*** value $<$ 0.05 were considered statistically significant. Standard statistical tests were used to determine differences between groups using Sigma, ST. Louis, Missouri, USA Stat (Jandel Corp., San Rafael, CA, USA).

III. Results

1. Agmatine protects many neurons in rat brains after transient global ischemia

To investigate the neuroprotective effect of agmatine on global cerebral ischemic injury, 4-vessel occlusion (4-VO) rat model was prepared. Agmatine (100 mg/kg of rat) was injected intraperitoneally into the rat brain ischemic injured. The effect of agmatine was measured by staining coronal section of rat brain with H&E.

Rat global cerebral ischemia model occluded for 20 min was damaged severely in the hippocampus among 10, 20 and 30min occlusion time. It was impossible to access an evaluation of neuronal damage in 30 min occlusion group because of its very high motality. H&E staining demonstrated that many neurons showed ischemic changes such as pyknosis and cell shrinkage in the hippocampal CA1 region after 20 min of occlusion (Figure 4). In the CA3 (not shown) and dentate gyrus (DG) region, no remarkable ischemic change was observed after transient global ischemia. On the other hand, agmatine treatment group have shown neuroprotective effect exposed to 20 min of occlusion (Figure 4).

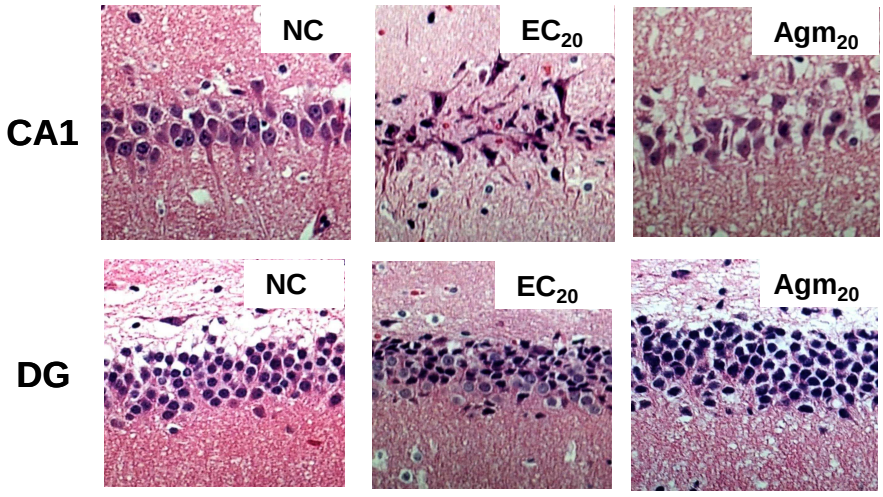


Figure 4. The effect of agmatine following different time of occlusion (original magnification, ×400). Cells in CA1 and CA2 (not shown) region were more sensitive that those in CA3 (not shown) and DG (dentate gyrus) to agmatine treatment after global ischemia. There were no significantly morphological differences in both CA3 and DG. NC; normal control, EC_{20}; 20 min of occlusion, Agm_{20}; EC_{20} with agmatine treatment.

2. The effect of agmatine on the delayed neuronal death after transient global ischemia

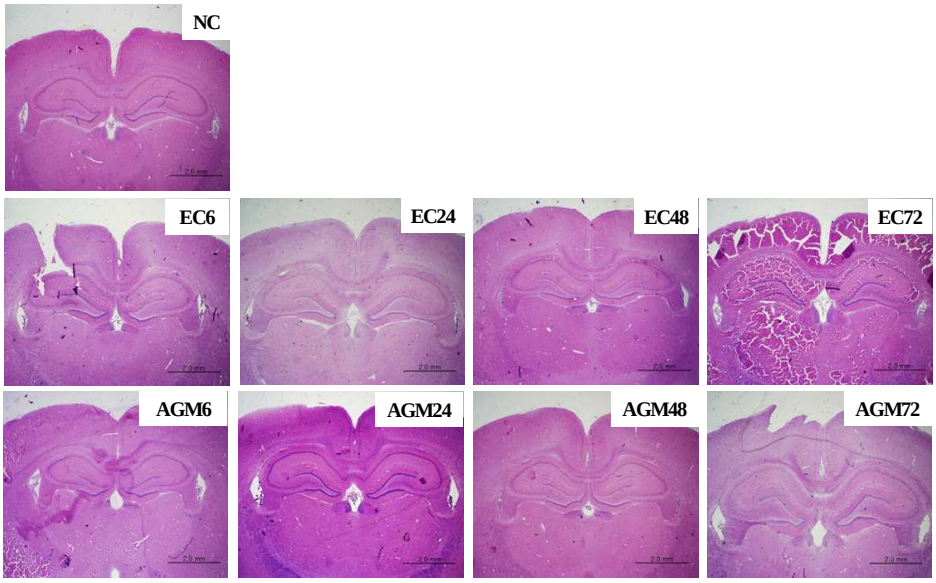
Ischemic neuronal damage in hippocampal neurons with agmatine treatment following different time of reperfusion in global cerebral ischemia, as evaluated by H&E stanining, was less than that in experimental control groups (Figure 5). H&E staining demonstrated that only a few neurons showed ischemic changes in EC6 . Almost all neurons showed ischemic changes in the CA1 region with time dependent manner and many neurons died in the CA1 subfield of EC72, whereas neurons of agmatine treatment groups were shaped like normal neurons (Figure 5B). In addition, the number of H&E positive cells was the highest in AGM24 (Figure 5A).

3. Long-term effect of agmatine after global ischemia

To investigate the side effect of agmatine, we studied long-term survival changes with and without agmatine treatment. For mortality assessment following to different time of occlusion, the animals were scrificed 10 min, 20 min, and 30 min of occlusion. For long- term effect of agmatine study, the animals were sacrificed 24, 72, and 168 hours (each n = 3) after reperfusion.

With 4-vessel occulsion, animal model was shown high mortality differently with other ischemic animal models (Table 2). The percentage of motality was 36.4, 45.5, and 66.7 % in $EC_{10,}$,$EC_{20,}$ and EC_{30} group respectively. In the other hand, the percentage of

A



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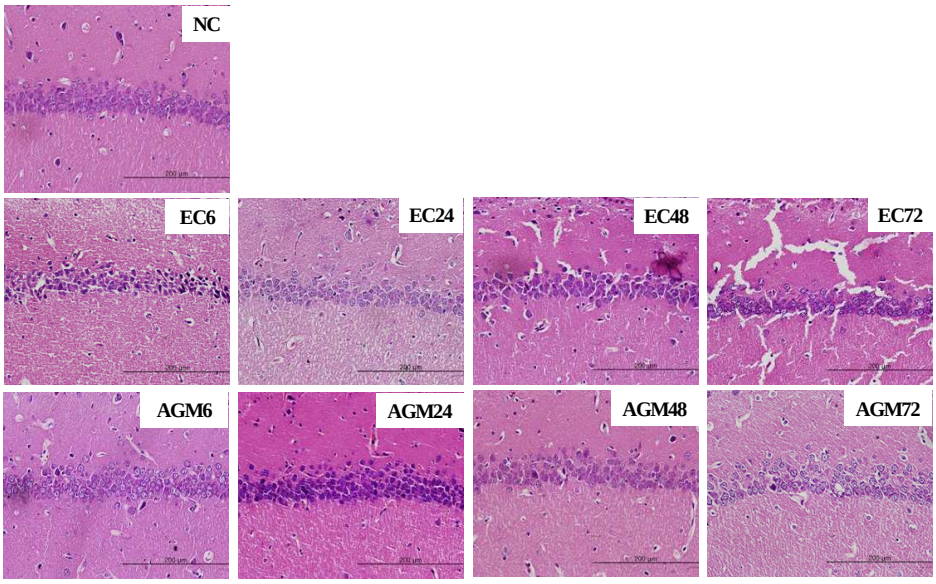

Figure 5. Histological evidence for agmatine mediated protection of CA1 hippocampal neurons following different time of reperfusion in

global cerebral ischemia. (A) Sections are shown at low (×20) power and (B) Sections are shown at high (×400) power, after the induction of ischemia without or with agmatine. NC; normal control, EC6; 6 hr after reperfusion, EC24; 24 hr after reperfusion, E48; 48 hr after reperfusion, EC72; 72 hr after reperfusion, AGM6; EC6 with agmatine treatment, AGM24; EC24 with agmatine treatment, AGM48; EC48 with agmatine treatment, AGM72; EC72 with agmatine treatment.

motality was 28.6, 37.5, 40 % in $Agm_{10,}$ $Agm_{20,}$ and Agm_{30} group respectively. Motality was higher in experimental control group than in agmatine treatment group. Agmatine reduced not only the neuronal death induced by 4-VO model following different time of occlusion and reperfusion, but also ameligrated the animal survival following different time of occlusion after transient global ischemia.

For long - term effect of agmatine study, agmatine was daily injected as 100 mg/kg by I.P. Agmatine treatment group was treated with agmatine at the same time of reperfusion at the first day, and it was given daily after 24 hours. Withdrawal from 24, 72, and 168 hours (each n = 3) of continuous agmatine administration had showed no remarkable changes as compared with normal control. The body weight was increased steadily both NC and agm group, and body temperature was always maintained within 37±0.5℃ (Figure 6). Agmatine did not affect food intake and body temperature of animals in all groups. It has been proven to exhibit

virtually low systemic toxicity.

Table 2. Mortality of 4-vessel model in transient global ischemia

Group	Total animal	survival animal	% of motality
EC_{10}	11	7	36.4
EC_{20}	11	6	45.5
EC_{30}	9	3	66.7
Agm_{10}	7	5	28.6
Agm_{20}	8	5	37.5
Agm_{30}	5	3	40

NC; normal control, EC_{10}, ,EC_{20}, EC_{30}; 10 min, 20 min, 30 min of occlusion respectively, Agm_{10}, Agm_{20}, Agm_{30}; EC_{10}, ,EC_{20}, EC_{30} with agmatine treatment.

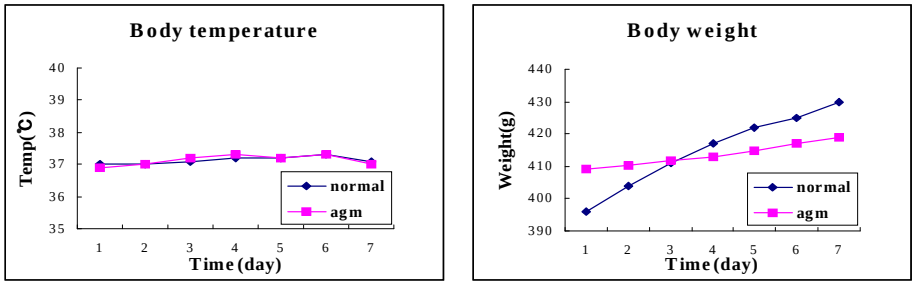

Figure 6. Long-term survival changes with and without agmatine treatment. Agmatine was daily injected as 100 mg/kg by I.P and the animals were sacrificed 24, 72, and 168 hours (each n = 3) after reperfusion. NC; normal control, agm; agmatine treatment.

4. Agmatine has shown the neuroprotective mechanism by suppressing the expression of nitric oxide synthase

The mechanism of neuroprotective effect by agmatine with nitric oxide was investigated in some ways; by assessing protein levels of three isoforms of nitric oxide synthase (NOS), by histological assessment of three isoforms of NOS and the formation of peroxynitrite by a reaction of NO.

To study the effect of agmatine on nitric oxide, the expression of nNOS, iNOS and eNOS was examined in the hippocampus and cerebral cortex of the rat brain after 20 min occlusion of 4-VO global model. Animals were killed and decapitated 6, 24, 48 and 72 hours (each n = 4) after exposing to global ischemia.

The level of nNOS in Agm group subjected to 6, 24, and 48 hr of reperfusion was higher than experimental control (EC) groups (Figure 7). However, its level was lowered 72 hr after reperfusion with agmatine treatment as compared to EC72 group. The level of iNOS in EC6 group increased 226 % of AGM6 group and remained higher than AGM24, AGM48 and AGM72 group. iNOS level of agmatine treatment was constantly decreased with the passage of time (Figure 7). In contrary, the level of eNOS was not significantly different from the normal control (NC). Its level was slightly higher in AGM48 and AGM72 group than in EC48 and EC72 group, respectively (Figure 7).

The level of nNOS was increased in AGM6 and AGM24 than in EC6 and EC24, respectively, whereas its level was decreased in

AGM48 and AGM72 compared to EC48 and EC72, respectively. iNOS protein level was induced after ischemia-reperfusion, agmatine treatment with 100 mg/kg, I.P blunted this induction especially in AGM72 group. On the other hand, eNOS protein level was similar to NC. Its level was slightly increased in AGM48 and AGM72 group than in EC48 and EC72 group, respectively (Figure 8).

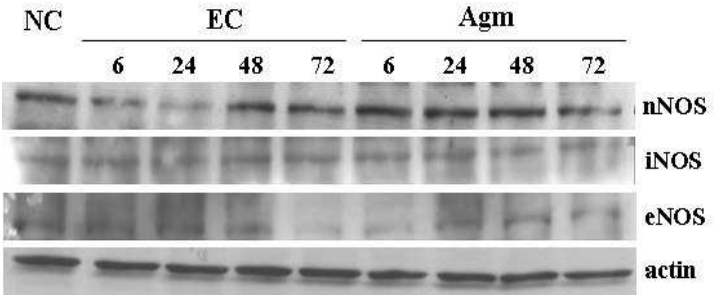

Figure 7. The expression of nNOS, iNOS and eNOS in the rat hippocampus using Western blot analysis. nNOS level was increased with agmatine (100 mg/kg, I.P), but its level was lower in AGM72 than in EC72. The level of iNOS was decreased by agmatine treatment following time course. eNOS level was more activated with agmatine in rat hippocampus after global ischemia.

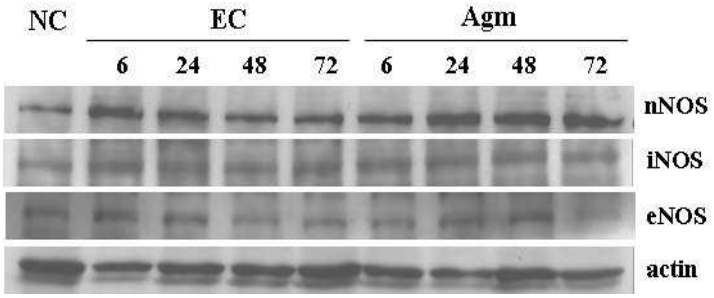

Figure 8. The expression of nNOS, iNOS and eNOS in rat cerebral cortex using Western blot analysis. The levels of nNOS and iNOS were shown similar patency in cerebral cortex compared to the rat hippocampus. On the other hand, eNOS protein level was expressed isocratically and slightly increased in AGM48 and AGM72 group than in EC48 and EC72 group, respectively.

5. Immunolocalization for nNOS, iNOS and eNOS after transient global ischemia

We investigated the immunoreactivity of nNOS, iNOS and eNOS in the hippocampus after transient global ischemia using immunohistochemistry.

The number of nNOS positive neurons was significantly increased in EC6 and decreased in AGM24, and there were only a few scattered nNOS positive neurons in AGM72 group in the hippocampal CA1 subregion (Figure 9). Similarly, occasional damaged and iNOS positive many neurons were observed in all experimental control groups. The number of iNOS positive cells was effectively decreased in AGM24 and AGM48 group (Figure 10). In contrast, the number of eNOS positive neurons was significantly unchanged in all groups (Figure 11). Intraperitoneal administration of agmatine (100 mg/kg) at the same time of reperfusion reduced immunoreactivity for nNOS and iNOS expression after ischemia; the maximun reduction of the immunoreactivity for active nNOS was obtained in AGM24, and the maximun reduction of the immunoreactivity for active iNOS was also shown in AGM24 group. Few neurons in the hippocampus showed immunoreactivity for active eNOS both experimental control groups and agmatine treatment groups.

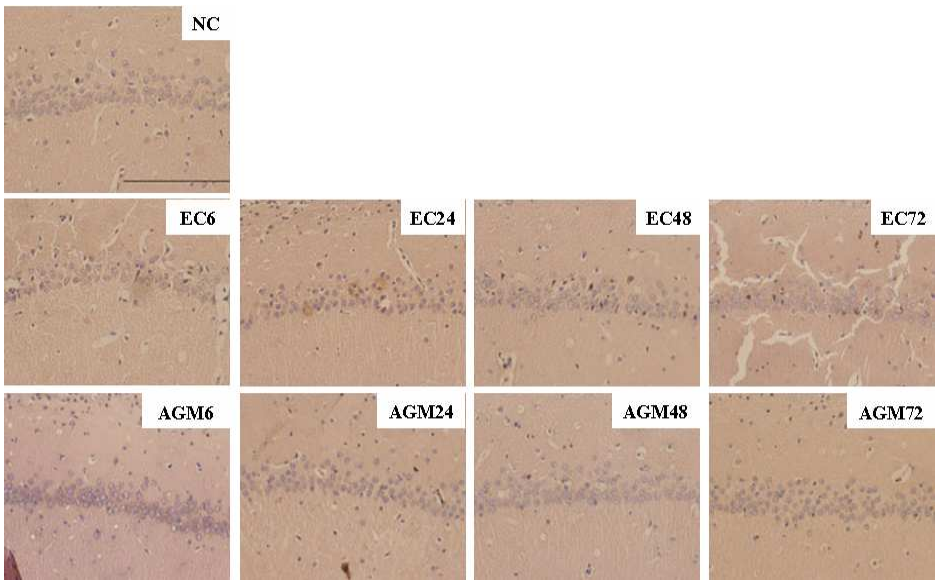

Figure 9. Representative photomicrographs of immunohistochemical study for nNOS in CA1 of the hippocampus after global brain ischemia following reperfusion time course. The number of nNOS positive neurons was significantly decreased in AGM24. Scale bar, 200 μm.

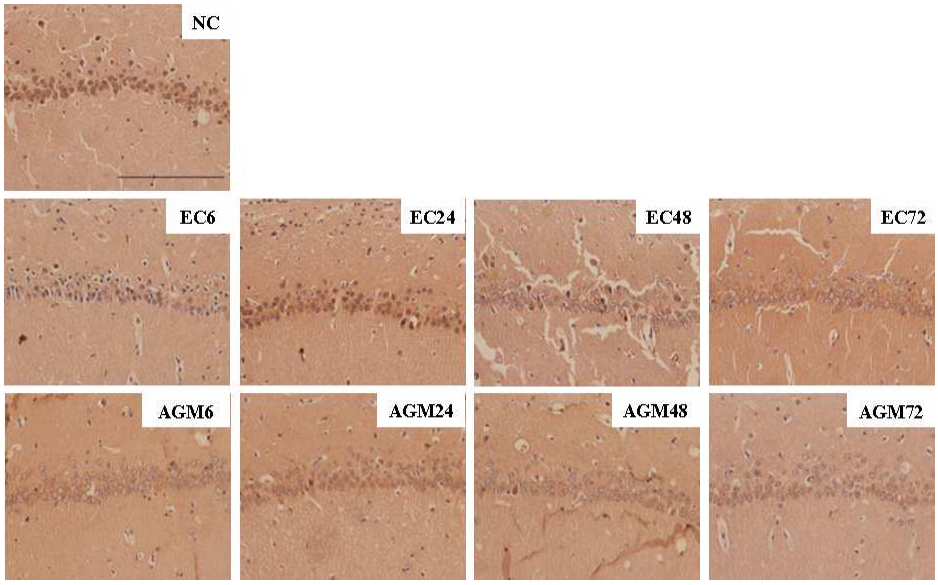

Figure10. Representative photomicrographs of immunohistochemical study for iNOS in CA1 of the hippocampus after global brain ischemia following reperfusion time course. The number of iNOS positive neurons was also significantly decreased in AGM24. Scale bar, 200 μm.

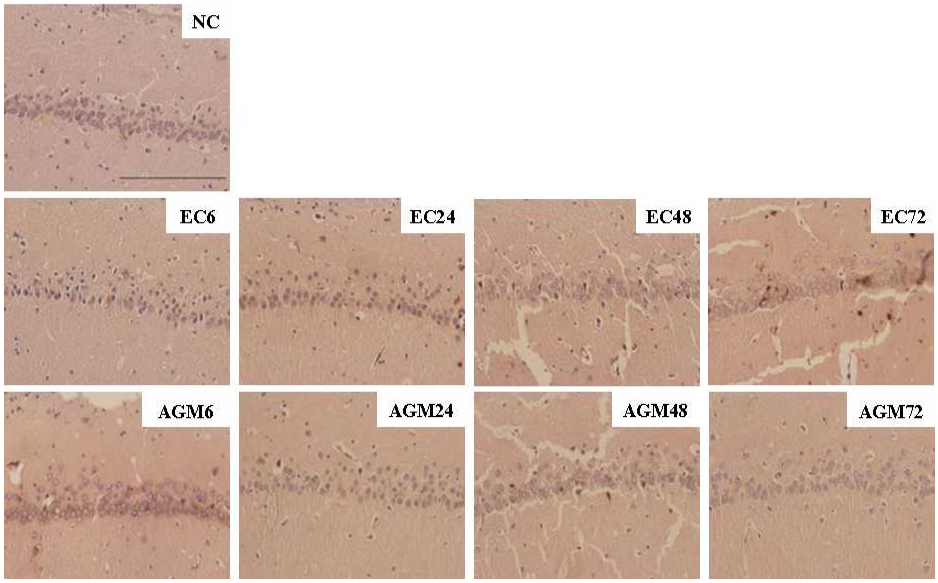

Figure11. Representative photomicrographs of immunohistochemical study for eNOS in CA1 of the hippocampus after global brain ischemia following reperfusion time course. Differently with nNOS and iNOS, the number of eNOS positive neurons was unchanged in all groups. Scale bar, 200 μm.

6. Blockade of nitrotyrosine production by agmatine after transient global ischemia

To assess an amount of NO production in tissue samples, We also examined the immunoreactivity for 3-nitrotyrosine, a marker of peroxynitrite, the reaction product of NO and superoxide.

Global ischemia induced a pronounced increase in nitrotyrosine expression in pyramidal neurons of CA1 in the hippocampus. Nitrotyrosine expression was significantly increased in CA1 neurons at 6 hr postischemia and was further increased at 24 hr after reperfusion, and maintained 48 and 72 hr postischemia in EC groups (Figure 12). On the contrary, the increase of nitrotyrosine expression was dramatically suppressed in agmatine treatment groups, particularly in AGM24, AGM72 in the hippocampus (Figure 12) and in the cerebral cortex (Figure 13), suggesting that agmatine attenuated ischemia/reperfusion injury through blockade nitric oxide production.

7. Agmatine has shown the neuroprotective mechanism by inducing the expression of NF-kB and Hsp70 and by suppressing MMP-9 expression after global ischemia

To examine whether the neuroprotective mechanism of agmatine regulated by NF-kB, MMP-2, MMP-9 and Hsp70 in rat brain after global ischemia, we performed an immunoblotting analysis and immunolabeling.

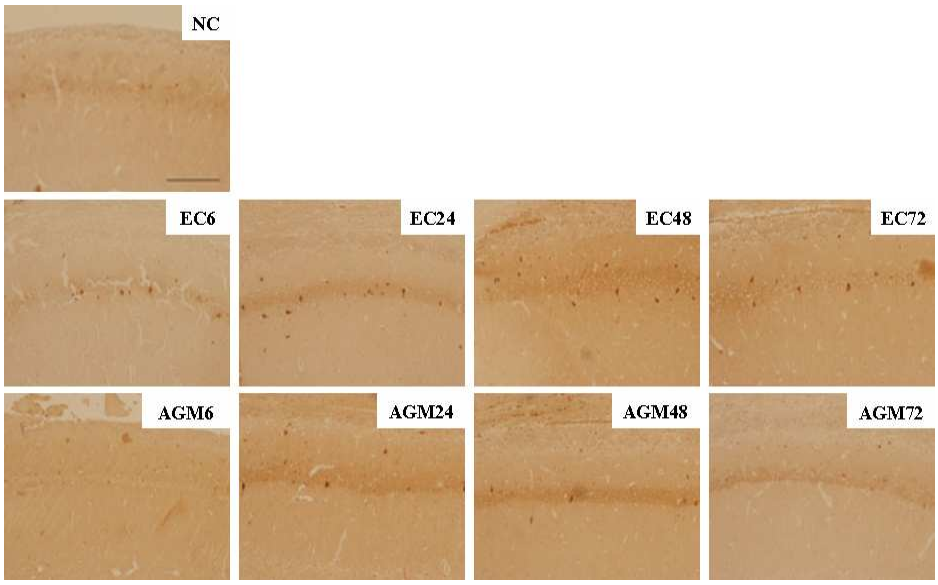

Figure 12. Nitrotyrosine immunostaining of postischemic CA1 neuron in rat hippocampus following reperfusion time course. Nitrotyrosine expression was significantly decreased by agmatine in AGM24, AGM72 group. Scale bar, 100 μm.

The level of NF-kB in Agm group subjected to 6, 24, and 48 hr of reperfusion was higher than experimental control (EC) groups especially in AGM6 and AGM24 (Figure 14A). However, its level was lowered 72 hr after reperfusion with agmatine treatment as compared to EC72 group. The level of MMP-2 was induced by global cerebral ischemia, but MMP-2 level was not significantly different among EC and AGM groups (Figure 14A). MMP-9 level was higher in EC48 than in AGM48 group, and its level was suppressed by agmatine in AGM72 group (Figure 14A). The level

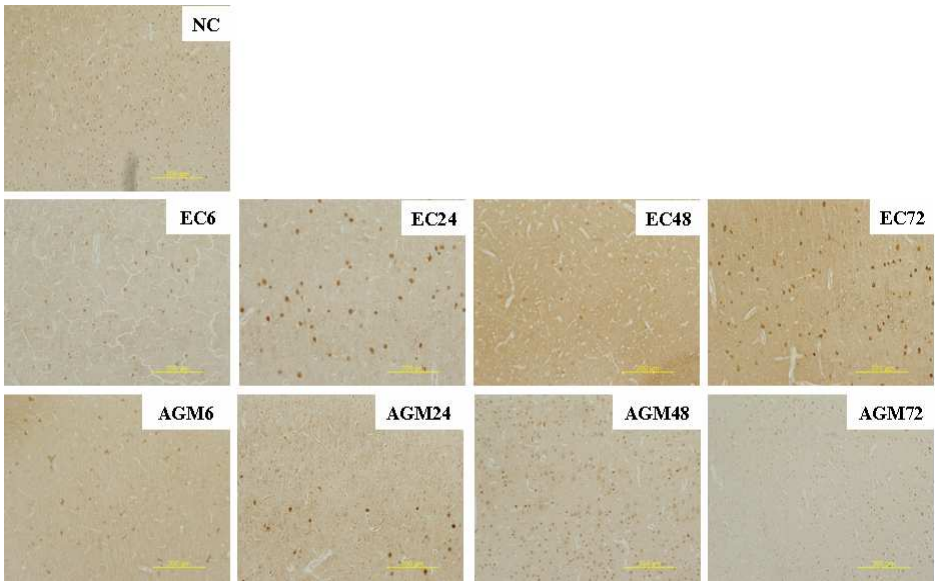

Figure 13. Nitrotyrosine immunostaining of postischemic CA1 neuron in the cerebral cortex following reperfusion time course. The increase of nitrotyrosine expression was dramatically suppressed by agmatine in AGM24, AGM72 group. Scale bar, 200 μm.

of Hsp70 was increased and highly maintained its level in all groups (Figure 14B).

The level of NF-kB was increased in EC6 and EC24 than in AGM6 and AGM24, respectively, while its level was decreased in EC48 and EC72 compared to AGM48 and AGM72, respectively in the cerebral cortex. There was no remarkable differences of MMP-2 protein level after ischemia-reperfusion. On the other hand, MMP-9 protein level was the highest in EC48 group and it was decreased steadily by agmatine treatment. The level of Hsp70 was strongly

high differently from other proteins, and it was higher in agmatine treatment groups than in experimental control groups, especially AGM48 and AGM72 groups.

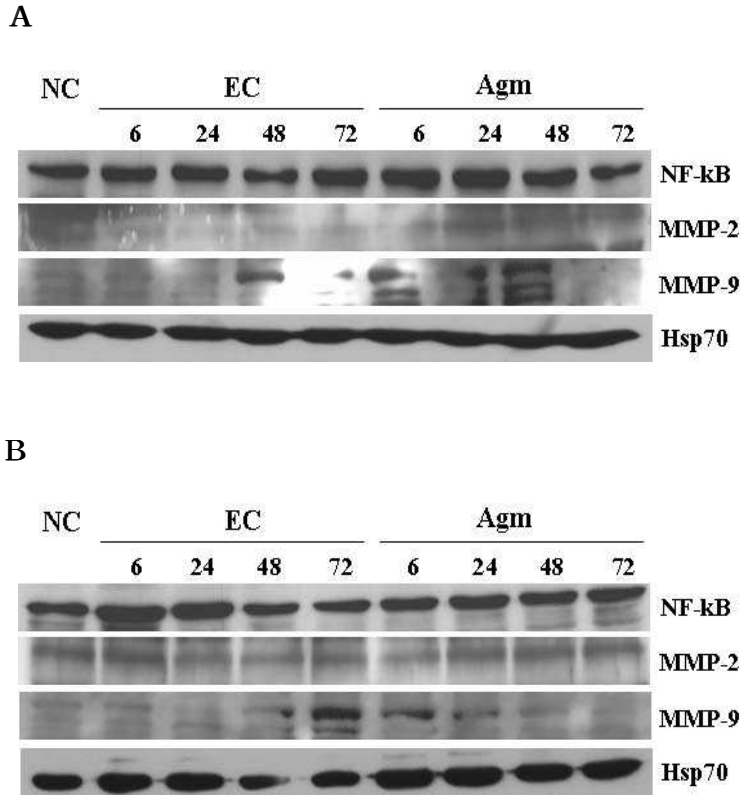

Figure 14. The expression of NF-kB, MMP-2, MMP-9 and Hsp70 in the rat hippocampus (A) and in the cerebral cortex (B) using Western blot analysis. (A) NF-kB level was increased in AGM6 and AGM24. The level of MMP-2 was not significantly different both EC and AGM groups. MMP-9 level was suppressed by agmatine in AGM72 and the level of Hsp70 was highly maintained in all groups. (B) The level of NF-kB was increased in EC6 and EC24, while it was decreased in EC48 and EC72. MMP-2 protein level was shown no changes after global ischemia. MMP-9 level was decreased steadily by agmatine treatment. The level of Hsp70 was higher especially in AGM48 and AGM72 groups.

8. Immunolocalization for Hsp70 in hippocampal CA1 neurons after transient global ischemia

Chaperones, especially the stress inducible Hsp70, have been studied for their potential to protect the brain from ischemic injury. The number of Hsp70 positive neurons was increased in EC6, and there were a few scattered Hsp70 positive neurons in AGM72 and EC72 group in the hippocampal CA1 subregion. Interestingly, AGM24 and AGM48 have shown a strongly positive immunireaction for Hsp70. In contrary, there were no Hsp70 positive cells in EC24 and EC48 group (Figure 15).

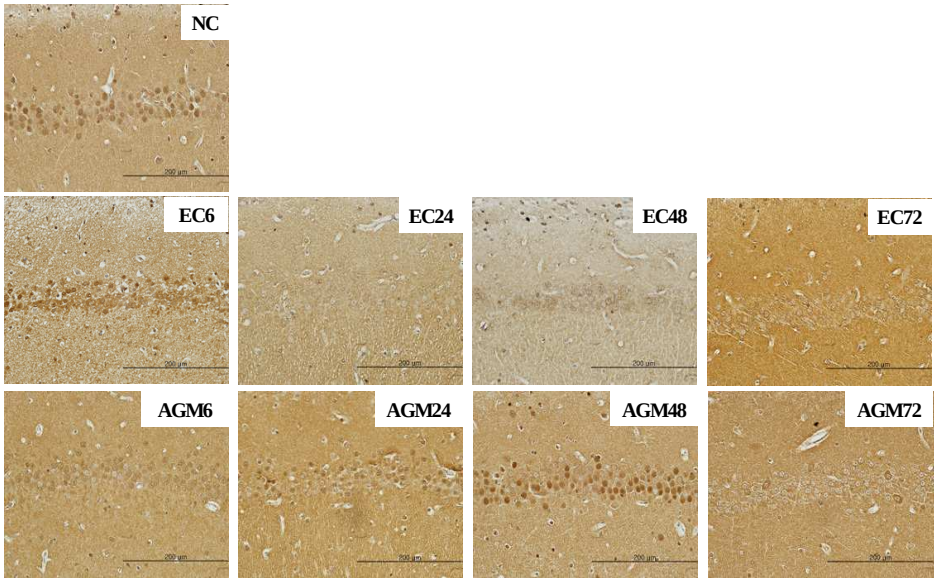

Figure 15. Immunohistochemical localization of Hsp70 in the hippocampal CA1 region following reperfusion time course. The number of Hsp70 positive neurons was increased in EC6. Interestingly, AGM24 and AGM48 have shown a strongly positive immunireaction for Hsp70. Scale bar, 200 μm.

9. Agmatine protects neurons from transient global ischemia concerned with ER stress-mediated apoptosis

To investigate the effect of agmatine related to ER stress, the expression of ER stress-mediated proteins, Hsp70, Grp78, CHOP and Bcl-2 were examined in the hippocampus and cerebral cortex of the rat brain after global ischemia by immunoblotting. The inhibiting effect of agmatine on ER stress-mediated neuronal apoptosis was further suppoeted by an electrom microscope study because changes of organelles in ER structure induced by ER stress did not exhibit any ultramicroscopic abnormalities.

The level of Hsp70 was increased and highly maintained its level in all groups (Figure 16A). The level of Grp78 in Agm group after 4-VO global ischemia was higher than experimental control (EC) groups especially in AGM6 and AGM24 (Figure 16A). The level of CHOP was induced by global cerebral ischemia, and CHOP level was more induced in AGM group (Figure 16A). Bcl-2 level was higher in EC48 than in AGM48 group.

The level of Hsp70 was strongly high differently from other proteins, and it was higher in agmatine treatment groups than in experimental control groups, especially AGM48 and AGM72 groups in the cerebral cortex (Figure 16B). The level of Grp78 was increased in agmatine treatment group and its level was the highest in AGM24. CHOP protein level was more induced in AGM group (Figure 16B). Bcl-2 level was higher in EC48 than in AGM48 group.

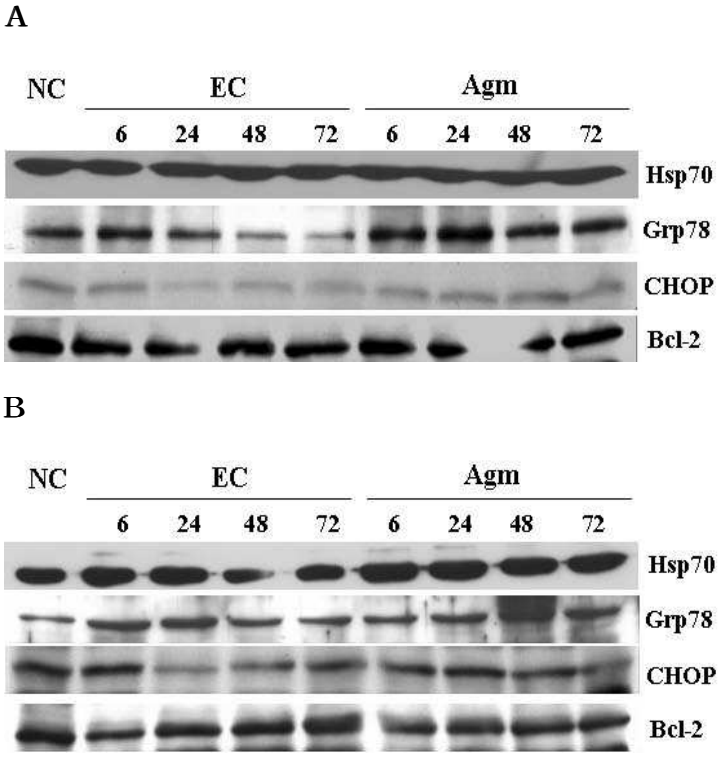

Figure 16. The expression of Hsp70, Grp78, CHOP and Bcl-2 in the rat hippocampus (A) and in the cerebral cortex (B) using Western blot analysis. (A) Hsp70 level was increased in all groups. Grp78 level and CHOP level were higher in Agm group. Bcl-2 level was higher in EC48 than in AGM48 group. (B) Hsp70 level was increased especially AGM48 and AGM72. Grp78 level and CHOP level were increased in Agm. Bcl-2 level was higher in EC48 than in AGM48 group.

10. Electron microscopic study mediated by ER stress for transient global ischemia

Next, we examined whether the effect of agmatine by global ischemia is observed with and without agmatine treatment 24 hr after reperfusion. We performed an electron microscopic analysis with the hippocampal CA1 pyramidal neurons of EC24 and AGM24.

Electron microscopy of normal control revealed that vivid chromatin, distinct nucleus membrane and strong ER structure and Golgi apparatus (Figure 17a, 17d, 17g).

Our EM study indicated significant morphological changes of ER and of mitochondria. In the experimental control, cells showed the typical apoptotic patterns of compaction and segregation of chromatin, destruction of ER lumen and disruption of the mitochondria. After 24 hr of reperfusion without agmatine, swollen rough ER was prevalent (Figure 17b, 17e, 17h). Swollen rough ER and free ribosomes that were released from the ER increased in EC, whereas the mitochondria and ER lumen remained morphologically normal at 24 h of agmatine treatment. Most of the cells in the agmatine-treated rat brain showed no significant morphological differences (FIgure 17c, 17f, 17i). These results suggest that suppression of ER stress by agmatine attenuates the apoptotic process.

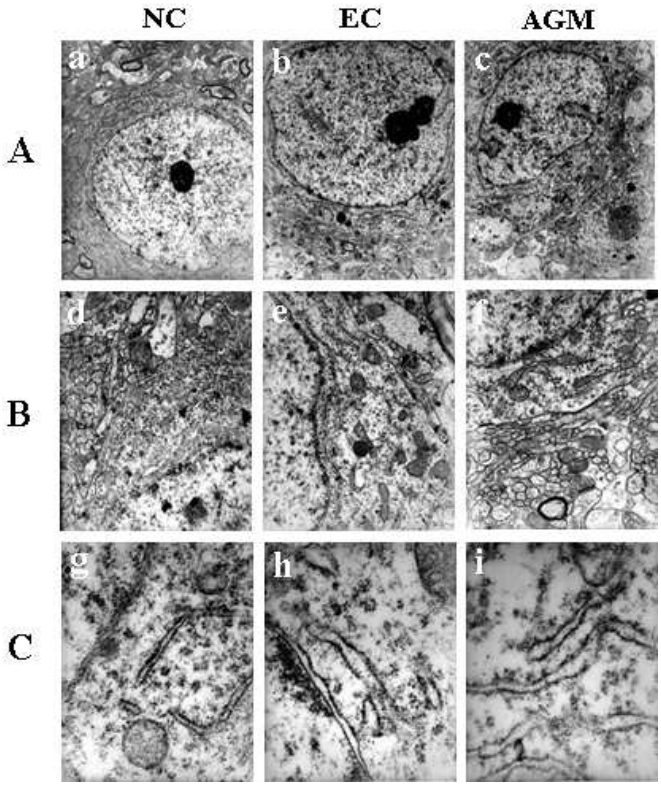

Figure 17. Electron microscopy of cell injury by ER stress following 24h reperfusion in the rat hippocampus obtained by global ischemia. Electron micrographs show ultrastructural alterations in individual neurons in the hippocampus. (a) The control hippocampal neuron had a large nucleus, relatively small cytoplasm, and intact mitochondria, ribosomes on the ER lumen and other organelles. (b) ER structure was severely destructed and many ribosomes were innervated in cytoplasm of EC group. Differently, ER structure of Agm was shown intactly being like normal group. A; ×15960, B; ×37200, C; ×124800 original magnification.

IV. DISCUSSION

In the present study, we used a rat model of transient global ischemia. Twenty minutes of ischemia produced markedly damage to the hippocampus as determined by H&E staining[37]. On the other hand, we showed that intraperitoneal administration of agmatine protected brain from ischemic injury as evidenced by decreasing the level of iNOS, MMP-9 and increasing the level of NF-kB and Hsp70. Simultaneously, we found that agmatine can effectively suppress ER dysfunction in vivo. Moreover, the present findings indicate that the protective effect of agmatine on ischemic injury may be medicated in part by restoration of ER dysfunction.

In cerebral ischemia, apoptosis is caused by excitotoxicity, ionic imbalance, oxidative/nitrosative stress and apoptotic-like pathways[1,4,5,39]. Correlated with theses events, there is an increase in the generation of nitric oxide (NO), resulting from activation of the nitric oxide synthase (NOS) on both resident and infiltrating cells[40]. Excessive generation of NO is thought to play an important role in ischemic injury[41]. Exogenous and endogenous radical scavengers have been demonstrated to suppress nitrosative stress and thus prevent ischemic injury[18,32,34]. Agmatine has been shown to exert a protective effect against cerebral ischemic injury by blocking NMDA receptor activation and inhibiting NOS activity[21,42]. On the other hand, ample evidence suggested that ER damage is

involved in neuronal cell death induced by cerebral ischemia[43,44,45]. Thus, in this study, we investigated the effect of agmatine on ER dysfuction under pathological conditions.

After 20 min occlusion of 4-VO global model, we observed an increase in the level of nNOS by 48 hr and a decrease in the level of nNOS 72 hr after reperfusion. Moreover, we also observed a decrease in the level of iNOS time-dependent manner and no significant change in the level of eNOS. Following our previous report, agmatine decreased infract sizes and appeared to reduce nNOS expression to a greater extent than iNOS in middle cerebral artery occlusion (MCAO) mouse model[27]. In addition, we observed a significant decrease in the number of positive cells of nNOS and iNOS after 24 hr with agmatine treatment.

There are at least three likely mechanisms for agmatine neurorotection. Agmatine has been shown to reduce excitotoxicity in vitro by blocking NMDA receptor activiation[21,42]. Agmatine, an α-2 adrenoceptor agonist has been shown to protect neurons from injury in vivo and in vitro[19,22]. Lastly, it is a NOS antagonist, and generation of NO has been implicated in ischemic brain injury[46].

Agmatine also has shown to be a competitive inhibitor of both nNOS and iNOS[27,47]. In the present study, we found that the induction of nNOS and iNOS by global ischemia was inhibited with the administration of agmatine. Agmatine also effectively reduced the production of nitrotyrosine in the hippocampus and cerebral cortex in this animal model.

An important source of oxidative stress-mediated brain damage is the oxidant reactions due to the formation of peroxynitrite, a powerful oxidant that results from the interaction between NO and superoxide. This anion has been shown to cause cell damage by several mechanisms that include lipid peroxidation, tyrosine nitration, sulfhydril oxidation and nitrosylation, and DNA breakage, etc[8,9].

In contrast to the toxic effects of nNOS and iNOS, eNOS mediates vasodilation, inhibition of platelet aggregation, and leukocyte adhesion to endothelium, all effects that would be protective following cerebral ischemia[6,7,41]. In our findings, there was no significant change in the level of eNOS in Western blot analysis and in the number of eNOS positive cells in immunoreactivity. The result confirms the neuroprotective role of NO from eNOS during cerebral ischemia.

We also observed a decrease in the level of MMP-9 and increased in the level of NF-kB and Hsp70 with agmatine treatment after global ischemia. Matrix metalloproteinases (MMPs) are a family of serine proteases involved in the remodeling of the extracellular matrix[48,49]. MMPs are activated under pathological conditions in the brain, and this can lead to disruption of the blood brain barrier (BBB), serum protein extravasation, and hemorrhage. In fact, MMP-9 was found to play a deleterious role in ischemic pathophysiology as MMP-9-deficient knockout mice had significantly smaller ischemic lesions than their wild type

littermates[50,51]. We previously reported Hsp70 suppresses MMP-2 and -9 expression following ischemia-like insults in cultured astrocytes[52]. In this study, we confirm not only the increase of Hps70 level and the immunoreactivity of Hsp70 to protect neurons from global ischemia, but the effective suppression of MMP-9 by Hsp70.

NF-kB is an inducible transcription factor composed of various combinations of NF-kB/Rel family members[53,54]. Following activation subsequent to cerebral ischemia and reperfusion injury, NF-kB acts on genes for cytokines, adhesion molecules, nitric oxide synthase, cyclooxygenase-2, MMP-9, and perhaps apoptotic genes. These genes have a variety of positive and negative influences on the outcome of brain injury. In neurons, NF-kB supported survival signaling by inducing the expression of anti-apoptotic factors, for example anti-apoptotic bcl-2 family members, manganese superoxide dismutase (MnSOD), and inhibitos of apoptosis (IAP)[55]. The effect of manipulation of NF-kB needs to be examined critically, as it pertains to these reactive genes[56].

In rats under twenty minutes of global ischemia followed by a given period of recovery, we observed an decrease in the level of the transcription factor CHOP and inactivation of Grp78 and Bcl-2 in the ischemic hippocampus and cerebral cortex. The results indicate that global ischemia caused severe ER damage and triggered ER stress-associated apoptosis. On the other hand, treatment with agmatine increased in the level of CHOP and

activated of Grp78 and Bcl-2 in the hippocampus and cerebral cortex. There is no report about neuroprotective mechanism of agmatine related to ER stress yet. These findings would culminate the first one concerning how agmatine protects neurons from ischemic injury associated with ER stress.

ER stress response activates two transcription factors, CHOP and NF-kB, whose role in the stress response has not been as yet fully clarified[15]. C/EBP homologous protein (CHOP), also known as growth arrest and DNA damage-inducible gene 153 (GADD153)[15] was first isolated in a screen aimed at identification of genes responsive to UV treatment, but subsequent studies have recognized this transcription factor to be most sensitive to the alterations in the ER culminating in UPR[57]. Expression of GADD153 is activated by all agents that up-regulate ER chaperons, and, like chaperones, it is induced by ATF6 and ATF4 transcription factors[57]. Several studies have shown a role for GADD153 in negative regulation of cell growth and differentiation, and it has also been implicated in apoptosis induction[58]. One study has indicated that GADD153 sensitizes cells to ER stress by down-regulation of Bcl-2 family and by depletion of glutathione[57,62]. However, it is largely unknown how the changes in expression of these target genes relate to apoptosis in ischemic injury.

Glucose-regulated protein (Grp) 78 is a molecular chaperone involved in the UPR, termed the unfolded protein response in the endoplasmic reticulum (ER) responded to all eukaryotic cells[59]. The

ER chaperone Grp78 binds to the ER luminal domains if both PERK and IRE1, which serves to repress activation of their cytosolic catalytic domains[60]. The increase in Grp78 provides significant cytoprotection against toxic agents[61], including thapsigargin.

Bcl-2 is one among any key regulators of apoptosis, which are essential for proper development, tissue homeostasis, and protection against foreign pathogens[14]. The anti-apoptotic function of Bcl-2 can also be regulated through proteolytic processing and phosphorylation. Bcl-2 may promote cell survival by interfering with activation of the cytochrome c/Apaf-1 pathway through stabilization of the mitochondrial membrane[63,64].

We demonstrated the neuroprotective effects of agmatine in rat global cerebral ischemia by using Western blot analysis, immunohistochemistry when we treated agmatine at the same time of reperfusion. Agmaine have many possible pathway to inhibit the neuronal cell death, block NMDA channel, competitive inhibitor of NOS, and so on. We focused on the relation of agmatine to NOS and NO. In addition, we focused on the possible protective effect of agmatine against ER dysfunction related NO. We obtained evidence that agmatine significantly inhibited ER stress-mediated apoptotic signals induced by transient global ischemia and attenuated ER stress in global ischemia.

Agmatine is an amine and organic cation formed by the decarboxlyation of L-arginine by the enzyme arginine

decarboxylase (ADC)[18]. While long recognized to be synthesized and stored in plants, bacteria, and invertebrate[18], agmatine and its biosynthetic enzyme were recently discovered in mammals[18,19,20], originally in rat brain and later in other tissues and serum. Agmatine binds to imidazoline and α2-adrenergic receptors and has been proposed as an endogenous ligand for imidazoline receptors[19,65]. Although the study of the possible physiological functions of agmatine in the brain is still in its infancy, accumulating evidence indicates several levels of pharmacological and physiological importance.

Agmatine has recently been shown to inhibit nitric oxide snthase (NOS) in isolated rat arota[23] and in rat brain[19,66]. In fact, the inhibitory effect of agmatine on NO synthesis has been implicated in its inhibitory effect on morphine abstinence syndrome and in its antidepressant effect[23,24]. Agmatine can also antagonize the N-methyl-D-aspartate (NMDA) receptor-induced effects in hippocampal neurons[21,42]. Apart from these neuromodulatory roles, agmatine is a metabolic precursor of polyamines, which by themselves induce various central effects[1].

It is well established that the rat transient global ischemia model mimics the condition after transient cardiac arrest and causes selective neuronal death in vulnerable regions, such as hippocampal CA1 pyramidal cells, Purkinje cells of the cerebellem, and neurons in the third to fifth layers of the cerebral cortex[30,31]. The vulnerability has been arrtibutes to many factors, such as

glutamate neurotoxicity, calcium, expression of cell suicide genes, activation of apoptotic proteins, mitochondrial dysfunction[32]. Global cerebral ischemia triggers activation of apoptotic pathways in neurons destined to die[34,67]. It has been emphasized the critical role of the mitochondrial dysfunction in theses mechanism after recirculation.

The major finding we report is that agmatine, an analog of L-arginine[68,69,] protects brain tissue against ischemic injury. This effect appears to be mediated through different inhibition of three isoforms of NOS, suppression of MMPs by activating Hsp70, induction of CHOP, Grp78 and Bcl-2 associated with ER stress in ischemic insults.

Agmatine, an endogeneous inhibitor of NOS, may be a promising therapeutic target for treatment of cerebral ischemia. Considering the findings of the present study, we speculate that agmatine has multiple effects, although the mechanism of its actions needs to be elucidated further.

V. CONCLUSION

In the present study, we demonstrated that agmatine has neuroprotective effects by inhibiting nitric oxide synthase and on ischemic injury and evaluated the effect of agmatine on ischemic injury in rat global cerebal ischemic model.

These conclusions are supported by the following obsevations.

1. Agmatine treatment attenuates neuronal damage throughout the hippocampus and cerebral cortex after an ischemic insults, persists in the selective vulnerable CA1 subfield, and recovers in the so-called "resistant" areas CA3 and dentate gyrus.
2. Agmatine decreased the level of nNOS and iNOS slightly in immunoblotting.
3. Agmatine suppressed the expression of nNOS and iNOS in immunohistochemical staining at 24 hr after reperfusin, but did not that of eNOS.
4. Agmatine reduced the formation of NO in the immunoreactivity of nitrotyrosine.
5. Agmatine suppressed the level of MMP-9, otherwise induced the level of NF-kB and Hsp70.
6. Agmatine increased the level of Grp78, CHOP and Bcl-2 associated with ER-mediated stress.

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ABSTRACT(IN KOREAN)

전뇌 허혈 동물 모델에서 아그마틴의 신경세포 보호 효과

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문 진 희

허혈성 뇌질환에서 세포사멸은 과독성, 이온 불균형, 산화/질산화 스트레스와 세포사멸 관련 기전에 의해 일어난다. 허혈성 질환이 발생한 후 유도되는 자유이온의 하나가 일산화질소이다. 일산화생성효소에 의해 생산된 일산화질소는 허혈에 따른 신경세포 저해를 일으킨다. 최근 몇 년 동안 소포체 관련 세포사가 전뇌허혈에 중요한 역할을 할 것이라고 보고되어 왔다.

아그마틴은 알기닌 탈탄산효소에 의해 L-arginine으로부터 합성되는 내재성 물질로, 포유류의 뇌 속에도 존재한다. 이전 연구들은 아그마틴의 신경세포보호작용을 세포성 모델과 허혈성 동물 모델로 제시해 오고 있다. 또한, 우리는 중간대뇌동맥결찰 마우스 모델을 제작, 그것을 통해 아그마틴이 허혈성 저해로부터 신경세포를 보호하고 있음을 이미 보고하였다.

4-VO 전뇌 허혈성 동물 모델은 1979년 Pulsinelli et al.의 모델을 참조하여 제작하였다. 동물은 이소푸르란을 이용하여 마취하였고, 아

그마틴은 100 mg/kg 양을 재관류 시작과 동시에 복강주사하였다. 일산화질소생성효소의 발현에 아그마틴이 미치는 영향을 알아내기 위해 재관류 시작 후 6, 24, 48, 72 시간이 경과하면 뇌를 적출하였다. 또한 소포체 관련 칼슘 분비와 아그마틴의 조절 관계를 확인하기 위해 조직면역화학염색법을 실시하였고, 일산화질소생성효소의 발현 정도를 알기 위해 immunoblotting을 수행하였다. 아그마틴 처리는 전뇌허혈성 저해로부터 해마 CA1 구역의 신경세포들을 보호하였고, 일산화질소생성효소의 발현도 저해시켰다. 한편, 아그마틴에 의한 일산화질소생성효소의 발현 조절은 뇌 지역에 따라 차이를 보였다. 아그마틴 처리는 NF-kB, Hsp70와 Grp78의 발현을 유도하는 반면, MMP-9과 MMP-2의 발현은 감소시켰다. 또한, 전자현미경적 분석을 통해 아그마틴 처리군은 실험 대조군보다 소포체와 골지체를 비롯한 세포소기관의 형태가 정상군에 보다 가까움을 알 수 있었다.

이상의 결과들은 아그마틴이 전뇌허혈성 동물 모델에서 3가지 일산화질소생성효소의 발현을 다르게 조절 및 감소시킴으로써 일산화질소의 생성을 억제함을 보여준다. 아그마틴은 전뇌허혈성 저해로부터 신경세포를 보호하기 위해 여러 경로를 통해 MMPs, Hsp70, NF-kB, Grp78 등 anti-apoptotic 단백질과 pro-apoptotic 단백질들의 발현을 조절한다. 또한, 아그마틴은 소포체성 세포사멸과 연관된 허혈성 저해로부터 신경세포를 보호한다.

핵심되는 말 : 아그마틴, 전뇌허혈, 질소생성효소, 신경세포사멸, 소포체성 세포사멸