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Protective Effects of Memantine in Experimentally Induced Cerebral Ischemia and Reperfusion Injury in Rats

Deneysel Serebral İskemi-Reperfüzyon Oluşturulmuş Ratlarda Memantinin Koruyucu Etkilerinin İncelenmesi

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Summary

Objective: The severity of apoptosis developing after hypoxia-ischemia and reperfusion is an indicator of cerebral injury. In cerebral ischemia, there are many factors initiating the events progressing to cell death. The most common leading cause is excessive increase in intracellular calcium concentration. Ion channels in NMDA receptors cause cell death by increasing Ca^{++} entries into the cell. Memantine is a non-competitive excitatory amino acid blocker of the NMDA receptor. Studies suggesting administration of memantine before and after ischemia decreasing the neural injury have been published. In this study we aimed to examine the memantine could have a decreasing effect on neuronal injury resulting with apoptosis especially in the penumbra region after ischemia and its effects on antioxidants and oxidants in brain tissues.

Material and Method: Experimental study was performed in three groups; each of them including 7 rats. The control group (without any intervention) was used for evaluation of the normal brain tissue. Transient focal cerebral ischemia was performed by clipping the right common carotid arteries of the rats in ischemia and ischemia-drug groups. Ten mg/kg intraperitoneal memantine was administered in ischemia-drug group 30 minutes after ischemia and for 5 days thereafter. All of the rats were sacrificed after the experiment. Antioxidant and oxidant levels of the cerebral tissues were measured. Apoptotic cells were determined immunohistochemically using TUNEL method.

Results: When the memantine administered group was compared with the ischemia group, we observed that memantine decreased apoptotic cells in the brain tissue and there was an improvement in the oxidant levels ($p<0.05$).

Discussion: In conclusion, memantine may be effective in prevention of apopitosis and neuronal injury in cerebral ischemic tissue via decreasing cerebral oxidant formations. *(Turkish Journal of Neurology 2013; 19:85-9)*

Key Words: Memantine, apoptosis, ischemic stroke, antioxidants, oxidants

Özet

Amaç: Hipoksi-iskemi ve reperfüzyon sonrasında gelişen apopitozisin şiddeti, beyin hasarının bir göstergesidir. Serebral iskemide hücre ölümüne uzanan olayları başlatan birçok faktör bulunmaktadır. En önemli faktörlerin başında hücre içi kalsiyum konsantrasyonundaki aşırı artış gelmektedir. N-metil-D-aspartat (NMDA) reseptörlerindeki iyon kanalları Ca^{++}'nın hücreye girişini artırarak hücre ölümüne neden olmaktadırlar. Memantin, NMDA reseptörünün nonkompetitif eksitatör aminoasid blokörüdür. İskemi öncesi veya sonrası memantin uygulamasının nöronal hasarı azalttığını ileri süren çalışmalar yayınlanmıştır. Bu çalışmada; memantinin iskemi sonrası, özellikle penumbra alanındaki apopitoz ile sonuçlanan nöronal hasarı azaltıcı ve beyin dokularında antioksidan ve oksidan düzeylerine olan etkilerinin araştırılması amaçlandı.

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Gereç ve Yöntem: Deneysel araştırma deney hayvanları her birinde 7 rat olacak şekilde üç grupta yapıldı. Kontrol grubuna hiçbir işlem yapılmadı ve normal beyin dokusunun değerlendirilmesi amacıyla kullanıldı. İskemi ve iskemi-ilaç grubundaki ratların sağ kommon karotis arteri kliplenerek geçici fokal serebral iskemi oluşturuldu. İskemi-ilaç grubuna iskemiden 30 dakika sonrasında ve 5 gün boyunca 10 mg/kg intraperitoneal memantin uygulandı. Deney sonunda tüm ratlar sakrifiye edildi. Beyin dokularında antioksidan ve oksidan düzeylerinin ölçümü yapıldı. Tüm örneklerde TUNEL yöntemiyle immunohistokimyasal olarak apopitotik hücreler belirlendi.

Bulgular: İskemi grubu ile karşılaştırıldığında, memantin uygulanan grupta beyin dokusundaki apopitotik hücrelerin azaldığı ve oksidan düzeylerinde de iyileşme olduğu görüldü ($p<0,05$).

Sonuç: Sonuç olarak, serebral iskemik dokuda memantin, serebral oksidan oluşumunu azaltarak apopitozun ve nöronal hasarın azalmasında etkili olabilir. *(Türk Nöroloji Dergisi 2013; 19:85-9)*

Anahtar Kelimeler: Memantin, apopitoz, iskemik inme, antioksidan, oksidan

Introduction

Ischemic stroke research has a lot of contribution in molecular, genetic, clinical and biochemical aspects in the recent years. Due to the decreased blood flow, a host of biochemical and immunological reactions take place during ischemic stroke (1). In the physiopathology of acute ischemic stroke, changes in ions, excitotoxicity, free radicals, nitric oxide damage and post-ischemic inflammatory reactions play important roles (1).

Apoptosis is one of the most important mechanisms in the reperfusion and the secondary damaging of the brain parenchyma following ischemia (1). There has been research with numerous agents to alleviate the negative effects of this activation (2). The ion channels in N-mthyl-D-aspartate (NMDA) receptors cause cell death by allowing Ca++ influx to the cell. It is known that the increased intracellular Ca++ concentration also causes an increase in free radicals and oxidants. The protective and apoptosis-preventing effects of NMDA receptor antagonists on ischemia-reperfusion pathway have been reported (3). However there has not been a study in the literature investigating the effect of NMDA receptor antagonists on the antioxidant and oxidant levels in the brain.

In the present study, we aimed to evaluate the effects of memantine, an NMDA antagonist, on both the apoptosis and the antioxidant and oxidant levels in the brains of rats with experimentally induced cerebral ischemia-reperfusion.

Materials and Methods

Following the ethical approval of our experimental study by the Institutional Board of Ethical Research on Experimental Animals, 3 experimental groups with 7, 200-220g female Wistar rats in each were formed by sampling randomly from the Institutional Experimental Animals Research Center.

Group 1 (C): Control group without ischemia (N=7)

Group 2 (IC): Ischemia-reperfusion control group that was kept alive for 5 days (N=7)

Group 3 (IM): Ischemia-reperfusion memantine group that was kept alive for 5 days (N=7)

The healthy rats in the control group only received a hypodermic incision. The cerebral ischemia model was not applied to the first group. They were sacrificed at the end of the 5th day. The other two groups, however, received the same surgical procedure to induce ischemia. The rats in the second group received 0.9% saline 10 mg/kg intraperitoneally during 5 days, starting 30 minutes after the release of clamps. In accordance with the previous studies, the rats in the third group received 10 mg/kg/day memantine intraperitoneally during 5 days, once a day at the same hour, starting at the 30th minute of the induced trauma (3). The rats in the second and third group were also sacrificed at the end of the 5th day.

Transient Focal Cerebral Ischemia Model

All of the animals received general anesthesia using 10 mg/kg xylazine and 50 mg/kg ketamine hydrochloride intramuscularly. The depth of anesthesia was evaluated by pain administration at every 15 minutes from the tail. The body temperature of the rats were kept at 37°C using a rectal thermometer. All of the surgical operations were done under microscope (Opmi 99, Carl Zeiss, Germany).

The rats in the second and the third group were fixed on the operating table in supine position. The medial neckline was then shaved. The medial line incision was made after disinfecting the operative area. After the surface microdissection, the right common carotid artery was reached by doing deep microdissection. Once the trachea was seen, common carotid artery was available for manipulation after dissecting paratracheal muscles. In order to gain proximal and distal control, 2 Yaşargil aneurism clips were placed on common carotid artery from 1cm to 3cm away from the carotid bifurcation. The clips were kept close for 10 minutes. In the first group of rats there was only hypodermic incision. No artery ligation was made. All of the open incisions were sutured after 10 minutes.

Biochemical Study

Total Antioxidant Status

Total antioxidant status (TAS) measurement was made using the total antioxidant activity method described by Özcan Erel (4). The resulting measurement is reported using µmol Trolox equivalent/L units.

Total Oxidant Status

Total oxidant status (TOS) measurement was made using the method described by Özcan Erel (5). The resulting measurement is reported using µmol Trolox equivalent/L units.

Oxidative Stress Index

Oxidative Stress Index (OSI) value is defined as the percentage ratio of TAS to TOS. First the TAS values were converted to mmol/L. OSI value was calculated using the Formula method (6). OSI(Arbitrary Unit) = TOS(mmol H2O2 Equiv./L)/TAS (mmol Trolox Equiv./L).

TUNEL Method

Five to six µm thick slices from paraffin blocks were transferred to polylysine plates. Following the producer's instructions, the cells undergoing apoptosis were identified using ApopTag plus peroxidase in situ apoptosis detection kit (Chemicon, cat no:

S7101, USA). The tissues that were deparaffinized with xylene were washed with phosphate buffered saline (PBS) after being treated with graded alcohol series. The tissues which were incubated with 0.05% proteinase K for 10 minutes were then incubated with 3% hydrogen peroxide for 5 minutes to prevent endogenous peroxidase activity. After being washed with PBS the tissues were incubated with equilibration buffer for 6 minutes and then incubated with wet medium solution (%70 µl Reaction Buffer + %30 TdT Enzyme) for 60 minutes. The tissues were treated with anti-digoxigenin-peroxidase for 30 minutes after being waited in Sop/Wash buffer for 10 minutes. The apoptotic cells were visualized with Diaminobenzidin (DAB) substrate. The slices were closed using appropriate closing solutions after Harris hematoxylin counterstaining. Breast tissue was used for positive control. The prepared dishes were inspected with the research microscope (Olympus BX–50) and photographed. In the TUNEL staining evaluation, the cells that were stained in blue with Harris hematoxylin were considered as normal, the ones with brown nuclear staining were considered apoptotic. At least 500 normal and apoptotic cells were counted in randomly selected frames in 10x enlarged slices. Apoptotic index (AI) was calculated as the ratio of apoptotic cells to total (normal + apoptotic) number of cells.

Statistical Analysis

The acquired data was represented as mean±standard deviation. Student t-test and ANOVA was used to determine the statistical differences. $P<0.05$ was accepted as a statistically significant difference.

Results

Biochemical Findings

TAS, TOS and OSI Values

The groups were not found to be significantly different from one another in when they were compared for TAS values. For TOS and OSI, however, Group 2 was significantly higher in both, compared to Group 1. Group 3, on the other hand, showed a decreased TOS compared to Group 2 (Table 1).

Discussion

Ischemic stroke remains as an important health issue due to its high mortality rate and its morbidity. Cerebral ischemia initiates the molecular processes that are triggered by the energy deficiency due to decreased blood flow to the brain. Changes in the glucose metabolism take place, energy metabolites such as ATP and phosphocreatine decrease while lactate levels increase. Metabolic imbalance occurs as a consequence. The decrease in ATP causes a disruption in membrane depolarization and permeability, therefore increasing sodium, calcium, chlorine concentrations within the cell, and potassium concentration outside the cell. Consequently, glutamate, glycine and GABA are released outside the cell (7, 8). Glutamate increases calcium influx by activating NMDA channels. Intracellular calcium accumulation stimulated the formation of free radicals by the activation of NOS pathway which leads to the formation of NO. The overactivation of the NMDA receptors triggers a chain of events that lead to apoptosis (9-11). Experimental studies have tried to diminish or slow down the apoptosis process using different drugs, and partially restoring certain functions with reperfusion. Memantine is often used as NMDA antagonist in the treatment of mild to severe cases of Alzheimer's disease. It acts by reducing the intracellular calcium accumulation caused by high glutamate levels (12).

Anoxic hypoxic neuronal culture studies showed that EAA that is being released in the synaptic gap reaches neurotoxic levels. This damage was alleviated by both competitive and noncompetitive NMDA antagonists (13, 14). The studies with NMDA antagonists on global ischemia models generally have given discouraging results. Focal ischemia, however, gave promising results (15). The physiopathogenesis in focal ischemia is completely different than that of global ischemia. Here there is infarcted tissue in the center, surrounded by partly preserved penumbra area. While the blood flow is completely cut off in the center, this is not necessarily the case for the periphery, which makes this region the prime target for intervention.

It has been possible to reduce the inflicted ischemic damage in focal cerebral ischemia models by 50% by using NMDA antagonists. The protection is maximal at the cortex and minimal

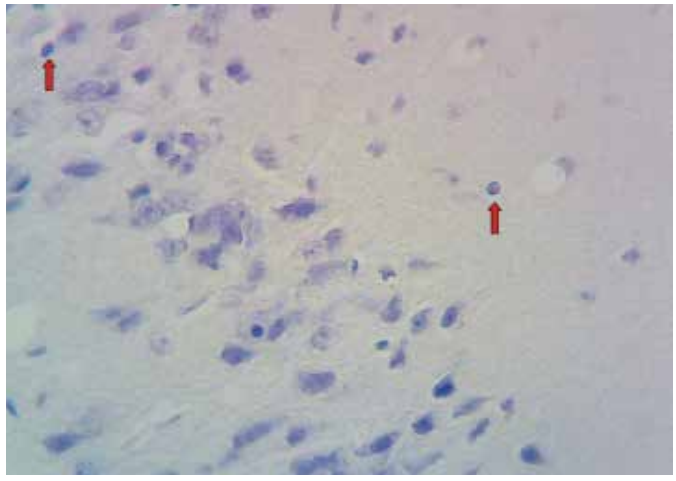

Figure 1: TUNEL positive cell in the brain cortex from control group (→) X200.

Table 1. TAS, TOS and OSI values

	Grup I (n=7)	Grup II (n=7)	Grup III (n=7)
TAS (µmol Trolox Equivalent/L)	0.39±0.03	0.48±0.03	0.45±0.03
TOS (mmol H2O2 Equivalent/L)	1.75±0.07	4.08±0.21[a]	2.64±0.08[b]
OSI (Arbitrary Unit)	4.55±0.35	8.59±0.56[a]	6.02±0.38[b]

aWhen compared to the control group (Group 1). ($p<0.05$).
bWhen compared to the ischemia group (Group 2). ($p<0.05$).

Table 2. Apoptotic index (%)

Group	Mean±SD
Group 1 (n=7)	0.33±0.52
Group 2 (n=7)	2.17±0.75[a]
Group 3 (n=7)	1.00±0.63[b]

The values are reported as mean±standard error.
a. significantly different from the control. p<0.01
b. significantly different from the ischemia. p<0.05

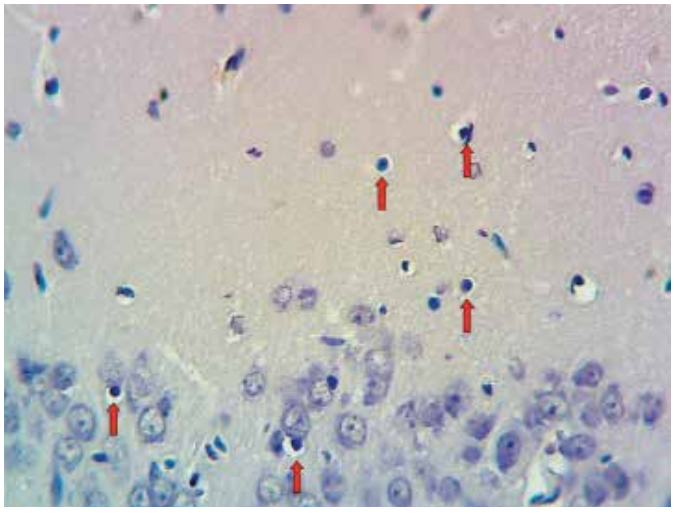

Figure 2: TUNEL positive cell in the brain cortex from ischemia group (→) X200.

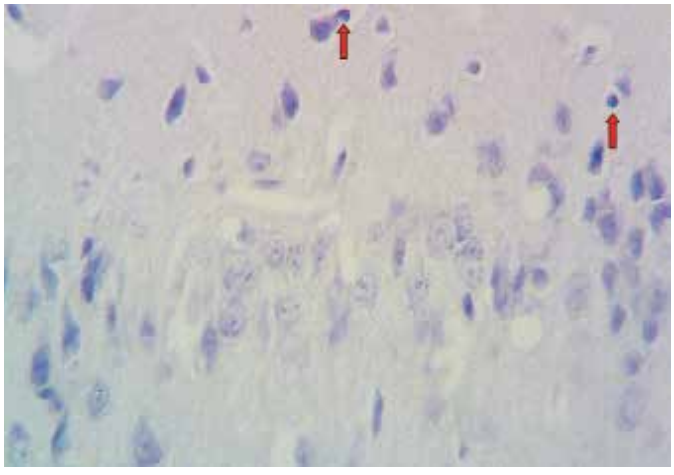

Figure 3: TUNEL positive cell in the brain cortex from ischemia + memantine group (→) X200.

in the striatum. This difference can be explained by the small number of NMDA receptors in the striatum compared to the cortex or that the damage is more pronounced in the striatum than it is at the cortex. The high affinity, non-competitive EAA antagonist dizocilpin (MK-801), the gold standard for the NMDA receptor antagonists, has yielded especially beneficial results. When dizocilpin was administered right before the middle cerebral artery blocking in rats, the ischemic area volume decreased by 76%. It reduced the permeability of blood-brain barrier and the ischemic edema (16).

Lorrio et al. evaluated the treatment effect of galantamine, memantine and their combination in their cerebral ischemia reperfusion model in gerbils. According to their assessment using TUNEL model, they observed that galantamine, memantine and their combination reduced apoptosis in their respective groups (17).

One of the most important outcomes of the increased intracellular calcium influx following NMDA channel activation in cerebral ischemia is the release of free radicals as a result of NOS pathway activation and the formation of oxidants (9, 10). In terms of understanding the role of NMDA antagonists on apoptosis, the literature will benefit from the investigation of their effects on brain tissue oxidant and antioxidant levels.

Numerous methods have been used in determining the oxidative stress in cerebral ischemia. There has been studies showing increased TOS and OSI values in cerebral ischemia models (17).

In our study, the TAS, TOS and OSI values were recorded for all groups at the beginning. In all groups, while TAS values seemed consistent, TOS and OSI values changed significantly. The TOS levels in the brain tissues of induced ischemia rats increased significantly compared to controls. In the memantine group, however, the values were close to normal. OSI, increased in ischemic control group but for memantine group it stayed close to control. These findings suggest that memantine reduces the damage inflicted by ischemia-reperfusion damage but it is unable to repair the damage already inflicted.

TUNEL staining was used for detecting the apoptotic cells in the rat's cerebral cortex. This method that enables detection of DNA fragmentation in situ is one of the most important identifier of apoptosis (18, 19). After TUNEL staining, the TUNEL positive cell counts noticeably increased in rats with ischemia reperfusion compared to controls. In the memantine treatment group, however, there was a significant decrease compared to control group.

The most important limitation of the present study are inability to monitor common carotid occlusion and inability to conduct a blind histopathological evaluation.

In summary, in addition to its already known effect of preventing apoptosis in cerebral ischemia, memantine can exert its neuroprotective influence by preventing the increase of oxidant levels in the brain tissues. Our findings, if supported by future studies, may indicate that memantine can also be useful in the treatment of acute stroke and/or other neurological or neurosurgical conditions accompanying cerebral ischemia.

References

1. Bielewicz J, Kurzepa J, Lagowska-Lenard M, Bartosik-Psujek H. The novel views on the patomechanism of ischemic stroke. Wiad Lek 2010;63:213-220.
2. Morris DC, Chopp M, Zhang L, Lu M, Zhang ZG. Thymosin beta4 improves functional neurological outcome in a rat model of embolic stroke. Neuroscience 2010;169:674-682.
3. Back T, Otto D, Kittner D, Schüler OG, Hennerici MG, Mennel HD. Failure to improve the effect of thrombolysis by memantine in a rat embolic stroke model. Neurol Res 2007;29:264-269.
4. Erel O. A novel automated method to measure total antioxidant response

against potent free radical reactions. Clin Biochem 2004;37:112-119.

5. Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem 2005;38:1103–1111.
6. Bolukbas C, Bolukbas FF, Horoz M, Aslan M, Celik H, Erel O. Increased oxidative stress associated with the severity of the liver disease in various forms of hepatitis B virus infection. BMC Infect Dis 2005;5:95.
7. Castellanos M, Serena J. Applicability of biomarkers in ischemic stroke. Cerebrovasc Dis 2007;24:7-15.
8. Chan PH. Reactive oxygen radicals in signaling and damage in the ischemic brain. J Cereb Blood Flow Metab 2001;21:2–14.
9. Faden AI, Lemke M, Simon RP. N-methyl-D-aspartate antagonist MK801 improves outcome following traumatic spinal cord injury in rats: behavioral, anatomic, and neurochemical studies. J Neurotrauma 1988;5:33-45.
10. Faden AI, Simon RP. A potential role for excitotoxins in the pathophysiology of spinal cord injury. Ann Neurol 1988;23:623-626.
11. Faden AI, Ellison JA, Noble LJ. Effects of competitive and non-competitive NMDA receptor antagonists in spinal cord injury. Eur J Pharmacol 1990;175:165-174.
12. Sonkusare SK, Kaul CL, Ramaro P. Dementia of Alzheimer's disease and other neurodegenerative disorders--memantine, a new hope. Pharmacological Research 2005;51:1-17.
13. Boast CA, Gerhardt SC, Pastor G, Lehmann J, Etienne PE, Liebman JM. The N-methyl-D-aspartate antagonists CGS 19755 and CPP reduce ischemic brain damage in gerbils. Brain Res 1988;442:345-348.
14. Happel RD, Smith KP, Banik NL. Ca2+-accumulation in experimental spinal cord trauma. Brain Res 1981;211:476-479.
15. Gill R, Foster AC, Woodruff GN. Systemic administration of MK-801 protects against ischemia-induced hippocampal neurodegeneration in the gerbil. J Neurosci 1987;7:3343-3349.
16. Yang G, Chan PK, Chen SF, Babuna OA, Simon RP, Weinstein PR. Reduction of vasogenic edema and infarction by MK-801 in rats after temporary focal cerebral ischemia. Neurosurgery 1994;34:339-345.
17. Uzar E, Acar A, Fırat U, Evliyaoğlu O, Alp H, Tüfek A, Yavuz C, Demirtaş S, Taşdemir N. Deneysel Serebral İskemi/Reperfüzyon Hasarında Kafeik Asit Fenetil Esterin Koruyucu Etkisi Turk Norol Derg 2011;17:131-136.
18. Lorrio S, Negredo P, Roda JM, García AG, López MG. Effects of memantine and galantamine given separately or in association, on memory and hippocampal neuronal loss after transient global cerebral ischemia in gerbils. Brain Res 2009;1254:128-137.
19. Kressel M, Groscurth P. Distinction of apoptotic and necrotic cell death by in situ labelling of fragmented DNA. Cell Tissue Res 1994;278:549-556.