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The Dose-Duration-Effect Profile of Agmatine on Rat Primary Neuron Cell Culture*

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Summary: Agmatine is a newly found neurotransmitter and its role in important modulations of central nervous systems how the pharmacological importance of agmatine. The aim of this study was to investigate the possible toxic and proliferative effects of agmatine, which is known to have endogenous neuroprotective effect, on primary rat neuron culture. In this context, it has been tried to determine the possible toxic and protective effects of agmatine at 8 different exposure times at 5 different doses. Neuron cultures obtained from brain cortex in the neonatal rats are treated with agmatine at 15 min, 30 min, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 24 hours, doses of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M. Then, the dose duration effect relationship was determined with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) proliferation kit. According to the results of the viability assay, a statistically significant neuroprotective effect was observed in 10^{-5} and 10^{-6} M doses, especially in 1 hour and 24 hours exposure periods. In addition, proliferation was observed only at a concentration of 10^{-4} M for 24 hours. However, no statistically significant difference in toxic effect was observed. Consequently agmatine showed neuroprotective effect in the primary rat neuron culture, especially at the lowest dose and the longest exposure.
Key words: Agmatine, cell death, neuronal cultures, neuroprotection

Sıçan Primer Nöron Kültüründe Agmatinin Doz-Süre-Etki Profili

Özet: Agmatinin yeni bir nörotransmitter adayı olması ve santral sinir sisteminde çok sayıda önemli modülasyonda görev alması farmakolojik önemini göstermektedir. Çalışmamızda endojen nöroprotektif etkisi olduğu bilinen agmatinin primer sıçan nöron kültüründe olası toksik ve proliferatif etkilerini ekzojen olarak araştırması amaçlanmıştır. Bu kapsamda agmatinin olası toksik ve protektif etkileri 5 farklı dozda ve 8 farklı maruziyet süresinde belirlenmeye çalışılmıştır. Yeni doğan sıçanların beyin korteksinden elde edilen nöron kültürlerine 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} ve 10^{-6} M konsantrasyonda agmatin uygulanarak 15 dk, 30 dk, 1 saat, 2 saat, 4 saat, 8 saat, 12 saat ve 24 saat süre ile maruz bırakılmıştır. Daha sonra doz süre etki ilişkisini belirlemek için MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) proliferasyon kiti kullanılmıştır. Yapılan canlılık testi analiz sonuçlarına göre 10^{-5} ve 10^{-6} M konsantrasyonda özellikle 1 saat ve 24 saat maruziyet sürelerinde istatistiksel olarak anlamlı nöroprotektif etki gözlenmiştir. Buna ilaveten sadece 10^{-4} M konsantrasyonda 24 saat maruziyet süresinde proliferasyon artışı gözlemlenmiştir. Ayrıca hiçbir zaman diliminde toksik etki gözlemlenememiştir. Sonuç olarak agmatin primer sıçan nöron kültüründe özellikle en düşük dozunda ve en uzun maruziyet süresinde nöroprotektif etki göstermiştir.
Anahtar kelimeler: Agmatin, hücre ölümü, nöronal kültür, nöroproteksiyon

Introduction

Biogenic amines are compounds formed by the decarboxylation of amino acids or by amination and transamination of aldehyde and ketones as a result of metabolic activity of animals, plants and microorganisms (1). The formation of biogenic amines increases the pH of the environment and protects the microorganisms from the effect of the acidic environment. According to many studies, different bacterial strains produce biogenic amines. Histamine, tyramine, serotonin, dopamine and agmatine are biogenic amines found in foods (8). Biogenic amines, found in high quantities in foods, are thought to be a significant health hazard in humans (13). Metabolites of important biogenic amine agmantin such as spermine and spermidine also act as biogenic amines. Agmatine is a molecule in the structure of endogenous guanidoamine, which occurs as a result of decarboxylation of the mitochondrial arginine of L-arginine, which is a semi essential amino acid. The formation of agmatine which was determined to be synthesized in mammals after 1990 depends on the existence of L-arginine. Agmatine is found in cell groups such as brain, kidney, endothelial, vascular smooth muscle

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cells and even astrocytes. In the central nervous system, agmatine is locally synthesized and stored in synaptic vesicles. Like other transmitters, it is released by calcium-dependent depolarization. Biological inactivation in the brain tissue is the result of enzymatic degradation of Agmatinease which is released from axon terminals via energy dependent mechanisms and contained in mitochonrial matrix, into synapticosomes. As a result of its destruction, diamine precursors are metabolized into putresine and urea, or broken down with diamin oxidase (18,23). Some of the agmatine is taken back to the presynaptic neuron and the reuptake can reach a high concentration of saturation. Agmatine NMDA (N-Methyl D-Aspartic Acid), plays an important role in the peripheral and central nervous system through receptors such as alpha adrenergic, opioid, imidazoline, nicotinic receptors, forming a very wide spectrum of pharmacological effects (7,14). Alpha 2 binds to all sub-types of adrenergic receptors with high affinity. On the other hand, it is able to inhibit one of the most important receptors of the glutamatergic system, NMDA receptors, even at micromolar concentrations. It also inhibits inducible nitric oxide synthase and neural nitric oxide synthase. The neuroprotective effects of agmatine were determined in 1995. Several studies have shown that there are cytoprotective effects, including especially neuroprotective, cardioprotective and nephroprotective effects. NMDA receptor inhibition after neurotrauma has led to a focus on the neuroprotective effect. This blockage is mainly due to the relationship between the guanido group and NMDA receptor channel pores (11,12). These effects have also been shown to have neuroprotective effects in situations such as stroke and neuropathic pain, occur through the modulation of neurotransmitter receptors such as NMDA, modulation of ion channels such as calcium, and inhibition of nitric oxide synthesis in paticular. For this reason, agmatine has been shown to be promising both as a preventive (neuroprotective) agent and as neurorecovery therapy after cerebrovascular events. To summarize the pharmacological effects of agmatine in general, antinociceptive, antinflammatory, neuroprotective, anticonvulsant, antidepressant, anxiolytic and inhibition of morphine dependence symptoms has been shown in studies conducted so far (2,17). The aim of this study is to show the possible protective and toxic effects of agmatine in primary neuron culture in terms of dose and duration and to present the effect profile. In addition, to determine the dose at which agmatine has a high neuroprotective effect and the duration in which this effect occurs.

Materials and Method

In this study, the provisions of the “Guidance on the Use and Care of Laboratory Animals” were complied with, and the study was approved by the Animal Experiments Local Ethics Committee of Kafkas University (HADYEK 2018/031).

Preparation of cell culture

Ten new born Sprague-Dawley rats were quickly decapitated and the cortex portion removed and placed in a prepared salt solution (Hanks’ Balanced Salt solution, Thermo Fisher, Germany). After macro shredding with a scalpel, 1:1 trypsin-ethylenediamintetra acetic acid (0.25% Thermo Fisher, Germany) was added for micro shredding, and after incubation for 30 min, DNase type 1 (Sigma Aldrich® Steinheim, Germany) was added. Centrifuge was performed 3 times at 1200 rpm for 5 min and supernatant was discarded each time and new medium was added. In a separate tube, 1000:1 Antibiotic (Penicillin–Streptomycin, Thermo Fisher, Germany) 50:1 B-27 (Suplament, Thermo Fisher, Germany) and 10:1 fetal bovine serum (Gibco, USA) was added to the neurobasal medium (88% NBM, Gibco, USA). Cells were added to the prepared medium. 150 microliters of medium were added to each well of 96 well plates. Cells were seeded to have 10^a (a=5) cells in 100 µL culture medium per 96 well plate. The cells were kept in the incubator for 10 days to adhere to the floor of the chamber and to cover the floor and to grow (6). After the neuron cells showed sufficient growth and covered the base of the well plates (Figure 1), the experimental study was started.

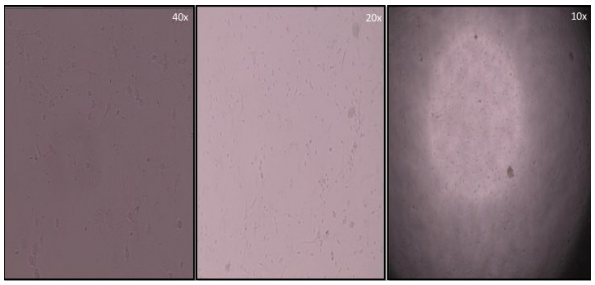

Figure 1. Inverted microscopy of primary neuron cultures after 10 days of incubation

Drug application

Forty one groups were created for dose time application and each group was allocated 10 wells (n=10) and 5 96 well plates were used. A total of 480 cultures were established. The established cortex primary culture was subjected to agmatine (CAS Number 0002482000, Gibco-Life Technologies, Australia) at 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M concentrations for 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h. Then, the dose duration effect relationship was determined with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) proliferation kit. The experimental groups were planned as follows (Table 1).

Table 1. Experimental groups

1.Group: Pure cell culture								
	15 min	**30 min**	**1 hour**	**2 hour**	**4 hour**	**8 hour**	**12 hour**	**24 hour**
10^{-2}M	2.Group	7.Group	12.Group	17.Group	22.Group	27.Group	32.Group	37.Group
10^{-3}M	3.Group	8.Group	13.Group	18.Group	23.Group	28.Group	33.Group	38.Group
10^{-4}M	4.Group	9.Group	14.Group	19.Group	24.Group	29.Group	34.Group	39.Group
10^{-5}M	5.Group	10.Group	15.Group	20.Group	25.Group	30.Group	35.Group	40.Group
10^{-6}M	6.Group	11.Group	16.Group	21.Group	26.Group	31.Group	36.Group	41.Group

Viability assay

MTT (Cayman Chemical, Ann Arbor, MI, USA) was used to determine the effects of agmatine on cell viability at the determined doses and periods. 10 µL MTT mixture was added to each well by pipette. Cells were incubated at 37° C for 3-4 hours in the CO_2 incubator. After incubation, the formazan in the cells was seen as dark crystals at the bottom of the wells. 100 µL of cistal Solvent Solution was added to each well. This solution dissolved formazan crystals and created an orange solution. Formazan absorbance was evaluated with ELISA reader (MicroQuant, Reader, BioTek, Winooski, VT, USA) at 570Nm wavelength (6,15).

Statistical analysis

The IBM 20.00 SPSS software was used for statistical analysis of the data obtained in the study. One-way analysis of variance was used in the analysis of more than two independent groups in terms of the variables, and the Tukey test was used in the evaluation of the groups that created the difference. Statistical significance was accepted as P<0.05.

Results

In this study, agmatine was applied in concentrations of 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}M to the neuron cultures prepared to determine the toxic and proliferative effects of agmatine on primary neurons in the context of dose and time. MTT test was performed at 8 different durations, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours and 24 hours. As shown in the results shown in Figures 2, 3, 4 and 5, a statistically significant proliferation was observed, especially with 24-hours exposure.

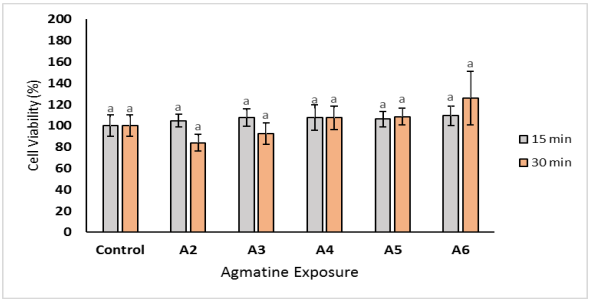

Figure 2. Results of viability test after 15 min and 30 min agmatine administration to primary neuron cultures. Means in the same column with the same letter are not significantly different; means in the same column with different letters indicate significant differences between the groups (P<0.05 was considered significant). C: Control, A2: 10^{-2} M Agmatine, A3: 10^{-3} M Agmatine, A4: 10^{-4} M Agmatine , A5: 10^{-5} M Agmatine, A6: 10^{-6} M Agmatine

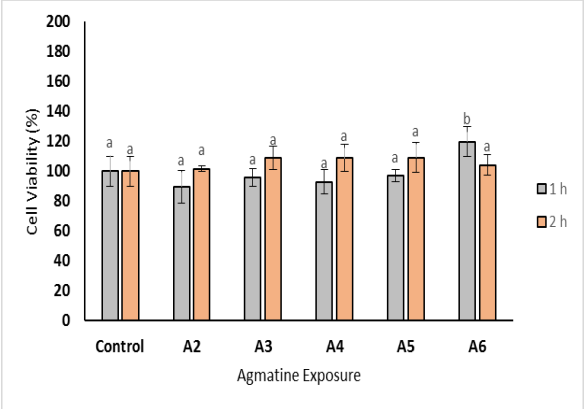

Figure 3. Results of viability test after 1 hour and 2 hours agmatine application to primary neuron cultures. Means in the same column with the same letter are not significantly different; means in the same column with different letters indicate significant differences between the groups (P<0.05 was considered significant). C: Control, A2: 10^{-2} M Agmatine, A3: 10^{-3} M Agmatine, A4: 10^{-4} M Agmatine , A5: 10^{-5} M Agmatine, A6: 10^{-6} M Agmatine

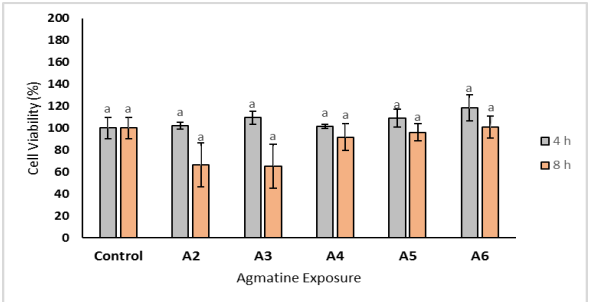

Figure 4. Results of viability test after 4 hours and 8 hours agmatine application to primary neuron cultures. Means in the same column with the same letter are not significantly different; means in the same column with different letters indicate significant differences between the groups (P<0.05 was considered significant). C: Control, A2: 10^{-2} M Agmatine, A3: 10^{-3} M Agmatine, A4: 10^{-4} M Agmatine , A5: 10^{-5} M Agmatine, A6: 10^{-6} M Agmatine

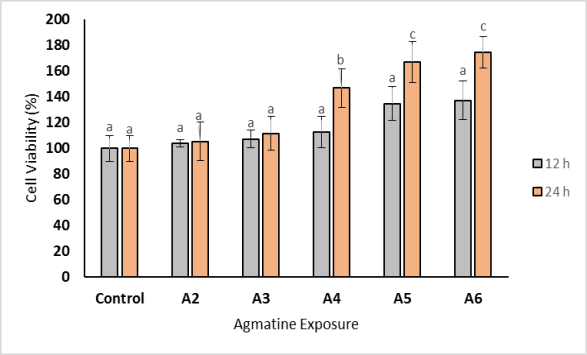

Figure 5. Results of viability test after 12 hours and 24 hours agmatine application to primary neuron cultures. Means in the same column with the same letter are not significantly different; means in the same column with different letters indicate significant differences between the groups ($P<0.05$ was considered significant). C: Control, A2: 10^{-2} M Agmatine, A3: 10^{-3} M Agmatine, A4: 10^{-4} M Agmatine, A5: 10^{-5} M Agmatine, A6: 10^{-6} M Agmatine

Statistical significance was observed in 10^{-4} 10^{-5} 10^{-6} M doses at 24 hours exposure duration (Figure 5). At 1 hour exposure (Figure 3), 10^{-6} M dose agmatine administration showed cell viability enhancing activity when compared to the control group. When the results were examined in general, it was observed that high doses of agmatine could not increase the proliferations in any period of time and, on the contrary, although not statistically significant, decreased cell viability compared to the control group. In this study, it was observed that agmatine has a proliferative effect in 10^{-5} and 10^{-6} M doses and this effect is evident with 24 hour exposure. Another important finding of this study was the statistically significant increase in proliferation at 10^{-6} dose and 1 hour exposure.

According to the MTT test results, it was observed that agmatine showed a high proliferative effect at the lowest doses, while high doses caused a decrease in cell viability. However, since this reduction was not statistically significant, it could not be said that agmatine caused toxicity. In order to determine the duration of proliferation by agmatine, a study was planned on a wide period of time. According to our results, when agmatine was administered for 1 hour, its proliferation was statistically significant only in the lowest dose of agmatine compared to other groups and times. In addition, with 24 hour application of agmatine, agmatine has been shown to make a significant difference in the minimum two concentrations compared to other times. The increased proliferation in other exposure periods did not make a statistically significant difference.

Discussion

Studies on agmatine remain important today. Our study was the first study to show the effect profile of agmatine, whose neuroprotective activity has been shown, on primary neuron culture. The study of primary neuron culture instead of neuron cell lines is the most accurate method in which the exogenous effects of agmatine can be demonstrated in vitro. In cell cultures prepared with cells isolated from various tissues, some common features show. The cells in the culture first enter a rapid division process called the growth phase. Then, the cells which come in contact with each other slow down the division and go through the process of differentiation to gain the characteristic of the tissue from which they are taken. When the neural cells obtained from the embryonic brain are cultured, the division stops completely. These cells extend their neurites and come into contact with each other to form synapses and thus become electrically active. These characteristics make neuron culture different from other cell cultures. Cell lines originating from these cells do not fully demonstrate neuronal properties. Therefore, 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 synthesize neurotransmitters, ion channels, receptors, and proteins specific to neurons, which are the characteristic of differentiated neurons, they are not a good model for working on a specific neuron phenotype. Even the PC12 cell closest to a differentiated nerve cell is unable to properly form axons and dendrites and can't establish synaptic connections. Therefore, currently known cell lines provide limited resources for studies on the central nervous system (9,22). Therefore, in this study, the effects of rat primary neuron culture method and agmatine were investigated instead of cell line. In particular, the preference of cell culture is important in terms of the number of experimental animals used. Thus, a wide range of studies can be performed with only 10 experimental animals, instead of using hundreds of experimental animals.

Agmatine, a polyamine, interacts with G proteins, protein kinases, nucleotide cyclase and receptors in the central nervous system, as well as with other systems such as catecholamines, gamma-aminobutyric acid, nitric oxide and glutamate. It has an important contribution to cell growth and regulation of cell membrane functions (including neurons) in mammals. It is known that agmatine, a biologically active substance, blocks glutamatergic NMDA receptors in rat hippocampal neurons (19). It is suggested that agmatine is an endogenous neurotoxicity inhibitor because of its NMDA receptor antagonist activity. Agmatine has been shown to have protective efficacy over neurotoxicity induced by glutamate (4). On the other hand, a study reported that agmatine produces schizophrenia-like effects in experimental animals

over a certain dose range (10). On the other hand, it has been suggested that the administration of agmatine in uncontrolled and high doses may cause polyamine stress. Polyamine stress has been associated with many mental disorders, primarily suicide (5). In addition to mental problems, polyamine stress has also been reported to be associated with many types of cancer (21). It has been reported that the use of agmatine as a food supplement by bodybuilders for pain relief effect can cause some negative effects due to its potential addictive effect (10). In a study of the effects of subcutaneously administered agmatine on the skin, it was observed that it caused late dermal reaction. On the other hand, intraperitoneal application has been shown to have no such effect. This finding suggests that prolonged subcutaneous agmatine administration in rats has toxic effects (24). In light of this information, it has been observed that agmatine should be used in the lowest dose for neuroprotective activity. The lowest dose having been found to be effective in this study supports findings in literature. On the other hand, the emergence of the strongest effect with a chronic exposure time of 24 hours has given rise to the idea that it may be unfavourable given the addictive effects of agmatine shown in literature. For this reason, it can be seen that it is more appropriate to apply agmatine for a 1-hour period which can be called acute exposure, where it has been shown to have other significant proliferative effects.

In vitro studies on rat hippocampal neurons investigated the protective efficacy of agmatine against neural damage caused by glucocorticoids. As a result of the study, agmatine was thought to be protective against neurotoxicity due to glucocorticoids, and this effect was due to antiapoptotic effect due to NMDA receptor antagonism (16). In a study to examine the effects of agmatine against neurotoxicity caused by anticancer drugs, promising results were again obtained (3). If we look at the relationship between cancer and agmatine, which increases healthy cell proliferation, unlike the polyamines of which it is a precursor, agmatine has been shown to prevent the proliferation of cancer non-intestinal mammalian cell models. Studies on the human colon adenocarcinoma cell line have shown that agmatine accumulated within the cell has a strong static effect (20). Whether the proliferative effect of low-dose agmatine as shown in our study is due to an NMDA-mediated mechanism or the antioxidant properties shown chemically by agmatine should be investigated. It has been shown in literature that it has a proliferative effect against toxicity, while it has an apoptotic activity on dividing cells such as cancer cells. In this study it was observed to have a proliferative effect on healthy neuronal cells. Although it is observed that high doses of agmatine has apoptotic activity, not statistically affecting cell viability is evidence that it is non-toxic.

In conclusion, in this study, the doses in which agmatine increased proliferation in primary neuron culture cells and the duration of exposure producing this effect were determined. It was observed that agmatine, especially the lowest concentration of 10^{-6} M, increased cell proliferation. Although other high concentrations partially decrease cell viability, it remained statistically insignificant. According to our results; agmatine administered for 1 hour (with acute effect) at the lowest dose increased proliferation to a statistically significant level compared to the other groups. In addition, the administration of agmatine for 24 hours (with chronic effect) has been shown to make a significant difference in the minimum two concentrations compared to the other periods. Our findings suggest that agmatine is a proliferative target that can be used in cases that result in neural death. Furthermore, the in vivo demonstration of this effect which has been put forward in vitro is sufficient to illuminate current debates on agmatine in literature.

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