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Agmatine and Glutamate Induced Primary Neuron Damage: In Vitro Study

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Abstract*—The aim of this study is to show the effects of agmatine, which is shown to be promising as a preventive (neuroprotective) agent on glutamate induced neuron damage in newborn rat brain cortex. The newborn 4 rats were rapidly decapitated and the cortex were taken surgically and stored immediately in the prepared neurobasal solution. Low doses of agmatine prevent cell death against glutamate and have a stronger neuroactive effect. Increased TOC level and decreased TAC level were significantly improved with low dose agmatine. The application of agmatinin brought caspase 3 levels closer to the control group. In particular, agmatine administration significantly downregulate caspase 9 mRNA expression compared to the control group. In our study, the best results were obtained with low dose of agmatine. In light of all this information, agmatine, a potent NMDA receptor antagonist, prevented glutamate-induced neurotoxicity.*

***Keywords*—** *Agmatine, Neuron, Glutamate, Rat, Oxidative Stress*.

I. INTRODUCTION

Agmatine, an endogenous substance known to modulate many functions in the nervous system, is an organic cation (1). Agmatin is synthesized from L-arginine by the decarboxylase enzyme of arginine, which is a specific enzyme in the nervous cell in the central nervous system. It is released from the presynaptic end as a result of Ca^{+2} dependent depolarization (2). With agmatinase enzyme, putresine is converted into polyamines such as spermidine, spermine. Agmatin has a wide spectrum of pharmacological effects by binding on various receptors and taking part in important functions in periphery and center (1, 3). Alpha 2 binds to all sub-types of adrenergic receptors with high affinity. On the other hand, it inhibits NMDA receptors, one of the most important receptors of the glutamatergic system, even at micromolar levels. To summarize the pharmacological effects of agmatine in general, antinociceptive, antiinflammatory, neuroprotective, anticonvulsant, antidepressant, anxiolytic and morphine addiction symptom inhibition effects have been shown in studies conducted so far (2, 4, 5).

The primary damage in the pathophysiology of the central nervous system involves ischemic and traumatic events, while secondary damage involves complex events that begin to develop within minutes at the cellular level. It is known that excitatory neurotransmitters play a role in the pathogenesis of secondary neural damage (6). Excitatory amino acids (EAA) such as glutamate and aspartate are the main neurotransmitters of the mammalian central nervous system. Glutamate directs the synaptic transmission and controls the ion passage, while forming the source of neurotoxicity (7, 8). Acute changes with EAA toxicity begin with the storage of neurons. Extracellular Cl^- moves into the cell. To maintain the ion balance, there is Na^+ enters and the cell swells. Neuronal calcium channels regulate the NMDA receptor. Glutamate leads to neural and glial death due to oxidative stress as a result of plasma membrane depolarisation and entry of Ca^{++} into the cell, activation of free radical-forming systems such as nitric oxide synthase. Ca increase in cells activates lipolytic enzymes. These enzymes cause the release of arachidonic acid from phospholipids in the neuron membrane. Prostaglandin, thromboxane and leukotrienes are synthesized. This continues with free radical and lipid peroxides until the death of the neuron (9). However, there is still no definitive treatment for neuronal damage that develops due to glutamate. Therefore, new clinical and experimental studies are continuing to clarify the mechanism of glutamate toxicity and improve preventive treatments.

The aim of this study is to show the effects of agmatine, which is shown to be promising as a preventive (neuroprotective) agent on glutamate induced neuron damage in newborn rat brain cortex.

II. MATERIALS AND METHODS

A. Animal Supply and Ethics Committee

A total of 4 newborn Spraque Dawley rats obtained from the experimental animal laboratory were used. This study was approved for ethical rules according to the the Local Ethics Committee of Kafkas University Animal Experiments.

B. Primary Neuron Culture

The newborn 4 rats were rapidly decapitated and the cortex were taken surgically and stored immediately in the prepared neurobasal solution. After adding 1:1 Thyripsine and waiting for 30 minutes in the incubator, then centrifuged three times, supernatant was discarded each time and new medium added. In a separate tube, a neurobasal medium prepared with 1000:1 penicillin 50:1 B27 and 10:1 FBS (fetal bovine serum) was prepared. Cells were added to the flask with prepared medium. 150 microliters of medium were added to each well of 96 well plates. 3 cc of medium was added to the 6-well plates. The cells were kept in the incubator for 10 days to adhere to the floor of the chamber and to cover the flask floor until confluence.

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C. Drug Application

Groups
Group 1 — Control
Group 2 — Glut 3x10^{-3} M (G1)
Group 3 — Glut 6x10^{-3} M (G2)
Group 4 — Ag 10^{-5} M
Group 5 — Ag 10^{-6} M
Group 6 — G1+ Ag 10^{-5} M
Group 7 — G1+ Ag 10^{-6} M
Group 8 — G2+ Ag 10^{-5} M
Group 9 — G2+ Ag 10^{-6} M

Described as above, Glutamate toxicity at 6x10-3 M and 3x10-3 M doses induced in each group (10 well per group). Agmatine (dissolved in medium) was administered at 10-5 M and 10-6 M 1 hour before the toxicity was established.

D. Vitality Tests

The number of dead / live cells was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) proliferation kit (Cayman Chemical, Ann Arbor, Mi, USA) described as previously (10).

E. Total Antioxidant Capacity (TAC) and Total Oxidant Capacity (TOC) Analysis

In glutamate toxicity, commercial kit was used to determine the TAC and TOC levels of neuroprotective endogenous molecule agmatine on primary neuron neuron culture cells (Rel Assay Diagnostics, Gaziantep, Turkey). The kit application is calibrated with a stable antioxidant, vitamin E analogy and called Trolox equivalent (Erel 2004; Erel 2005).

A commercial TOC kit was used to determine the TOC levels of neuroprotective endogenous molecule agmatine on primary neuronal culture cells in glutamate toxicity (Rel Assay Diagnostics, Gaziantep, Turkey).

F. Molecular Analysis

RNA Isolation and RT-PCR Measurements

In our study, caspase 3 and caspase 9 mRNA expression levels were compared between groups. RNA extraction from cells: When the cells were plated on 6-well plates, drug administration procedures were performed and cells were removed by removing the medium from the plates. Tissue LYSER II (Qiagen) cells were homogenized and RNA extraction was performed with Qiacube. Using the RNeasy Mini Kit (Qiagen), the total RNA isolation phases from cell samples using the the Qiaqube RNA isolation device (Qiagen) were continued as recommended by the manufacturer. Total mRNA was measured at 260nm with nano drop spectrophotometry (EPOCH, Biotek) (lit ekleee.)

Reverse Transcriptase Reaction and cDNA Synthesis: CDNA synthesis from total RNA was performed using Roche cDNA Reverse Transcription Kit enzyme. Each reaction was performed with 10µl of RNA and the cDNA synthesis was performed with Veriti 96 Well Roch Light Cycler according to the following temperature values. The amount of cDNA was measured by nano drop spectrophotometry (EPOCH Plate, Biotek). The quantitative determination of real time PCR and mRNA expressions was performed using the Roche Gene Expression Master Mix kit. Amplification and quantification was performed on the Roche Light Cycler. The 100 ng cDNA was pipetted as shown in the table below withRoche Gene Expression Assays and conducted with 40 cycles. Ct values were automatically converted to delta Ct on device

G. Statistical Analysis

BM SPSS 20.00 package program was used statistical analyses of the study. One way ANOVA test multiple comparison test was used for the significance of the groups (posthoc the Tukey test). Mean ve SD value were used for comparison of the data. P <0.05 was considered significant.

III. Results

A. Prolifeation Analyses

Glutamate induced primary neuron damage were determined according to the MTT test (Fig. 1). Low dose agmatine administration alone increased the cell proliferation compared to the control group (p<0.05). However, cell viability decreased dose dependent in glutamate groups (p<0.05). Agmatin showed a stronger neuroprotective effect on both doses of glutamate induced neurotoxicity. In particular, low doses of agmatine prevent cell death against glutamate and have a stronger neuroactive effect (p<0.05).

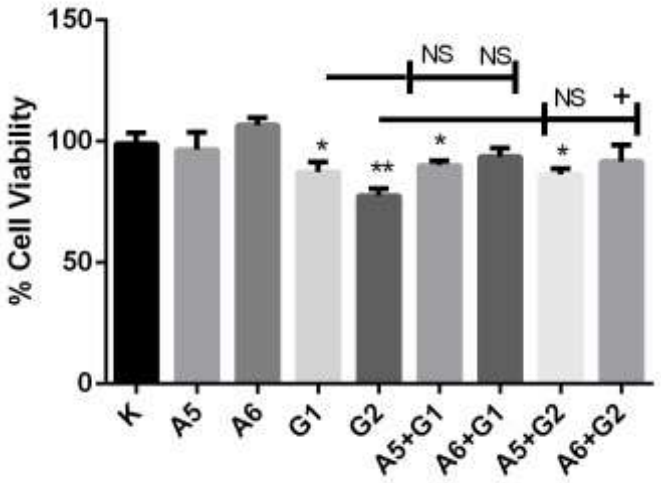

Fig. 1. Demonstration of the effects of agmatine on neuronal cell viability by MTT analysis. (p<0.05 was considered significant).

B. Oxidative Stress Results

According to the total antioxidant capacity (TAC) and total oxidant capacity (TOC) measurements (Table I), in neuron culture medium exposed to glutamate for 24 hours increased TOC level and decreased TAC levels were significantly improved with low dose agmatine (Table I) (p<0.05). In addition, low dose agmatine alone decreased TOC level compared to the control group (p<0.05).

TABLE I. The effects of agmatine on neuronal oxidative system

Groups	TOC mmol/L		TAC mmol/L	
Control	1,57 ±	0,10	2,78 ±	0,19
G2	2,84 ±	0,34**	1,51 ±	0,22**
A6	1,37 ±	0,04*++	2,99 ±	0,17+
A6+G2	1,58 ±	0,27+	2,1 ±	0,2*

TOC: total oxidant capacity, TAC: total antioxidant capacity
*Compared to the control, +compared to the G2

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C. Apoptosis Findings

Caspase 3 Expression Levels significantly increased in cisplatin glutamate group compared to the control group (Fig. 2) ($p<0.05$). The application of agmatinin brought the caspase 3 levels closer to the control group ($p<0.05$). Caspase 9 Expression Levels increased in glutamate group compared to the control group ($p<0.05$). Agmatine administration significantly downregulate caspase 9 mRNA expression compared to the control group and glutamate (Fig. 3) ($p<0.05$).

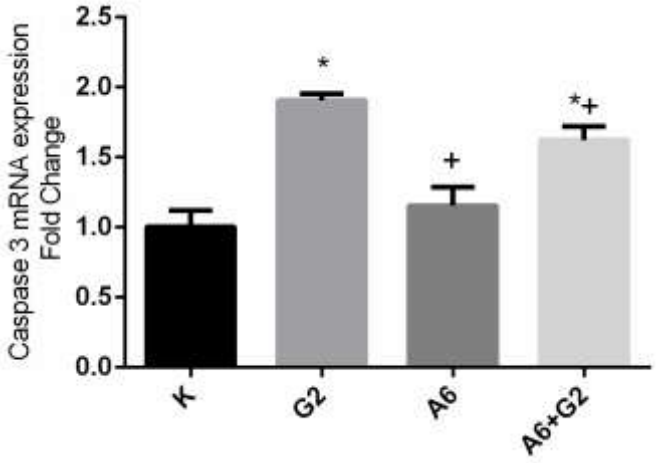

Fig. 2. The effects of agmatine on caspase 3 mRNA expression against glutamate neurotoxicity. ($p<0.05$ was considered significant).

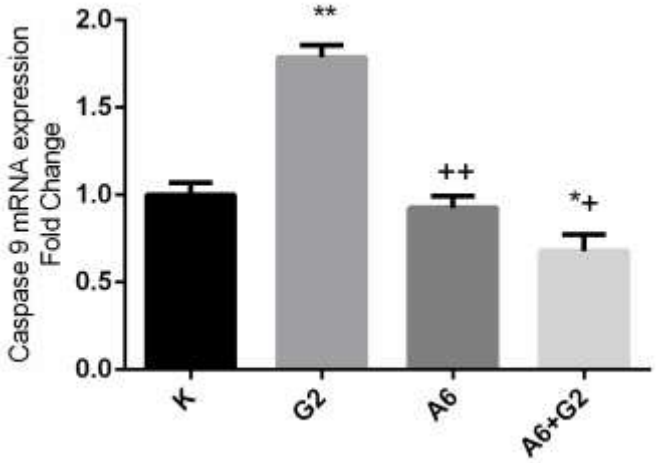

Fig. 3. The effects of agmatine on caspase 9 mRNA expression against glutamate neurotoxicity. ($p<0.05$ was considered significant).

IV. Discussion

In our study, we performed a vitality test to determine the possible toxic and protective effects of agmatine. It has been observed that a low dose of agmatine has an activity that increases cell viability. In addition, two different doses of glutamate were studied for toxicity and both doses showed that glutamate has a serious toxic effect, particularly in high doses. While the doses of agmatine were administered against low dose toxicity of glutamate, all agmatine doses were administered against high dose glutamate toxicity. According to these results, high dose glutamate toxicity and low dose agmatin doses were found to be effective.

Glutamate is the anion of glutamic acid and acts as neurotransmitter in neuroscience. Glutamate is one of the stimulant neurotransmitters released from the basal ganglia. It is the most abundant neurotransmitter in the vertebrate nervous system. Excessive activation of glutamate leads to exocytosis (11, 12). In chronic neurodegenerative diseases, exitotoxic findings may occur as a result of an abnormality in stimulating amino acid receptors and/or disruption of energy metabolism. Glutamate is known to play a role in sinaptogenesis, learning and memory, as well as in many pathological conditions such as ischemia, epilepsy and neurodegenerative diseases (13, 14). Glutamate is converted enzymatically into glutamine after it is taken by neurons or gliales, thus it can be stored in the cell until it is converted to glutamate again. In stress conditions such as trauma or central nervous system disorders, glutamate is increased in synaptic intervals, either through direct release from neurons or by the closing of the glial glutamate carriers. Excessive levels of extracellular glutamate can lead to negative effects on cell health and life due to excessive stimulation of neurotransmitter receptors. Oxidative stress is an important role of glutamate neurotoxicity (15-17). In addition, a high level of extracellular glutamate inhibits the function of glutamate/cystine anti-oxidants, inhibiting the formation of glutathione, resulting in cellular free radical increase and cellular toxicity (7, 8). In current study, glutamate significantly increased primary neuron damage by increased doses and led to inhibitor effect on cell proliferation during 24 h. However, agmatine administration showed protective effect against glutamate induced neuronal damage by decreased oxidative stress and increased antioxidant defence system.

Agmatine act as a free radical scavenger and protective against oxidative stress (18). Previously, Agmatine and its role of oxidative stress showed in mouse cortical neural stem cells (NSCs) (19). Agmatine improved the NADPH oxidase activity, led to oxidative inactivation (20), and decreases OH- in vitro under high glucose conditions (21). In our study, Agmatine administration significantly reduced oxidative stress compared to the control. In additon, Agmatine exert neuroprotective effect against glutamate by decreasing oxidative stres and incresing antioxidant system.

Glutamate acts by activating iGluR and mGluR. mGluR provides the control of intracellular enzymes by the G protein mediated pathway (22). iGluR is responsible for most of the excitotoxic neurotransmission in CNS by controlling ion channels. iGluR is divided into two as NMDAR and non-NMDAR receptors (22). The molecular mechanism of neuronal death that occurs through iGluR in CNS has becoming better understood. Apoptosis cause neuronal cell death as a result of glutamate toxicity which activates caspase protein cascades. Caspases are the main proteins which regulate apoptotic signals (23). Moreover, increased oxidative stress promotes apoptosis by stimulating caspase 3 and 9 activity (24). Firstly, Caspase 9 have role of initiating apoptosis and lead to pro-caspase 3 and Caspase 3 synthesis

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which acts as an apoptotic executor in the second phase of apoptosis. Similarly, current study showed that glutamate significantly up regulated caspase 3 and 9 mRNA expression and lead neuronal cell death. However, agmatine improved these effects of glutamate and downregulated caspase 3 and 9 mRNA expression.

Besides the antioxidant properties of agmatine, its effects on neurons are possibly associated with inhibition of apoptotic protein cascades. Zhu et al. showed that agmatine induces prevention of caspase-3 activation against dexamethasone (25). In addition, Moosavi et al. demonstrated agmatine administration prevented streptozotocin-induced hippocampal apoptosis (caspase-3 and Bax/Bcl-2 ratio) (26). Agmatine is also able to downregulate apoptotic protein synthesis in retinal ganglion cells by inhibiting TNF-a (27, 28). An antioxidant effect of agmatine also confers resistance to neuronal apoptosis both preventing oxidative stress and blocking the intrinsic apoptosis (29). Current study findings provide a biochemical and molecular basis for the neuroprotective actions of agmatine.

V. CONCLUSION

In our study, glutamate reduced cell viability in increased doses, while agmatine showed the best neuroprotective effect in low doses. Increased total oxidant capacity due to toxicity was significantly improved by agmatine. Again, the antioxidant capacity decreased in the toxicity group showed improvement with the application of agmatine. At the same time, increased Caspase 3 and Caspase 9 mRNA expressions after glutamate application significantly decreased with agmatine application. In our study, the best results were obtained with low dose of agmatine. In light of all this information, agmatine, a potent NMDA receptor antagonist, prevented glutamate-induced neurotoxicity.

VI. LIMITATIONS

Cell cultures prepared with cells isolated from various tissues show some common features. The use of primary neuron cultures is especially important in the investigation of neurodegeneration mechanisms. One problem in the use of continuous cell lines developed for use in neurobiological research is that these cells cannot perform some basic events of neuronal differentiation. Although they are cell lines that express neurotransmitters, ion channels, receptors and neuron specific proteins that are characteristic of differentiated neurons, they are not a good model for studying a specific neuron phenotype. Therefore, currently known cell lineage provides limited resources for studies on the central nervous system (21).

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Conflict of Interest

None of the conflict of interest for all authors.

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