Neuroprotective effect of agmatine on the murine cortical neurons from ischemia-like condition.

Jae Hwan Kim

Department of Medical Science
The Graduate School, Yonsei University

Neuroprotective effect of agmatine on the murine cortical neurons from ischemia-like condition.

Directed by Professor Jong Eun Lee

The Master's Thesis submitted to
the Department of Medical Science, the Graduate School
of Yonsei University in partial fulfillment of the
requirements for the degree of Master of Medical Science

Jae Hwan Kim

December 2002

This certifies that the Master's Thesis
of Jae Hwan Kim is approved.

Thesis Supervisor : Jong Eun Lee

[Won Taek Lee : Thesis Committee Member#1]

[Soo Chul Park : Thesis Committee Member#2]

The Graduate School

Yonsei University

December 2002

ACKNOWLEDGEMENTS

긴 시간 동안의 노력 끝에 이 논문이 나오기까지 많은 격려를 해주신 아버지와 어머니 그리고 멀리 있는 동생에게 고마움을 전합니다.

항상 옆에서 같이 해준 나의 아내에게도 감사합니다.

늘 함께 해준 친구에게 꼭 감사의 말을 하고 싶습니다.

마지막으로 해부학교실 조직학실험실에서 지난 2년간 함께 해온 실험실 사람들(이성호, 홍종순, 황혜진, 최은정, 윤연정, 안수경, 정효석, 김연정, 최윤정, 최수경, 김종열)에게 감사합니다.

여러 가지로 많은 도움을 주신 신영호 선생님께 감사 드립니다.

보이지 않게 격려를 보내주신 박경아 교수님과 이원택 교수님께 감사를 드립니다.

지금까지 열심히 지도해 주신 이종은 교수님께 감사드립니다.

지금의 내가 있게 해준 모든 것에 진정으로 감사합니다.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT ---------- 1

Ⅰ. INTRODUCTION ---------- 3

Ⅱ. MATERIALS AND METHODS ---------- 5

1. Animals ---------- 5

2. Cell culture ---------- 5

3. Oxygen-glucose deprivation (OGD) ---------- 5

4. Agmatine treatment ---------- 6

5. Evaluation of injury ---------- 7

6. TUNEL staining ---------- 8

7. Concentration of Nitrite (NO_2^-) and Nitrate (NO_3^-) ---------- 8

8. NADPH-diaphorase/NOS activity staining ---------- 9

9. Immunocytochemical staining for nNOS and iNOS ---------- 9

10. Stroke model ---------- 10

11. Assessment of ischemic injury ---------- 12

12. Measurement of cerebral blood flow ---------- 12

13. Statistical analysis ---------- 13

Ⅲ. RESULTS ---------- 14

1. Neuroprotective effect of agmatine *in vitro* system ---------- 14

(1) Measurement of LDH activity ---------- 14

(2) Hoechst-PI nuclear staining ---------- 16

(3) TUNEL staining ---------- 17

(4) Concentration of Nitrite (NO_2^-) and Nitrate (NO_3^-) ---------- 17

(5) NADPH-diaphorase / NOS activity staining ---------- 19

(6) Immunocytochemical staining for nNOS and iNOS ---------- 21

2. Neuroprotective effect of agmatine *in vivo* system ---------- 24

(1) 2,3,5-triphenyltetrazolium chloride (TTC) staining ---------- 24

(2) Hematoxylin-Eosin(H-E) staining ---------- 24

(3) Analysis of infarct area ---------- 25

(4) Measurement of cerebral blood flow (CBF) ---------- 25

Ⅳ. DISCUSSION ---------- 30

Ⅴ. CONCLUSION ---------- 37

REFERENCES ---------- 38

ABSTRACT (IN KOREAN) ---------- 47

LIST OF FIGURES

Figure 1. The scheme of experimental schedule for oxygen-glucose deprivation-- 6

Figure 2. The scheme of experimental schedule for animal ischemic model. ------ 11

Figure 3. LDH assay in primary cultured cortical neurons ---------- 15

Figure 4. Hoechst-PI staining---------- 16

Figure 5. TUNEL staining ---------- 18

Figure 6. Concentration of Nitrite (NO_2^-) and Nitrate (NO_3^-) ---------- 19

Figure 7. NADPH-diaphorase/NOS positive cells staining ---------- 20

Figure 8. nNOS positive cells staining ---------- 22

Figure 9. iNOS positive cells staining ---------- 23

Figure 10. TTC staining ---------- 26

Figure 11. H-E staining ---------- 27

Figure 12. Percentage of infarct area ---------- 28

LIST OF TABLES

Table 1. Relative cerebral blood flow (rCBF) ---------- 29

Abstract

Neuroprotective effect of agmatine on the murine cortical neurons from ischemia-like condition.

Jae Hwan Kim

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Jong Eun Lee)

Agmatine is a primary amine formed by the decarboxylation of L-arginine and is an endogenous clonidine-displacing substance synthesized in mammalian brain. To investigate the neuroprotective effect of agmatine on the transient focal ischemic injury *in vitro* and *in vivo* system, we prepared pure cortical neuronal cell cultures and MCAO suture animal model. Agmatine was treated at 30minutes before ischemic injury (Pre), the same time with ischemic injury (Co), the start time of reperfusion (Post0h), 2hours after the start time of reperfusion (Post2h), and 5hours after the start time of reperfusion (Post5h). Cultured cortical neurons were treated with agmatine (100 μM) as three different conditions-Pre, Co, Post0h- and brain ischemic injured mice were injected with agmatine (100 ㎎/㎏ of mouse, IP) as five different conditions-Pre, Co, Post0h, Post2h, Post5h. Agmatine significantly protect the

cortical neurons from ischemia-like injury (OGD) in the Hoechst-PI nuclear staining and TUNEL staining. The number of nNOS positive cells was decreased after co-treatment with agmatine in the ischemia-like injury (OGD). We have demonstrated that Pre-, Co-, Post0h, and Post2h-treated agmatine reduced the infarct area after ischemic injury in the MCAO suture animal model. The cerebral blood flow (CBF) was monitored during ischemia with and without agmatine. There was no change in the CBF during ischemia with and without agmatine. In these results, agmatine may act as a competitive inhibitor of NOS and can protect the neuron from the ischemic injury *in vitro* and *in vivo* system.

Key words: Agmatine, Ischemia, Neuronal cell death, Primary neuronal culture, Nitric oxide synthase.

Neuroprotective effect of agmatine on the murine cortical neurons from ischemia-like condition.

Jae Hwan Kim

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Jong Eun Lee)

I. Introduction

Agmatine, [$(NH_2(CH_2)_4NH_2C(NH=)NH$], a naturally occurring guanidino compound found in abundance in bacteria and plants[1] was recently identified in mammals[2,3], where it is expressed in the central nervous system. Agmatine is formed by the decarboxylation of L-arginine by the arginine decarboxylase (ADC)[1], first discovered in 1910 and hydrolyzed to putresicine and urea by agmatinase (agmatine uryl-hydrolase)[4,5,6]. In brain, Agmatine meets most of the criteria of a neurotransmitter/neuromodulator[7]: it is synthesized,

stored, and released from specific networks of neurons[8,9], is inactivated by energy-dependent reuptake mechanisms[10], is degraded enzymatically[11], and binds with high affinity to α_2-adrenergic and imidazoline (I_1) receptors[2,12]. Also, agmatine antagonizes nicotinic[13] and *N*-methyl-D-aspartate receptors (NMDAR)[14]. Agmatine can interfere with second messenger pathways by acting as an adenosine diphosphate (ADP)-ribose acceptor thereby inhibiting ADP-ribosylation of proteins[15], but the mode and sites of action have not been fully defined. Considering the close structural similarity between L-arginine and agmatine, agmatine is a competitive nitric oxide synthases (NOSs) inhibitor[16,17]. NOSs generate nitric oxide (NO) by sequential oxidation of the guanidinium group in L-arginine[18]. Since agmatine is an L-arginine analogue with a guanidinium group, the biochemistry and physiology of agmatine have to be reconsidered as an endogenous regulator of NO production in mammals. NO has been demonstrated to cause either apoptosis or necrosis in cell culture. And also agmatine is neuroprotective against glutamate-induced necrotic neuronal cell death *in vitro* and that this effect is mediated through NMDA receptor blockade by interacting with a site located within the NMDA channel pore. Based on this evidence, we hypothesized that agmatine may have neuroprotective effect on ischemic injury. The purpose of the present study was to evaluate the effect of agmatine on ischemic injury both *in vitro* using primary cultured cortical neurons and *in vivo* against transient focal ischemia MCAO suture animal model.

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 the NIH guidelines

2. Cell culture

Primary cortical neurons were cultured from fetal mice (15 days after gestation) with 5% fetal bovine serum and 5% equine serum and plated in 24-well plates coated with poly-D-lysine and laminin at a density of 3.55 hemispheres per plate. Twenty four to thirty hours after plating, 60% of the culture medium was replaced with glial conditioned medium (GCM), and cytosine arabinoside (Ara-C, 3 μM). These cultures were used on 10-11 days.

3. Oxygen-glucose deprivation (OGD)

Cultures were transferred into an anaerobic chamber (Forma Scientific Co.) (O_2, tension < 0.2%), washed three times with deoxygenated,glucose-free balanced salt solution ($BSS_{0.0}$) containing (in mM) NaCl 116, $CaCl_2$ 1.8, $MgSO_4$ 0.8, KCl 5.4, NaH_2PO_4 1, $NaHCO_3$ 14.7, *N*-(2-hydroxyethyl)piperazine-*N*'-ethanesulfonic acid (HEPES) 10; and phenol red 10

mg/l at pH 7.4 and incubated into $BSS_{0.0}$ at 37°C within the anaerobic chamber for 1 hour. OGD was ended by adding glucose to culture medium to a final concentration of 5.5 mM and returning cultures to a normoxic incubator (Forma Scientific Co.) for 11 hous.

4. Agmatine treatment

Agmatine (100 μM) were treated at 30 minutes before insults (Pre-treated group), the same time with OGD (Co-treated group) and the start time of reperfusion (Post-treated group) in the primary cultured cortical neurons following the scheme (Fig. 1). Experimental control was treated with same volume of PBS (Non-treated control).

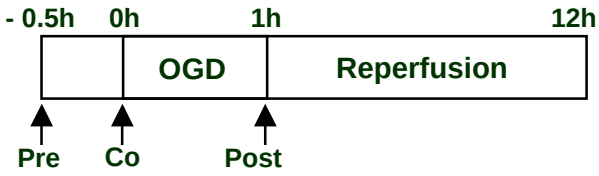


Figure 1. The scheme of experimental schedule for Oxygen-glucose deprivation (OGD). Agmatine (100 μM) were treated at 30 minutes before OGD (Pre), at the same time with OGD (Co), at the start time of reperfusion (Post). Experimental control was treated with same volume of PBS (Non-treated control).

5. Evaluation of injury

(1) Measurement of LDH activity

Cell lysis was quantified by assay of lactate dehydrogenase (LDH) activity released into the culture medium[19]. Total LDH release corresponding to complete neuronal death was determined at the end of each experiment following freezing at -70 ^{o}C and rapid thawing.

(2) Hoechst-PI nuclear staining

The cell death was evaluated by staining of non-viable cells with propidium iodide(Sigma, ST. Louis, Missouri, USA), and live cells with Hoechst 33258 dye (Sigma, ST. Louis, Missouri, USA)[20]. Staining with the fluorescent dyes propidium iodide and Hoeschst 33258 allows discrimination of apoptotic from non-apoptotic cells on the basis of nuclear morphology and evaluation of membrane integrity. Hoechst dye was added to the culture medium to a final concentration of 2–5 μg/ml and the cultured cells were kept at 37°C for 30 minutes. Propidium iodide solution was then added (final concentration 2–5 μg/ml) just before observation in a Olympus microscope equipped for epifluorescence with UV filter block.

6. TUNEL staining

For TUNEL staining, we used in situ cell death detection kit (Roche, Manhiem, Germany). Cultured cells were fixed with paraformaldehyde solution (4% in PBS, pH 7.4) for 1 hour at room temperature. The cultured cells were rinsed 3 times with PBS and incubated in permeabilisation solution(0.1% Triton X-100, 0.1% sodium citrate in PBS) for 30minutes at 4℃. The cultured cells were rinsed twice with PBS and 50 μl TUNEL reaction mixture were added on them following the protocol from Roche Molecular Biochemicals. The cultured cells were incubated in a humidified chamber for 1 hour at 37°C. After rinsing the cultured cells 3 times with PBS, they added 50 μl Converter-AP on were incubated in a humidified chamber for 30 minutes at 37℃. After rinsing, they were added 500 μl Fast Red (1mg/ml, Zymed, S. San Francisco, California, USA) on and incubated for 10 minutes at room temperature. The cultured cells were analysed under light microscope (Olympus, Tokyo, Japan).

7. Concentration of Nitrite (NO_2^-) and Nitrate (NO_3^-)

To assess NO production, the measurement of the stable end-productions of NO metabolism, nitrite (NO_2^-) and nitrate (NO_3^-), was used based on the Griess reaction[17,21].

In brief, 100 μl of media were added to 96well plate in duplicate. 100 μl of Griess reagent(Sigma, ST. Louis, Missouri, USA) was added to each well. The plate was mixed

gently, and allowed to incubate in dark room for 15minutes at room temperature. The absorbance of the reaction product was measured at 540 nm. Each experiment was repeated over 12 times with cells from 3 different preparations.

8. NADPH-diaphorase / NOS activity staining

For NADPH-diaphorase histochemical staining, cultured cells were incubated with reaction solution containing 0.1% β-NADPH (Sigma, ST. Louis, Missouri, USA), 0.01% nitro blue tetrazolium (NBT, Sigma, ST. Louis, Missouri, USA) and 0.1 M phosphaste buffer at 37℃ for 3-4 hours. The course of the reaction was controlled under a microscope. This stain demonstrates the presence of functional NOS protein[22].

9. Immunocytochemical staining for nNOS and iNOS

Cultured cells were fixed with paraformaldehyde solution (4% in PBS, pH 7.4) for 1 hour at room temperature. They were rinsed 3 times with PBS and incubated in permeabilisation solution(0.1% Triton X-100 in 10% FBS / PBS) for 30minutes at 4℃. The cultured cells were rinsed 3 times with PBS. They were incubated in blocking solution(10% FBS / PBS) for 30 minutes at room temperature. They were incubated with the nNOS (1 : 200, Upstate, Lake Placid, NY, USA) and iNOS (1 : 500, Calbiochem, San Diego, CA, USA) primary antibody for 2hours at room temperature and rinsed 3 times with PBS. They were

also incubated with biotinylated secondary antibodies for 1 hour at room temperature. They were rinsed 3 times with PBS and then placed in Avidin conjugated peroxidase for 1 hour at room temperature. Following several rinses with PBS, they were placed in 0.05% diaminobenzidine (DAB; Sigma, ST. Louis, Missouri, USA) solution containing 0.01% hydrogen peroxide for 10 minutes and washed 3 times with PBS and 3 times with distilled water. Controls were prepared using incubation solution without primary antibodies. All incubation steps were performed in a humidified chamber.

10. Stroke model

Male ICR mice weighing 38-40 gm were subjected to transient middle cerebral artery occlusion (MCAO). Animals were anesthetized with 4% chloralhydrate, i.p. Rectal temperature, respirations, heart rate were monitored and maintained in the physiologic range throughout the surgery. Ischemia was induced using an occluding intra luminal suture[23]. In brief, a cervical midline incision was made and the left carotid artery and branches were isolated. An uncoated 15 mm long segment of 6-0 Dermalon suture (blue monofilament nylon DG, Ethicon, Somerville, NJ) with the tip rounded by a flame was inserted into the arteriotomy and advanced under direct visualization into the internal carotid artery (ICA) approximately 11 mm from the bifurcation in order to occlude the ostium of the middle cerebral artery (MCA). At the end of the ischemic period, the suture was removed and

surgical incisions were closed and the animals were allowed to recover. Twenty-two hours later, animals were sacrificed. Agmatine (100 mg/kg of mouse, IP) were injected following the scheme (Fig. 2).

The brain was removed and 2 mm thick blocks were cut in the coronal plane, then stained with 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, ST. Louis, Missouri, USA) to delineate regions of infarction. Brain sections were then placed in 10% paraformaldehyde, and embedded in paraffin. After postfixing, 6-μm sections in the coronal plane were taken and stained with hematoxylin and eosin (H & E). 10 additional animals measured cerebral blood flows using Laser-Doppler [24].

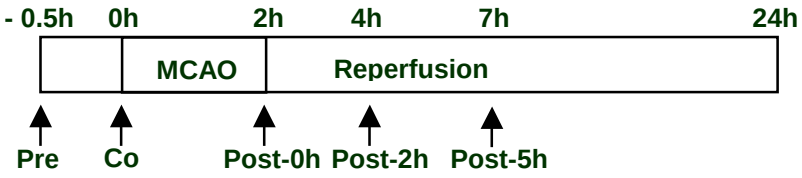


Figure 2. The scheme of experimental schedule for animal ischemic model. Agmatine (100 mg/kg of mouse, IP) were injected 30 minutes before MCA occlusion (Pre), at the same time with ischemic injury (Co), at the start time of reperfusion (Post0h), 2hours after reperfusion (Post2h) and 5hours after reperfusion (Post5h). Experimental control was treated with same volume of saline(Non-treated control). Twenty-two hours after MCA occlusion, Animals were sacrificed for the study.

11. Assessment of ischemic injury

Infarct areas from 5 pre-selected 2,3,5-triphenyltetrazolium chloride (TTC) stained sections and 5 brain sections of each group stained with hematoxylin and eosin (H&E) were determined as previously described using a computer assisted image analysis system and corrected for the presence of edema (MCID, St. Catherines, Ontario, USA) [25]. In brief, animals were sacrificed, and the brains were quickly removed and sliced into 2-mm thick coronal sections. The brain slices were incubated in 2% TTC for 15 minutes at 37℃ and fixed in paraformaldehyde solution(10% in PBS, pH 7.4)[26]. After paraffin embedding, paraffin blocks were cut into 6 μm sections and these sections were stained with H&E.

Infarct was evaluated by light microscopy in a blinded fashion. Histologic criteria for infarct included areas of pan-necrosis with shrunken dark neurons and glial pallor. The area of infarct was expressed as a percentage of the total area of left hemisphere.

12. Measurement of cerebral blood flow

The CBF was measured using Laser-Doppler velocimetry at three different times: during MCAO (2h) and 1h, 2h after reperfusion with and without agmatine. Agmatine was treated in the same time to put the suture into the MCA (Co-treated group) and to take it out from the vessel (Post0h-treated group). In this technique a laser beam of low-energy light is placed over a micro-blood vessel. The moving blood cells scatter the incident light, so that

there is a frequency shift between incident and reflected light (the Doppler shift). The range of frequency shifts was measured[24].

13. Statistical analysis

Data are expressed as the mean ± SEM. Standard statistical tests were used to determine differences between groups using SigmaStat (Jandel Corp., San Rafael, CA, USA). ANOVA followed by a multiple comparisons procedure, the Student's t-test was used for the experiments *in vitro*. For the stroke models *in vivo*, Student-Newman-Keul's test was used in the case of continuous data (infarct size).

III. Results

1. Neuroprotective effect of agmatine *in vitro* system

In the primary cultured cortical neurons, cells were incubated with 100 μM of agmatine as four different conditions, pre-, co-, post-treated group and non-treated control (experimental control) as described in methods.

(1) Measurement of LDH activity

Primary cultured cortical neurons subjected to oxygen-glucose deprivation(OGD) were also protected by addition of agmatine (100 μM) to the medium during OGD. We measured the LDH release (%) to know the degree of cell death as four different agmatine treatment conditions (Pre, Co, Post, and Non) as described in methods. Pre-treated group was 60.3±3.3%, Co-treated group was 42±4.7%, Post-treated group was 58.6±3.4%, and Non-treated control was 67.4±1.9%. Agmatine co-treated group reduced OGD injury by 25.4% compared to non-treated control culture by LDH release. However, pre- and post-treated agmatine were not effective *in vitro* (Fig. 3).

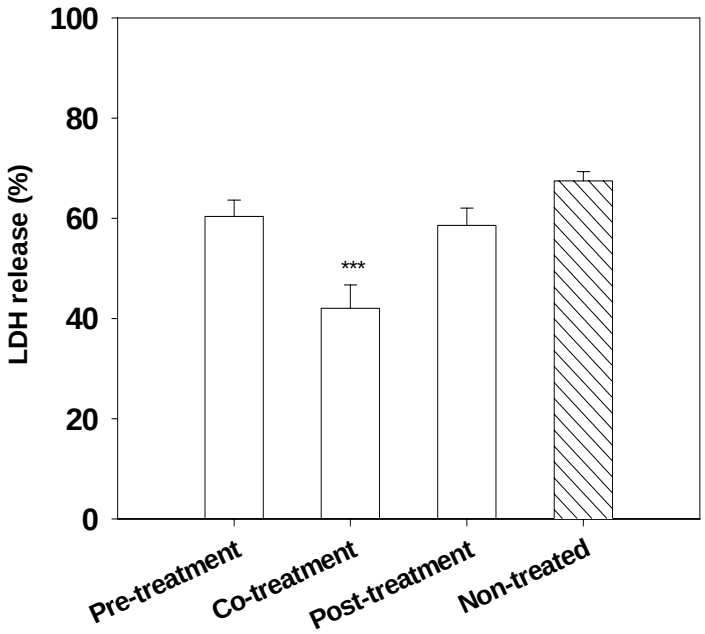


Figure 3. Effect of agmatine (100 μM) on the primary cultured cortical neurons after ischemia-like injury(OGD). LDH release was assayed for relative total cellular death. Data are expressed as mean ± SEM. Co-treated agmaitne significantly reduced the LDH release compared to non-treated control by student t-test (***: $p < 0.001$).

(2) Hoechst-PI nuclear staining

We appraised the cell death using Hoechst-PI nuclear staining. Non-viable cells stained with propidium iodide were detected as red color and live cells stained with Hoechst 33258 dye were detected as blue color. Agmatine co-treated group had many blue colored live cells compared to non-treated control, but pre- and post-treated group had red colored non-viable cells as much as non-treated control (Fig. 4).

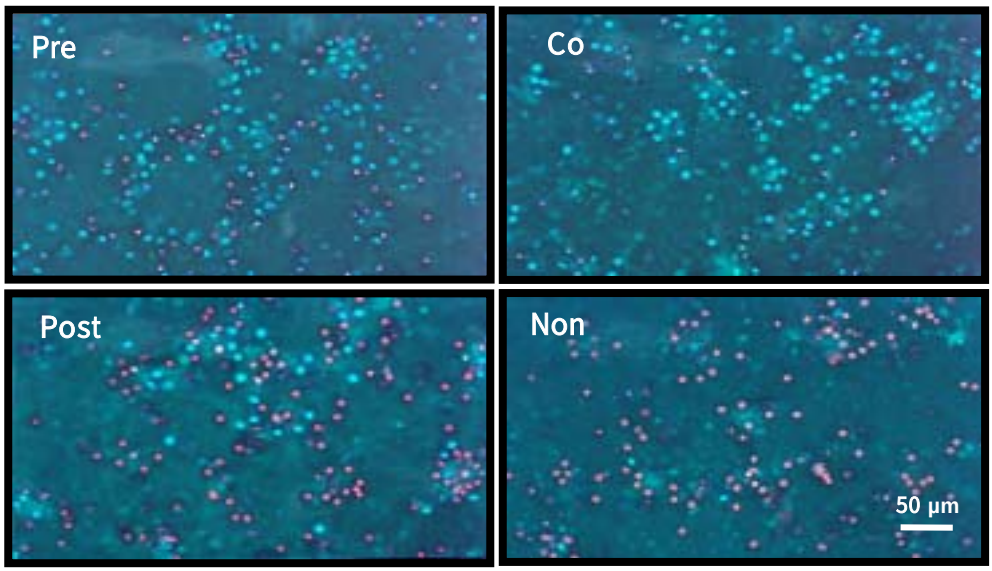


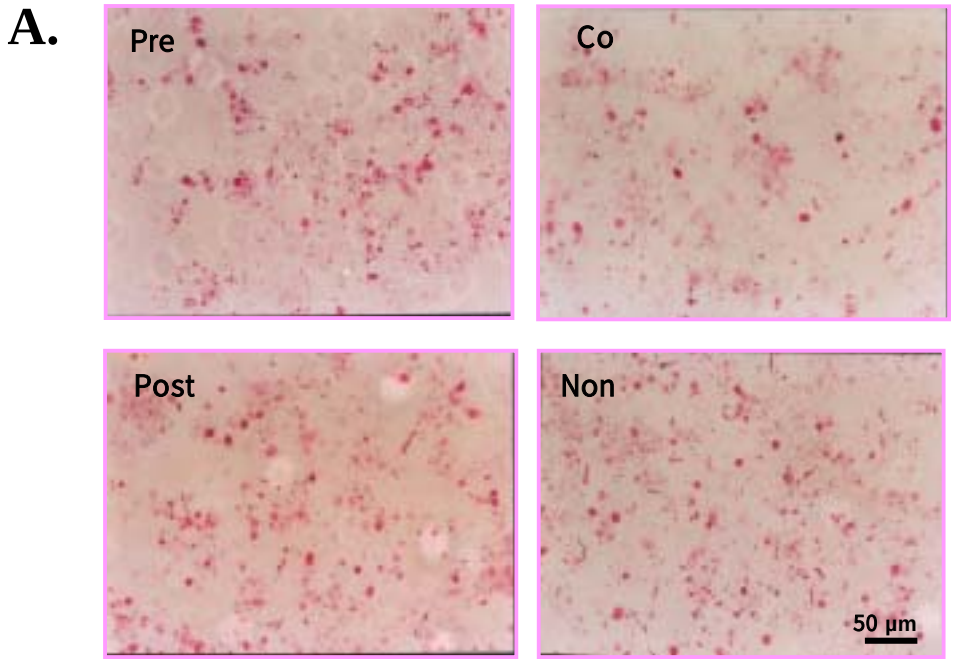
Figure 4. The macrograph of primary cultured cortical neurons stained with Hoechst-propidium iodide 24hr after ischemia-like injury (OGD) with and without agmatine (100 μ M). x 200

(3) TUNEL staining

To further characterize the neuroprotective effect of agmatine, TUNEL staining was performed (Fig. 5-A). In the ischemia-like injured cells with non-treated agmatine, there were many TUNEL-positive cells (56.8±1.8%). The number of TUNEL-positive cells were decreased in the co-treated group (29.5±2.7%) about a half of that of non-treated control, but not much in pre-(60.1±2.5%), and post-treated group (60.8±3%) (Fig. 5-B).

(4) Concentration of Nitrite (NO_2^-) and Nitrate (NO_3^-)

The nitrite and nitrate concentration in the medium of cells was measured by Griess reaction method. The nitrite and nitrate concentration of pre-treated group was 1.09 μM, co-treated group was 0.68 μM, post-treated group was 0.81 μM, and non-treated control was 1.00 μM. The concentration of nitrite and nitrate in the co-treated group was reduced compared to the the non-treated control (31.89%). That was reduced 19.68% in the post-treated group (Fig. 6).



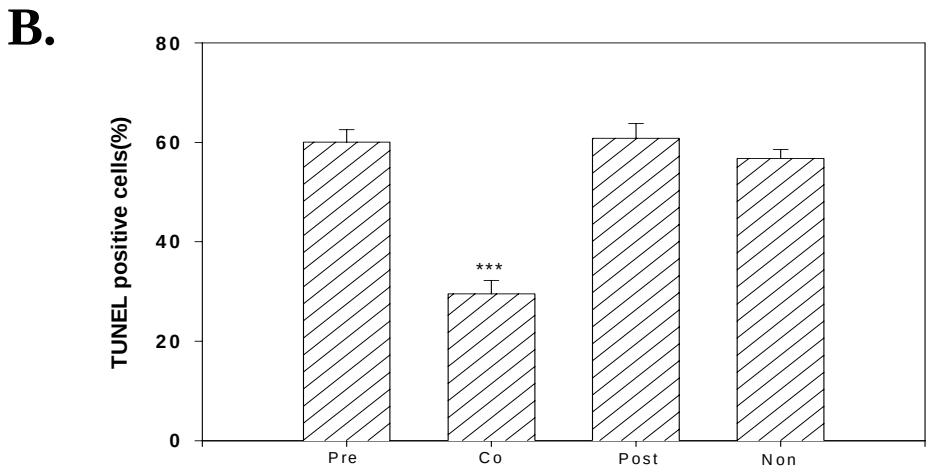


Figure 5. TUNEL-positive cells after ischemia-like injury (OGD) with and without agmatine (100 μM). x 200, Data are expressed as mean ± SEM. Co- treated agmatine significantly decreased the number of TUNEL-positive cells compared to non-treated control by student t-test (***: $p < 0.001$).

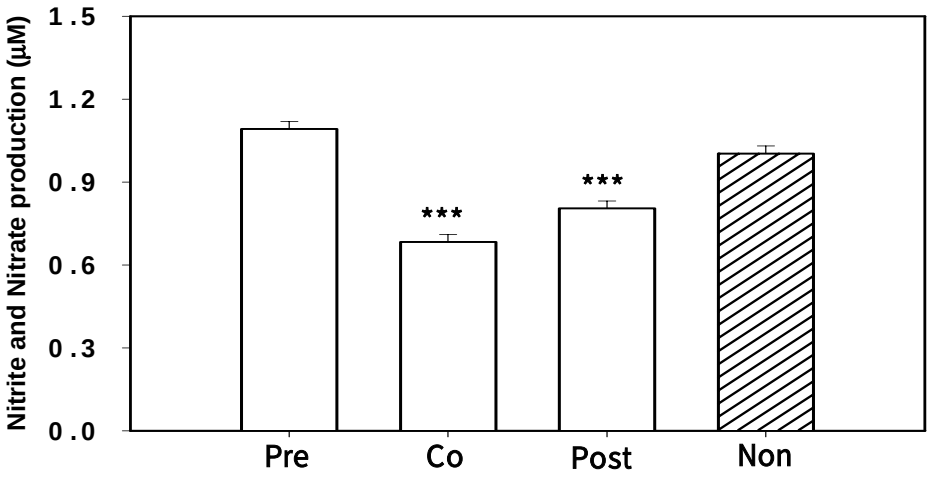


Figure 6. Concentration of Nitrite (NO_2^-) and Nitrate (NO_3^-) in the primary cultured cortical neurons after ischemia-like injury (OGD) with and without agmatine (100 μM). Data are expressed as mean ± SEM. Co- and Post-treated group significantly decreased the nitrite production compared to non-treated control by student t-test (***: $p < 0.001$).

(5) NADPH-diaphorase / NOS activity staining

We investigated the activity of nitric oxide synthase (NOS) in OGD injured cells using NADPH-diaphorase activity staining. NADPH-diaphorase/NOS positive cells were

stained to deep blue color. The number of NADPH-diaphorase/NOS positive cells was not changed significantly in either the pre- and post-treated groups compared to the non-treated control. However, the number of NADPH-diaphorase/NOS positive cells was reduced in the agmatine co-treated group (Fig. 7). This did not reflect a loss of cells, since cell number was not reduced when viewed by phase contrast microscope (data not shown) , rather it reflected a loss of NADPH-diaphorase activity in the cells.

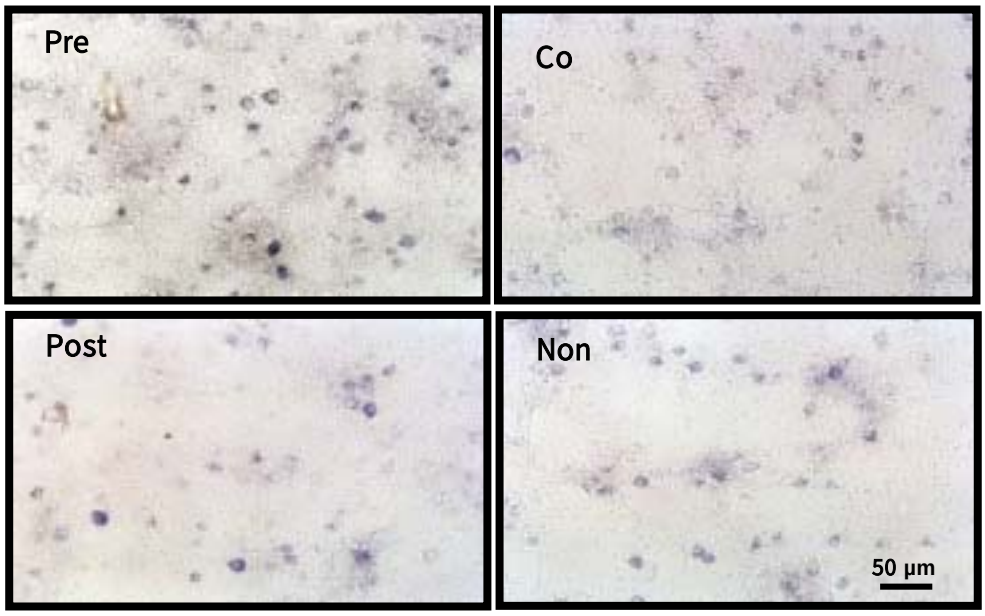


Figure 7. NADPH-diaphorase/NOS positive cells after ischemia-like injury(OGD) with and without agmatine (100 μM). x 200.

(6) Immunocytochemical staining for nNOS and iNOS

NOSs include three isoforms that are nNOS, iNOS, and e-NOS. Among isoforms of NOS, the expression of nNOS in neuron is constitutive, and that of iNOS is inducible. We investigated the effect of agmatine on nNOS and iNOS expression by immunocytochemical staining for nNOS (Fig. 8-A) and iNOS (Fig. 9-A).

The number of nNOS positive cells was 28.2±1.4% in pre-treated group, 14.5±1.1% in co-treated group, 23.5 ±3.7% in post-treated group, and 30.6±1.2% in non-treated control. The number of nNOS positive cells decreased in the co- and post-treated groups, but not significantly in the pre-treated group (Fig. 8-B).

The number of iNOS positive cells was 31.5±5.8% in pre-treated group, 29.8±3.2% in co-treated group, 31.2±6.3% in post-treated group, and 30.6±1.2% in non-treated control. There was no difference in the number of iNOS positive cells among pre-, co-, post-treated group, and non-treated control (Fig. 9-B).

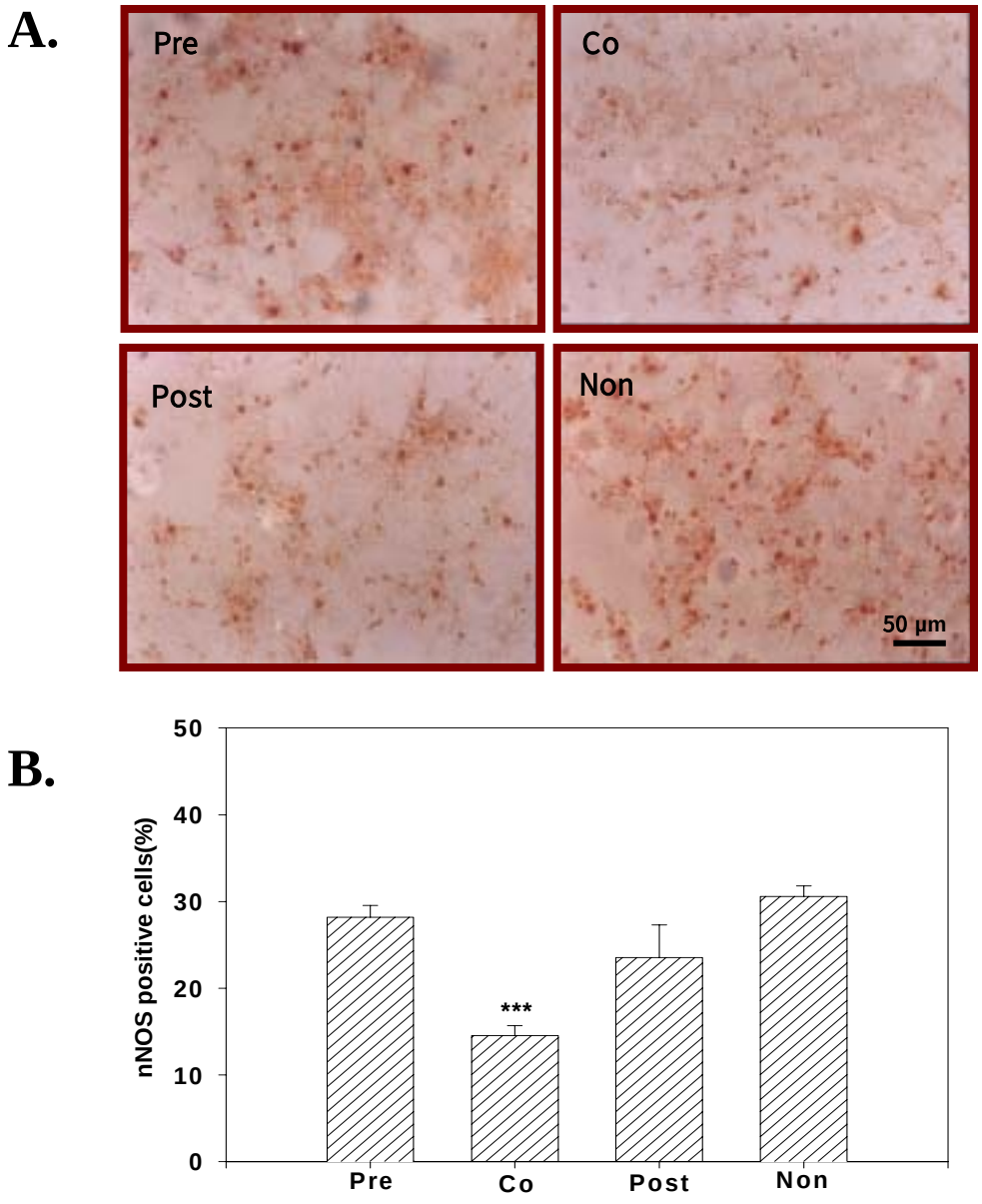


Figure 8. nNOS positive cells after ischemia-like injury (OGD) with and without agmatine (100 μM). x 200, Data are expressed as mean ± SEM. Co-treated agmatine significantly decreased the number of nNOS positive cells compared to non-treated control by student t-test (***: $p < 0.001$).

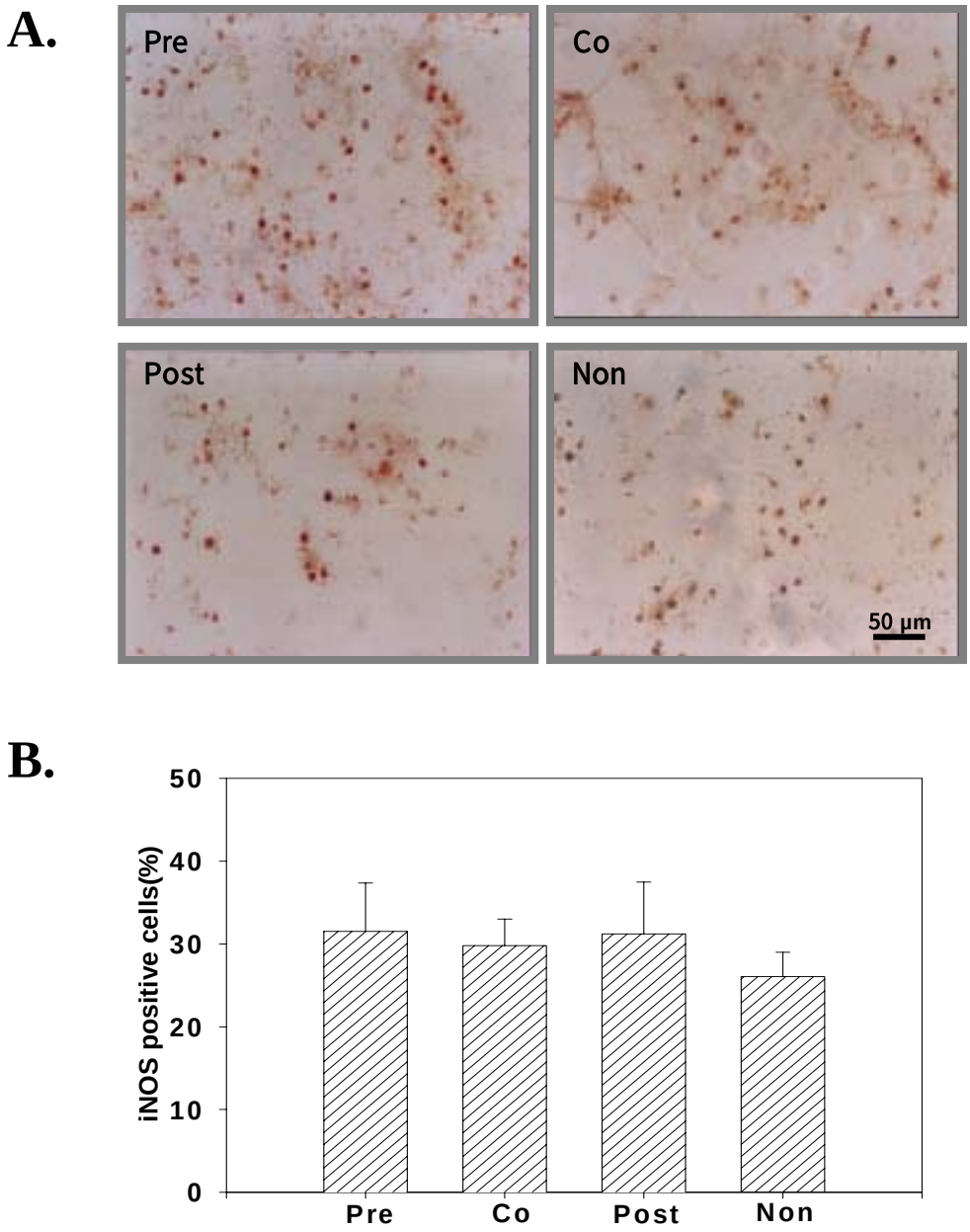


Figure 9. iNOS positive cells after ischemia-like injury (OGD) with and without agmatine (100 μM). x 200, Data are expressed as mean ± SEM. All agmatine treated groups have no difference about the number of iNOS positive cells compared to non-treated control.

2. Neuroprotective effect of agmatine *in vivo* system

We investigated the effect of agmatine in brain ischemic injured mice by MCAO (middle cerebral artery occlusion). Agmatine(100 mg/kg of mouse) were injected intraperitoneally into the brain ischemic injured mice as the above. The effect of agmatine was measured by staining coronal section of mouse brain with TTC and H&E and by analyzing infarct sizes. In addition, we measured the change of CBF.

(1) 2,3,5-triphenyltetrazolium chloride (TTC) staining

Assessment of infarct arear are after MCAO revealed marked protection by agmatine. Administering agmatine before, during, immediately after and 2hours after occlusion was highly effective at protecting from MCAO (Fig. 10), while the protection was no longer present when agmatine was given at 5hours of reperfusion

(2) Hematoxylin-Eosin(H-E) staining

The effect of agmatine was showed by staining coronal section of brain with H-E in the brain ischemic injured mice. Nuclei in all agmatine treated groups, except post5h, were similar to nuclei in normal control in shape, but post5h-treated group had a large number of condensed nuclei, that was similar to non-treated control (Fig. 11).

(3) Analysis of infarct area

Histopathology revealed over 80% reduction in the infarct area of agmatine-treated groups compared to non-treated control. The pre-, co-, and post-treated agmatine reduced the infarct area after ischemic injury in the MCAO suture animal model (Fig. 12).

In these results, TTC staining, infarct analysis, and hematoxylin-eosin staining, we could know that agmatine had the neuroprotective effect on the ischemic injury in pre-,co-,post0h, and post2h-treated group.

(4) Measurement of cerebral blood flow (CBF)

We measured the change of cerebral blood flow(CBF) before, during MCAO, and during reperfusion in co-treated group and non-treated control by using Laser-Doppler velocimetry. The value of CBF had no difference between co-treated group and non-treated control when measured before and during MCAO. However, in co-treated group, agmatine increased about 9.4% in the value of CBF during reperfusion (Table 1).

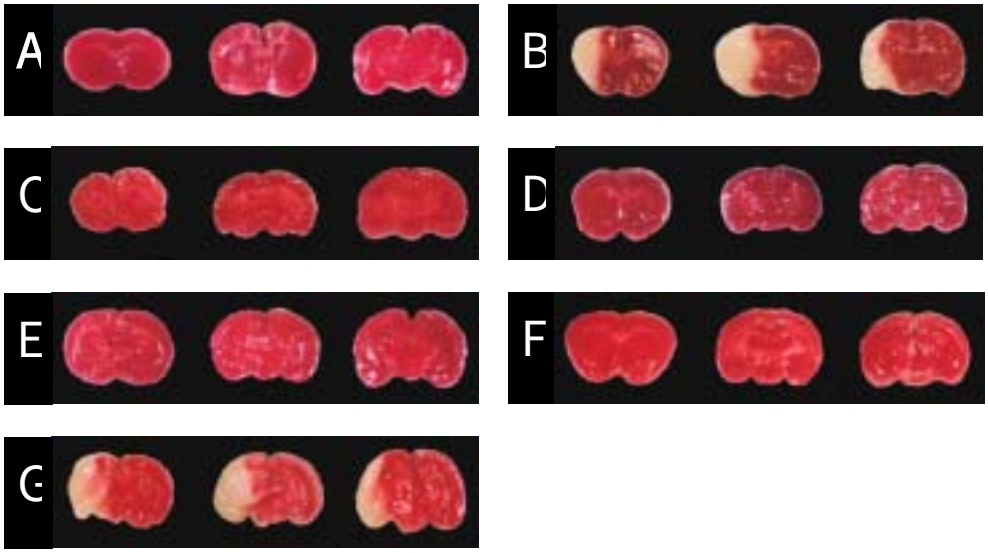


Figure 10. TTC staining of the ischemic injured brain with and without agmatine (100 ㎎/㎏ of mouse, IP).

A: normal mouse brain, B: experimental control (saline injected), C: agmatine injected brain 30 minutes before the start of MCA occlusion, D: agmatine injected brain at the same time of the start of MCA occlusion, E: agmatine injected brain 0 hour after the end of MCA occlusion, F: agmatine injected brain 2 hours after the end of MCA occlusion, G: agmatine injected brain 5 hours after the end of MCA occlusion.

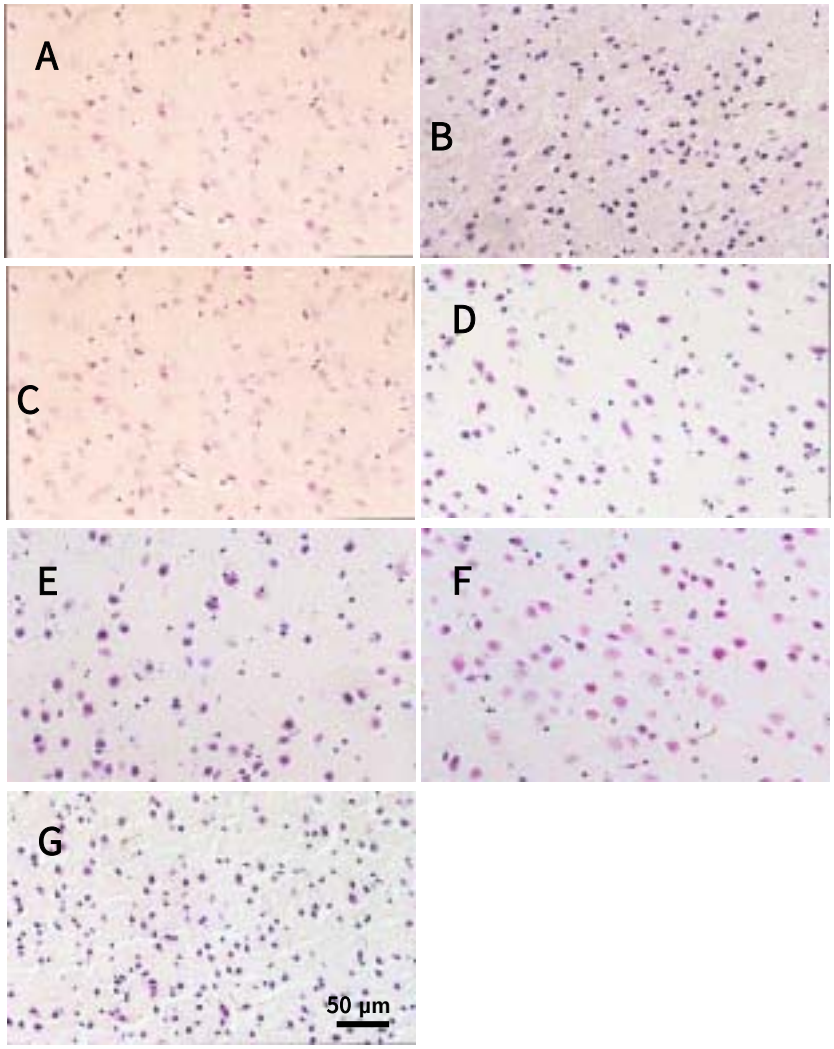


Figure 11. H-E staining of infarct brain with and without agmatine (100 ㎎/㎏ of mouse, IP). x 200, A: normal mouse brain, B: experimental control (saline injected), C: agmatine injected brain 30 minutes before the start of MCA occlusion, D: agmatine injected brain at the same time of the start of MCA occlusion, E: agmatine injected brain 0 hour after the end of MCA occlusion, F: agmatine injected brain at 2 hours after MCA occlusion, G: agmatine injected brain 5 hours after the end of MCA occlusion.

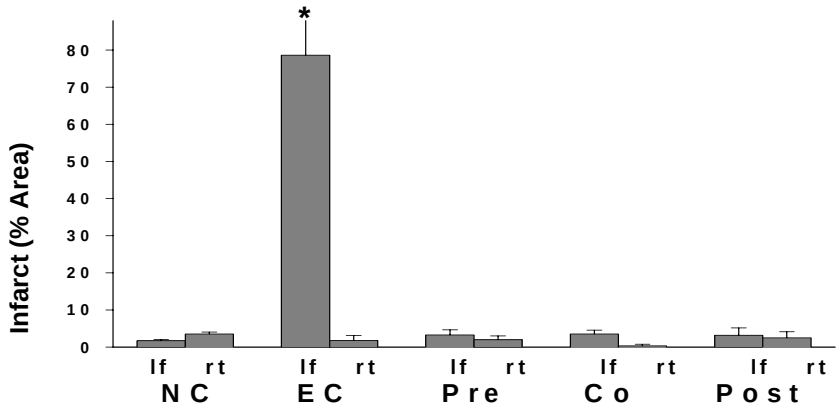


Figure 12. Percentage of infarct area for the hemisphere at 22 hours post-ischemia with 2 hours MCA occlusion with and without agmatine (100 mg/kg of mouse, IP). Data are expressed as mean $\pm$ SEM. The agmatine-treated animals significantly reduced the infarct area compared to non-treated control (EC) by one-way ANOVA and Student-Neuman-Keul's test (*: $p < 0.01$).

lf: Infarct side(left), rt: lateral side(right), NC: normal control, EC: experimental control, Pre: agmatine injected brain at 30 min before the start of MCA occlusion, Co: agmatine injected brain at the same time of the start of MCA occlusion, Post: agmatine injected brain 0 hour after the end of MCA occlusion.

Table 1. Relative cerebral blood flow (rCBF) as measured by Laser-Doppler velocimetry after MCA occlusion with and without agmatine (100 mg/kg of mouse, IP).

	Non-treated control Group (n = 3)	Agmatine co-treated Group (n = 3)
Baseline	100	100
Occlusion	54.66±1.96	53.52±1.96
Reperfusion	70.44±0.09	79.84±1.96

IV. Discussion

Agmatine is an endogenous substance synthesized from arginine by arginine decarboxylase and hydrolyzed to putrescine and urea by agmatinase. It is present in the brain of mammals including the rat, bovine and human. The level of agmatine in rat brain has been reported as 0.2–0.4 μg/g tissue by mass spectroscopy[2] and as 0.331–1.105 μg/g tissue by high-performance liquid chromatography[27]. The presence of agmatine in astrocytes and neurons has been demonstrated by immunohistochemical examination with anti-agmatine antibody[8,28],. Electron microscopic examination has demonstrated that agmatine is present in axon terminals associated with synaptic vesicles[8,9]. In addition, agmatine is released from synaptosomes or brain slice in response to depolarizing stimuli and is taken up into synaptosomes via a Na^+-independent transport system[10]. These observations suggest that agmatine functions as a neurotransmitter or neuromodulator in the brain.

There are at least three likely mechanisms for agmatine neuroprotection. Agmatine has been shown to reduce excitotoxicity in vitro[29] by blocking NMDA receptor activation . agmatine is an α-2 agonist and another α-2 agonist, dexmedetomidine has been shown to protect neurons from injury *in vivo* and *in vitro*[30]. Lastly, agmatine is an NOS antagonist, and generation of NO has been implicated in ischemic brain injury[31].

Since agmatine is structurally analogous to the NOS substrate L-arginine, it is possible that agmatine modulates the production of NO. Indeed, agmatine has been reported to inhibit the activity of iNOS, but not constitutive NOS, in rat aorta, to inhibit the activity of NOS purified from brain, macrophages and endothelial cells, or to activate NOS in endothelial cells. And also agmatine is neuroprotective against glutamate-induced necrotic neuronal cell death *in vitro* and that this effect is mediated through NMDA receptor blockade by interacting with a site located within the NMDA channel pore [29]. several lines of evidence support that agmatine may have neuroprotective effect in brain injury, such as neurotrauma and ischemia[32,33].

We demonstrated the neuroprotective effect of agmatine in primary cultured cortical neurons by using LDH assay, Hoechst- propidiumiodide staining, and TUNEL staining when we treated agmatine at the same time of the start of MCA occlusion. Agmatine have many possible pathway to inhibit the cell death, NMDA channel blocker, competitive inhibitor of NOS, and so on. We focused on the relation of agmatine to NOS and NO.

Nitric oxide (NO) has been recognized as an important messenger molecule in the nervous system[34]. NO is produced from the conversion of L-arginine to citrulline by the enzyme NO synthase (NOS) [35,36]. Three isoforms, representing the products of three distinct genes, have been identified. Two constitutive (cNOS) calcium-dependent forms of the enzyme are expressed: one first identified in neurons, nNOS; and the other in endothelial cells,

eNOS. Both eNOS and nNOS require tetrahydrobiopterin, FAD, FMN, NADPH, O_2, Ca^{2+}, and calmodulin. The neuronal form is both cytosolic and particulate and has an absolute dependence on Ca^{2+} and calmodulin. eNOS is very similar to nNOS with the exception that it contains a myristylation site which targets it to the plasma membrane. The calcium-independent isoform, immunologic NOS (iNOS), is not expressed in healthy tissue but can be expressed following transcriptional induction in a wide variety of cells including glia, macropharges and cerebellar neurons following exposure to endotoxins or cytokines. iNOS has cofactor requirements identical to those of nNOS and eNOS except that it does not require Ca^{2+} for activation. It was reported that NO is a major mediator of NMDA neurotoxicity in hippocampal[37] and striatal[38] slices and a variety of culture systems[39]. Moreover neuroprotective effects of drugs that are indirect inhibitors of NOS like certain gangliosides, calmidazolium, and FK506 may arise from blockade of nitric oxide formation through modulation of calmodulin and /or other Ca^{2+}-dependent processes[40].

It was suggested that NO may be neuroprotective or neurodestructive depending upon its oxidative/reductive status, with the NO free radical (NO·) being toxic and the nitronium form of NO (NO^+) being neuroprotective[41]. This may account for some of the earlierwork which indicated that inhibition of NOS was not neuroprotective. Some of the neuroprotective effects of NO could result from down-regulation of NMDA-receptor activity with the subsequent inhibition of calcium influx by reaction with the thiol group(s) of the

NMDA receptor's redox modulatory site[42]. However, some studies indicate that NO is inhibiting NMDA-receptor currents by forming complexes with cations and acts as a channel blocker[43].

Ischemia increases ROS, mainly during the reperfusion period and infarct size is reduced by the administration of free radical scavengers. Marked increases in NO production in the brain also occur during focal ischemia. Low doses of nonselective NOS inhibitors are neuroprotective . However high doses of NOS inhibitors may enhance the size of the infarct area developed after focal ischemia due to alterations in cerebral blood flow through inhibition of eNOS. In the early phase of cerebral ischemia the vasodilator action of NO seems to be beneficial to the tissue surrounding the ischemic core. Since NO is a strong cerebral vasodilator, activation of eNOS during cerebral ischemia may facilitate collateral flow and improve microcirculation in the penumbral area. A marked induction of iNOS also occurs after focal cerebral ischemia and inhibition of iNOS ameliorates cerebral ischemic damage. Because the activity peaks more than 24 hours after ischemic insult, it is unlikely that iNOS-generated NO could be responsible for the brain damage occurring within the first few hours of ischemia but it may play a role in the late effects that occur. During the initial phase of the ischemia enhanced NO generated by eNOS maintains cerebral blood flow, while NO derived from nNOS is neurotoxic. The induction of iNOS and consequent sustained production of NO can participate in the neuronal damage occurring in the late phase of focal

cerebral ischemia.

iNOS could also play a role in neuronal damage through inflammatory processes that occur during the activation of brain microglia and the subsequent production of cytokines. Cerebellar granule cell neurons in culture can also be stimulated to express iNOS by interferon-gamma and LPS indicating that iNOS expression is not limited to glial cells. Neurotoxicity induced by immunostimulated microglia is largely NO-mediated[44].

In our study, it was shown that agmatine inhibits the activity of NOS through the NADPH-diaphorase/NOS activity staining in primary cultured cortical neurons when we treated agmatine at the same time of the start of MCA occlusion. And also we knew that agmatine reduces the expression of nNOS by immunocytochemical staining for nNOS, but agmatine could not reduce that of iNOS. The result that agmatine had effect on the expression of nNOS has not been reported before. But agmatine had no effect on the expression of iNOS, which was suggested by Abe (2000). He suggested that agmatine had no effect on the expression of iNOS, but significantly suppressed the LPS-induced NO production in a concentration-dependent manner in microglia. We also evaluated the neuroprotective effect of agmatine on ischemic injury by MCAO suture animal model. All agmatine treated groups except Post5h have dramatic neuroprotective effects. That was shown that infarct area was decreased to almost normal brain state in same groups through TTC staining and infarct analysis, and that condition of cells of agmatine treated groups

except Post5h is similar to normal control cells using H-E staining. It was very special that Post0h- and Post2h- treated groups had neuroprotective effect. We could predict that the neuroprotective effect of agmatine on neuronal cells occurs during not only ischemic injury but also the early period of reperfusion. That is known that Ischemia increases ROS during the reperfusion period[45] and this ROS cause ischemic brain damage. Even if agmatine increased the value of CBF about 9.5% compared to non-treated control in co-treated group during reperfusion in CBF data, agmatine displayed dramatic neuroprotective effect on ischemic injury in co-treated group. This may be that agmatine already protected damage caused by MCA occlusion and mouse had no damage at the time of the start of reperfusion, that is, mouse was in a good state of health as normal control. So, increased blood flow might help to return to normal state as soon as possible.

Agmatine has been reported to inhibit the activity of iNOS, but not constitutive NOS, in rat aorta[16], to inhibit the activity of NOS purified from brain, macrophages and endothelial cells[17], or to activate NOS in endothelial cells[21]. The complex neuroprotective mechanism of agmatine was not defined. Based on the previous findings and our results, we think that agmatine inhibits the activity of nNOS and iNOS and activates that of eNOS, so that it suppressed neurotoxic NO derived from nNOS and iNOS, while enhanced neuroprotective NO generated by eNOS.

Our study suggests that agmatine may be a promising therapeutic target for treatment of cerebral ischemia. In the further study, we should define the neuroprotective mechanism of agmatine.

V. Conclusion

We hypothesized that agmatine may have neuroprotective effects on ischemic injury and evaluated the effect of agmatine on ischemic injury in primary cultured cortical neurons *in vitro* and MCAO suture animal model *in vivo*.

When agmatine (100 μM) was treated at the start time of OGD *in vitro*,

1. Agmatine reduced the degree of cell death compared to experimental control in LDH assay, Hoechst-PI staining, and TUNEL staining.
2. Agmatine reduced the formation of NO in Griess reaction..
3. Agmatine suppressed the expression of nNOS in immunocytochemical staining, but didn't that of iNOS.

When agmatine (100 mg/kg of mouse, IP) was injected *in vivo,*

4. Agmatine significantly reduced the ischemic brain damage, and the infarct area in TTC staining, Infarct analysis, and H-E staining, except Post5h-treated group.

In these results, we knew that agmatine has the neuroprotective effects on ischemic injury and thought that some of the protective mechanisms of agmatine are the inhibition of NOS activity and the suppression of nNOS expression.

References

1. Tabor CW, Tabor H. Polyamines. Annu Rev Biochem 1984; 53: 749–790.

2. Li G, Regunathan S, Barrow CJ, Eshraghi J, Cooper R, Reis DJ.

Agmatine: an endogenous clonidine-displacing substance in the brain.

Science 1994 Feb 18; 263(5149): 966-9.

3. Raasch W, Regunathan S, Li G, Reis DJ. Agmatine, the bacterial amine, is widely distributed in mammalian tissues. Life Sci 1995; 56(26): 2319-30.

4. Yang XC, Reis DJ. Agmatine selectively blocks the N-Methyl-D-Aspartate subclass of glutamate receptor channels in rat hippocampal neurons.

J Pharmacol Exp Ther 1999; 288: 544-549.

5. Gilad GM, Wollman Y, Iaina A, Rabey JM, Chernihovsky T, Gilad VH. Metabolism of agmatine into urea but not into nitric oxide in rat brain. NeuroReport 1996; 7: 1730–1732.

6. Sastry M, Regunathan S, Reis DJ. Enzymatic hydrolysis of agmatine to putrescine in rat brain. Soc Neurosci Abstr 1995; 21: 2059.

7. Reis DJ, Regunathan S. Agmatine: an endogenous ligand at imidazoline receptors is a novel neurotransmitter. Ann N Y Acad Sci 1999 Jun 21; 881: 65-80.

8. Otake K, Ruggiero DA, Regunathan S, Wang H, Milner TA, Reis DJ. Regional localization of agmatine in the rat brain: an immunocytochemical study. Brain Res 1998 Mar 16; 787(1): 1-14.

9. Reis DJ, Yang XC, Milner TA. Agmatine containing axon terminals in rat hippocampus form synapses on pyramidal cells. Neurosci Lett 1998 Jul 10; 250(3): 185-8.

10. Sastre M, Regunathan S, Reis DJ. Uptake of agmatine into rat brain synaptosomes: possible role of cation channels. J Neurochem 1997 Dec; 69(6): 2421-6.

11. Sastre M, Regunathan S, Galea E, Reis DJ. Agmatinase activity in rat brain: a metabolic pathway for the degradation of agmatine. J Neurochem 1996 Oct; 67(4): 1761-5.

12. Piletz JE, Chikkala DN, Ernsberger P. Comparison of the properties of agmatine and endogenous clonidine-displacing substance at imidazoline and alpha-2 adrenergic receptors. J Pharmacol Exp Ther 1995 Feb; 272(2): 581-7.

13. Loring RH Agmatine acts as an antagonist of neuronal nicotinic receptors. Br J Pharmacol 1990; 99: 207–211.

14. Reynolds IJ. Arcaine uncovers dual interactions of polyamines with the N-methyl-D-aspartate receptor. J Pharmacol Exp Ther 1990; 255: 1001–1009.

15. Murayama T, Tsai S-C, Adamik R, Moss J, Vaughan M. Effects of temperature on ADP-ribosylation factor stimulation of cholera toxin activity. Biochemistry 1993; 32: 561–567.

16. Auguet M, Viossat I, Marin JG, Chabrier PE. Selective inhibition of inducible nitric oxide synthase by agmatine. Jpn J Pharmacol 1995 Nov; 69(3): 285-7.

17. Galea E, Regunathan S, Eliopoulos V, Feinstein DL, Reis DJ. Inhibition of mammalian nitric oxide synthases by agmatine, an endogenous polyamine formed by decarboxylation of arginine. Biochem J 1996 May 15; 316 (Pt 1): 247-9.

18. Mayer B, Schmidt K, Humbert P, Bohme E. Biosynthesis of endothelium-derived relaxing factor: a cytosolic enzyme in porcine aortic endothelial cells Ca2+-dependently converts L-arginine into an activator of soluble guanylyl cyclase.
Biochem Biophys Res Commun 1989 Oct 31; 164(2): 678-85.

19. Koh JY, Choi DW. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay.
J Neurosci Methods 1987 May; 20(1): 83-90

20. Lee JE, Kim CY, Giaccia AJ, Giffard RG. The E6 and E7 genes of human papilloma virus-type 16 protect primary astrocyte cultures from injury.
Brain Res 1998 Jun 8; 795(1-2): 10-6

21. Morrissey JJ, Klahr S. Agmatine activation of nitric oxide synthase in endothelial cells.
Proc Assoc Am Physicians 1997 Jan; 109(1): 51-7.

22. Lee SH, Lee WT, Park KA, Lee JE. The possibility that NO mediate the apoptosis induced by capsaicin in primary cultured neuron of various region.
Society for Neuroscience 2000: abstract p2073.

23. Lee WT, Lee JE, Lee SH, Jang HS, Giffard RG, Park KA.

Human papilloma virus type 16 E7 genes protect astrocytes against apoptotic andnecrotic death induced by hydrogen peroxide. Yonsei Med J 2001 Oct; 42(5): 471-9

24. Kohner EM. The problems of retinal blood flow in diabetes.

Diabetes 1976; 25(2 SUPPL): 839-44

25. Mitsuyama T, Kawamata T, Yamane F, Awaya A, Hori T.

Role of a synthetic pyrimidine compound, MS-818, in reduction of infarct size and amelioration of sensorimotor dysfunction following permanent focal cerebral ischemia in rats.

J Neurosurg 2002 Jun; 96(6): 1072-6

26. Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartkowski HM.

Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. Stroke 1986 Nov-Dec; 17(6): 1304-8.

27. Feng Y, Halaris AE, Piletz JE. Determination of agmatine in brain and plasma using high-performance liquid chromatography with fluorescence detection.

J Chromatogr B Biomed Sci Appl 1997 Apr 11; 691(2): 277-86.

28. Regunathan S., Feinstein DL, Raasch W, Reis DJ. Agmatine (decarboxylated arginine) is synthesized and stored in astrocytes. Neuroreport 1995; **6** : 1897–1900.

29. Olmos G, DeGregorio-Rocasolano N, Paz Regalado M, Gasull T, Assumpcio Boronat M, Trullas R, et al. Protection by imidazol(ine) drugs and agmatine of glutamate-induced neurotoxicity in cultured cerebellar granule cells through blockade of NMDA receptor. Br J Pharmacol 1999 Jul; 127(6): 1317-26.

30. Wei H, Jyvasjarvi E, Niissalo S, Hukkanen M, Waris E, Konttinen YT, et al. The influence of chemical sympathectomy on pain responsivity and alpha(2)-adrenergic antinociception in neuropathic animals. Neuroscience 2002; 114(3): 655-68.

31. Iadecola C. Bright and dark sides of nitric oxide in ischemic brain injury. Trends Neurosci 1997 Mar; 20(3): 132-9.

32. Gilad GM and Gilad VH. Polyamines in neurotrauma. Ubiquitous molecules in search of a function. Biochem Pharmacol 1992 Aug 4; 44(3): 401-407.

33. Gilad GM, Salame K, Rabey JM and Gilad VH. Agmatine treatment is neuroprotective in rodent brain injury models. Life Sci 1996; 58(2):PL 41-6.

34. Dawson TM, Snyder SH. Gases as biological messengers: nitric oxide and carbon monoxide in the brain. J Neurosci 1994 Sep; 14(9):5147-59.

35. Ketteler M, Noble NA and Border WA. Cytokines and L-arginine in renal injury and repair. Am J Physiol 1994 Aug; 267(2 Pt 2): F197-207.

36. Reyes AA, Karl IE and Klahr S. Role of arginine in health and in renal disease. Am J Physiol 1994 Sep; 267(3 Pt 2):F331-46.

37. Izumi Y, Benz AM, Clifford DB, Zorumski CF. Nitric oxide inhibitors attenuate N-methyl-D-aspartate excitotoxicity in rat hippocampal slices. Neurosci Lett 1992 Feb 3; 135(2): 227-30.

38. Kollegger H, McBean GJ, Tipton KF. Reduction of striatal N-methyl-D-aspartate toxicity by inhibition of nitric oxide synthase. Biochem Pharmacol 1993 Jan 7; 45(1): 260-4.

39. Ciani E, Groneng L, Voltattorni M, Rolseth V, Contestabile A, Paulsen RE.
Inhibition of free radical production or free radical scavenging protects from the excitotoxic cell death mediated by glutamate in cultures of cerebellar granule neurons.
Brain Res 1996 Jul 22; 728(1): 1-6.

40. Dawson TM, Hung K, Dawson VL, Steiner JP, Snyder SH.
Neuroprotective effects of gangliosides may involve inhibition of nitric oxide synthase.
Ann Neurol 1995 Jan; 37(1): 115-8.

41. Lipton SA, Sucher NJ, Kaiser PK, Dreyer EB. Synergistic effects of HIV coat protein and NMDA receptor-mediated neurotoxicity. Neuron 1991 Jul; 7(1): 111-8.

42. Lei SZ, Pan ZH, Aggarwal SK, Chen HS, Hartman J, Sucher NJ, Lipton SA.
Effect of nitric oxide production on the redox modulatory site of the NMDA receptor-channel complex. Neuron 1992 Jun; 8(6): 1087-99.

43. Fagni L, Olivier M, Lafon-Cazal M, Bockaert J.
Involvement of divalent ions in the nitric oxide-induced blockade of N-methyl-D-aspartate receptors in cerebellar granule cells. Mol Pharmacol 1995 Jun; 47(6): 1239-47.

44. Facchinetti F, Dawson VL, Dawson TM. Free radicals as mediators of neuronal injury.

Cell Mol Neurobiol 1998 Dec; 18(6): 667-82.

45. Nelson CW, Wei EP, Povlishock JT, Kontos HA, Moskowitz MA.

Oxygen radicals in cerebral ischemia.

Am J Physiol 1992 Nov; 263(5 Pt 2): H1356-62.

국문요약

뇌허혈 손상시 아그마틴의 신경세포 보호작용

김 재 환

연세대학교 대학원 의과학사업단

(지도교수: 이 종 은)

아그마틴은 L-알기닌의 탈탄산화로 인해 생기는 일차 아민이며, 포유류의 뇌에서 생성되는 내재성 클로니딘 대체물질이다. 본 연구에서는 일시적 국부 뇌허혈 손상시 생체 안에서와 세포배양에서의 아그마틴의 신경세포 보호작용을 조사하기 위해 순수 대뇌피질 신경세포를 배양하고 쥐에 중간대뇌동맥 결찰술(MCAO)을 시행하였다. 아그마틴은 뇌허혈 손상 시작 30분전과 시작과 같이 그리고 재관류가 시작됨과 동시에 처리하였고, 생체실험에서는 재관류 시작 후 2시간 후와 5시간 후에도 아그마틴을 처리하였다. 배양된 신경세포에는 1리터당

100마이크로몰의 농도로 처리하였고, 쥐에는 1킬로그램당 100밀리그램의 농도로 복강을 통해 주사하였다. 그 결과 세포배양실험에서, 아그마틴은 매우 좋은 신경보호효과를 가지고 있음을 Hoechst-PI 염색과 TUNEL 염색을 통해 알 수 있었다. 또한 N-일산화질소 생성효소(nNOS)의 활성을 조사하기 위해 NADPH-diaphorase/NOS 활성 염색을 시행한 결과 NADPH-diaphorase에 양성을 보이는 세포의 수가 뇌허혈 손상 시작과 같이 아그마틴을 처리한 동시처리군에서 실험군에 비해 약 절반으로 감소하였다. NOS의 발현 정도에 대한 조사결과, 역시 아그마틴을 동시에 처리한 실험군에서 N-일산화질소 생성효소(nNOS)에 대해 면역양성반응을 보이는 세포의 수가 크게 감소함을 알 수 있었으나, I-일산화질소 생성효소(iNOS)에 대해 면역양성반응을 보이는 세포의 수에는 큰 차이가 없었다. 생체실험에서는 재관류가 시작된 이후 2시간까지 아그마틴을 처리한 경우에는 모두 뇌허혈 손상 부위의 크기가 상당히 감소되는 것을 확인하였다. 아그마틴 처리시 중간대뇌동맥 결찰술(MCAO)을 시행할 때 아그마틴을 처리하지 않은 군과 혈류의 양이 차이가 없음을 뇌혈류량을 측정함으로써 확인하였다. 이 모든 것을 볼 때 아그마틴은 일산화질소 생성효소(NOS)의 작용을 억제하고 뇌허혈 손상시 신경세포를 보호할 것이라 생각된다.

핵심되는 말: 아그마틴, 신경세포 사멸, 일차 신경세포 배양, 일산화질소 생성효소