Özgün Deneysel Araştırma

The Effect of Magnesium, Memantine and Combination of Magnesium and Memantine on Blood Brain Barrier Permeability and Brain Oedema After Experimental Traumatic Diffuse Brain Injury

Murat İMER [1], Ajlan UZUNKOL [1], Bülent Sacit OMAY [1], Pulat Akın SABANCI [1], Altay SENCER [1], Tülin ERDEM [2], Aykut KARASU [1], Mehmet KAYA [3]

[1] Department of Neurosurgery, [2] Anesthesiology, [3] Physiology, Istanbul University, Istanbul

✔ **Objective:** It has been shown that excess amount of glutamate released after primary neuronal injury by head trauma causes secondary injuries in the adult brain. Aiming to prevent this secondary injury a wide range of N-Methyl-D-aspartate receptor antagonist drugs have been investigated in experimental studies. Magnesium sulphate and memantine are pharmacological agents widely used clinically for this specific indication; prevention of secondary neural injury. According to current literature although these two drugs have been used alone or in combination with different drugs, they have not been tried together in combination before. This experimental study is aimed to investigate the effect of magnesium, memantine and the combination of two agents on the permeability of blood brain barrier (BBB) and the quantity of diffuse cerebral oedema after experimentally induced traumatic brain injury.
Methods: A standard traumatic brain injury was induced in Sprague-Dawley rats by a controlled impact device using a mass free falling from a certain height. Animals were introduced one of the following drugs of the same amount, intraperitoneally 30 minutes after traumatic brain injury; saline (1 ml/kg), magnesium sulphate (270 mg/kg), memantine (10 mg/kg), and magnesium sulphate plus memantine (270 mg/kg+10 mg/kg). To determine the brain oedema, the specific gravity of the injured brain tissue was evaluated. The changes in permeability of the BBB were investigated by Evans Blue dye that is known to bind to serum albumin.
Results: In treatment groups, the specific gravity values significantly increased when compared with the control group. Evans blue dye content in the brain tissue was significantly reduced with respect to the control group.
Conclusion: In preventing the secondary insult occurring after traumatic brain injury, NMDA receptor antagonists: Magnesium sulphate, memantine and combination of magnesium sulphate and memantine are shown to be effective by decreasing diffuse cerebral oedema and restoring the BBB.

Key words: Diffuse brain injury, blood-brain barrier, brain oedema, magnesium, memantine

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Deneysel Travmatik Yaygın Beyin Hasarı Sonrası Magnezyum, Memantin ve Magnezyum ile Memantin Kombinasyonunun Kan Beyin Bariyeri Geçirgenliğine Etkisi

✔ **Amaç:** Erişkinlerde kafa travması nedeniyle oluşan primer nöronal hasar sonucu aşırı miktarda glutamat salınımının ikincil hasarlara neden olduğu daha önce kanıtlanmıştır. Farklı deneysel çalışmalarla oluşan bu ikincil hasarı engellemek amacıyla çeşitli N-Metil-D-aspartat(NMDA) reseptör antagonisti ilaçlar araştırılmıştır. Bu amaçla klinik olarak en sık kullanılan farmakolojik ajanlar; magnezyum sülfat ve memantindir. Güncel literatüre göre bu ajanlar tek başlarına veya başka bir takım ilaçlarla kombine edilerek denenmelerine rağmen her ikisi kombine olarak kullanılmamıştır. Bu deneysel çalışmada; deneysel travmatik beyin hasarı sonucu oluşan beyin ödemi miktarına ve kan beyin bariyeri(KBB) geçirgenliğine magnezyum, memantin ve bu iki ajanın kombinasyonunun etkisinin araştırılması amaçlanmıştır.
Yöntem: Belli bir yükseklikten kontrollü serbest ağırlık düşmesi yöntemiyle Sprague-Dawley farelerinde standart travmatik beyin hasarı oluşturuldu. Deneklere travmatik beyin hasarından 30 dakika sonra periton içerisine sırasıyla; salin(1 ml/kg), magnezyum sülfat(270 mg/kg), mamantin (10 mg/kg) ve magnezyum sülfat ile memantin(270 mg/kg+10 mg/kg) verildi. Beyin ödemini belirlemek amacıyla hasar görmüş beyinlerin özgül ağırlıkları değerlendirildi. KBB geçirgenliğindeki değişiklikler ise serumdaki albumini bağladığı bilinen Evans mavisi kullanılarak araştırıldı.
Bulgular: Tedavi grupları, kontrol gruplarıyla karşılaştırıldığında özgül ağırlıkların anlamlı derecede arttığı özlenmiştir. Evans mavisi boyası ise kontrol grupla kıyaslandığında tedavi grubunda anlamlı derecede azalmış olarak bulunmuştur.
Sonuç: Travmatik beyin hasarı sonrası ikincil etkinin engellenmesinde, NMDA reseptör antagonistleri: Magnezyum sülfat, memantin ve bu iki ajanın kombinasyonunun beyin ödeminin azaltılması ve KBB'nin yeniden şekillendirilmesinde etkili olduğu gösterilmiştir.

Anahtar kelimeler:

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The initial event in craniocerebral trauma involves direct impact injury to the brain that produces parenchymal contusions and shearing injury to the axons in the white matter of the cerebral hemispheres and brain stem [22]. Primary injury triggers a sequence of secondary alterations in brain metabolism, ion homeostasis, intracranial hemodynamics and brain water compartmentation that develop during the hours following traumatic brain injury (TBI). The evolving pathophysiological processes can produce changes in the intracranial pressure-volume relationship with resulting intracranial hypertension and brain herniation. Satisfactory outcome for the patient often requires recognition and successful treatment of these secondary derangements [22].

The neurotransmitter glutamate is one of the key molecules linking primary neuronal injury to secondary neuronal injury [3,4,14]. After well known consequences of the primary neuronal injury excess amount of glutamate is released, and also reuptake is blocked so that the highly increased amount of glutamate can bind and activate the NMDA receptors leading to increased influx of calcium into neurons that have high density of those receptors. An excessive calcium ion in the cell stimulates Ca-dependent enzymes, such as phospholipases and proteases leading to cell death. This is the theoretical background of the idea that NMDA antagonists can protect the brain from secondary injury by inhibiting NMDA receptor mediated cell death [3,4,18,22,25].

Magnesium is a natural NMDA antagonist which has been used on many clinical grounds including prevention of secondary brain injury. Its effect on decreasing the intensity of posttraumatic brain oedema and repairing BBB has been shown [1,2,12,13,14].

Memantine (1-amino-3, 5-dimethyladamntane hydrochloride) is a drug, which has been used as an anti-parkinsonian agent. It is also a reversible open-channel blocker of NMDA receptors, which is shown to be effective in the prevention of secondary neuronal injury [6,13]. It has been thought that the blocking of Ca influx through the NMDA-operated Ca channel may be the most important mechanism for the neuroprotective effect of memantine [1,2,7,20] although its theoretical role in restoring the BBB and in decreasing the brain oedema after TBI has not yet been proved in any study.

This experimental study aims to investigate the effects of these two potent NMDA receptor blockers when both acting alone and together in preventing the secondary events in neural tissue after TBI.

MATERIALS and METHODS

The animals were cared for in accordance with National Institutes of Health Guide for the care and use of laboratory animals. All procedures were reviewed and approved by the Institute for Experimental Medicine and Research (DETAE), Istanbul University.

Surgical preparation

Male Sprague-Dawley rats (n=80) weighing 350–400 gr were fed and watered ad libitum before being initially anaesthetised with enflurane (2-5%), oxygen (40%) and air (60%) delivered in chamber and allowed to breathe spontaneously. Maintenance of adequate anaesthesia for the experimental procedure was confirmed by the loss of corneal reflex and no movement to painful stimuli.

Closed head injury

Traumatic brain injury was induced using the closed head injury model of diffuse axonal injury as described by Marmarou et al. [10,17]. After exposing the skull through a midline incision, stainless steel disc 10 mm in diameter and

3 mm in depth was cemented centrally along the coronal suture between the lambda and bregma by cyanoacrylate. The animals were put on prone position on a foam bed and dropping a 450 g brass weight from a distance of 2 m induced injury. Then animal's skin was sutured. Animals were intubated right after the trauma and mechanical ventilation was performed to maintain adequate oxygenation and CO_2 elimination. Tracheal extubation occurred following establishment of spontaneous respiration. This model of cranial injury has been used in multiple previous studies [12,13,14,24,26].

Physiological monitoring

Right femoral artery catheterisation was performed for hemodynamic measurements and oxygenation parameters in animals for each group. Rectal temperatures were maintained with a thermostatically controlled heating cover blanket set at 37 °C during the experimental procedure.

Experimental protocol

Seventy rats that survived out of 80 were randomly grouped into 4 categories; control, magnesium, memantine, and magnesium plus memantine. Each group of animals were introduced to one of the following drugs intraperitoneally 30 minutes after traumatic brain injury; saline (1 ml/kg), magnesium (270 mg/kg), memantine (10 mg/kg) and magnesium plus memantine (270 mg/kg+10 mg/kg). In the two-hour period following the trauma, the animals were kept under supervision and were returned to their cages only after they were stabilized. The 64 rats that survived 24 hours post injury were anaesthetised and sacrificed.

Determination of cerebral oedema

32 rats (8 rats in control group, 8 rats in magnesium group, 8 rats in memantine group, and 8 rats in magnesium plus memantine group) were decapitated at 24 hours after the TBI and their brains were rapidly removed for specific gravity determination. The specific gravity of the brain tissue was determined by the method described by Marmarou et al. [16,26]. Samples of 1 mm3 from the same region of each hemisphere were hand cut and placed into linear gradient columns of kerosene and bromobenzene. A calibration curve was determined for each column using anhydrous K2SO4 solutions of known specific gravity (1.045, 1.040, 1.035, and 1.025).

Determination of BBB integrity

32 rats (8 rats in control group, 8 rats in magnesium group, 8 rats in memantine group, and 8 rats in magnesium plus memantine group) were sacrificed 24 hours after TBI to evaluate the BBB integrity by the method described by Chan et al. [5]. Evans blue (EB) dye 4 ml/kg in 2% saline was administered from a penile vein and allowed to circulate for 60 min. The chest walls of the rats were opened under anaesthesia and 0.2 ml of blood were collected from the heart. Then the animals were perfused with saline through the left ventricle at 110 mm Hg pressures until colourless fluid was obtained from the right atrium. After this washout, the brains were removed and dissected into right and left hemispheres. Both hemispheres were examined for EB albumin extravasations and for the extent and the intensity of the EB staining. Each region was weighed and the samples were then homogenized with 3,5 ml phosphate-buffered saline (PBS) and mixed with a vortex for 2 min after the addition of 2.5 ml of 60% trichloroacetic acid to precipitate protein. The samples were then cooled for 30 min and centrifuged for 30 min at 1000 rpm. The absorbency of the supernatant for EB dye was measured at 610 nm using a spectrophotometer. EB dye content is expressed as μg/mg of brain tissue against a standard curve.

Statistical analysis

The results are expressed as mean ± SD and median values. Mean arterial blood pressure (MAP), partial arterial oxygen pressure (PaO_2 mmHg) and partial arterial carbon dioxide ($PaCO_2$ mmHg) pressure were compared among the four group's basal, post-injury and post-treatment values using Kruskall-Wallis analysis of variance followed by Dunn's test as post-hoc analysis. Friedman nonparametric repeated measures test was used for group comparisons. The probability value less than 0.05 was considered statistically significant. EB dye content in the brain tissue and brain tissue specific gravity values were compared among the four group's left and right hemispheres using Kruskall-Wallis analysis of variance followed by Dunn's test as post-hoc analysis. Mann-Whitney U test was used for intragroup comparisons. The probability value less than 0.05 was considered statistically significant.

RESULTS

Ten of the 80 rats were excluded because of skull fracture and focal injury, and six more rats were died following 24 hours after impact.

Physiological variables

The physiological data for the groups are presented in Table 1. There were no statistical differences in arterial pressure and oxygenation parameters in either group. No hemodynamic instability was observed with drug administration during the experimental period of the monitored animals.

Table 1. Data expressed mean ± SD (median) values and showing physiological parameters for the groups.

Variable	Groups	Basal mean ± SD (median)	Post-injury mean ± SD (median)	Post-treatment mean ± SD (median)	Fr	P value
MAP (mmHg)	Control	85 ± 3,8 (85)	90,5 ± 7,8 (91,5)	83,1 ± 4,8 (84)	1,862	0,3553
	Magnesium	82,5 ± 2,8 (82)	90,1 ± 4,6 (90,5)	81,6 ± 3,5 (82)	12,452	0,0003
	Memantine	81,9 ± 1,6 (81,5)	90,6 ± 6,6 (91)	83,1± 5,5 (84)	7,750	0,0179
	Mg&Memantine	81,6 ± 2,1 (81)	90,5 ± 7,5 (92)	83,5 ± 4,3 (84)	4,710	0,1197
	KW	5,343	0,1311	1.067		
	P	0,1483	0,9879	0,7852		
PaO_2 (mmHg)	Control	93,8 ± 3,8 (93,5)	93,25 ± 4,3 (93,5)	94,4 ± 95,5 (95,5)	0,8667	0,6543
	Magnesium	94,6 ± 4,9 (96)	94,4 ± 5,5 (96)	94,5 ± 3,3 (94,5)	0,2000	0,9674
	Memantine	93,3 ± 3,8 (94,5)	93,9 ± 4,5 (92,5)	92,1 ± 5 (92)	0,06452	0,9674
	Mg&Memantine	93,4 ± 3,8 (93)	93,6 ± 4,5 (92,5)	92,3 ± 5,6 (91,5)	0,2500	0,9674
	KW	0,8609	0,6035	1,639		
	P	0,8349	0,8956	0,6506		
$PaCO_2$ (mmHg)	Control	30,8 ± 4,1 (32)	31,8 ± 1,5 (31,5)	32,3 ± 1,9 (32)	0,2069	0,9674
	Magnesium	31,9 ± 1,4 (31,5)	31,6 ± 1,7 (31,5)	33,1 ± 1,7 (34)	4,345	0,1197
	Memantine	32,9 ± 1,1 (33)	32,8 ± 1,8 (33)	32,8 ± 1,9 (33,5)	0,2143	0,9674
	Mg&Memantine	32,9 ± 1,5 (33)	32,3 ± 1,8 (32,5)	32,5 ± 1,8 (32,5)	3,739	0,1495
	KW	3,772	2,225	1,015		
	P	0,2871	0,5270	0,7976		

Table 2. Mean values and standard deviations of brain tissue specific gravity of control and treatment groups are presented.

Groups (n)	Left Hemispheres (median)	Right Hemispheres (median)	U'	Two tailed p value
Control (8)	1,034 ± 0,004 (1,034)	1,034 ± 0,004 (1,034)	33	0,9591
Magnesium (8)	1,044 ± 0,001 (1,043)	1,440 ± 0,001 (1,044)	35	0,7984
Memantine (8)	1,044 ± 0,001 (1,043)	1,044 ± 0,001 (1,044)	39	0,5054
Mg&Memantine (8)	1,045 ± 0,001 (1,045)	1,045 ± 0,001 (1,045)	32	>0,9999
KW	20,719	20,060		
P	<0,0001	<0,0001		

Table 3. Mean values and standard deviations of amount of extravasations of EB dye indicating BBB permeability of control and treatment groups are presented.

Groups (n)	Left Hemispheres (median)	Right Hemispheres (median)	U'	Two tailed p value
Control (8)	0,00530 ± 0,00086 (0,00543)	0,00533 ± 0,00085 (0,00545)	38,5	0,5054
Magnesium (8)	0,00133 ± 0,00039 (0,00138)	0,00134 ± 0,00039 (0,00138)	32,5	0,9591
Memantine (8)	0,00039 ± 0,00011 (0,00405)	0,00032 ± 0,00013 (0,00029)	46,5	0,1304
Mg&Memantine (8)	0,00042 ± 0,00018 (0,00037)	0,00033 ± 0,00017 (0,00027)	42	0,3282
KW	26,057	26,099		
P	<0.001	<0.001		

Brain oedema

Mean values and standard deviations of brain tissue specific gravity of control and treatment groups are presented in Table 2. In treatment groups, the specific gravity values of brain tissue were significantly increased with respect to control group (p<0,05) (Figure 1). Intragroup comparisons showed no significance in this parameter.

Blood-brain barrier permeability

BBB permeability was assessed with intravenous injection of Evans blue dye in the rats. Mean values and standard deviations of amount of extravasations of EB dye of control and treatment groups are presented in Table 3. The extravasations of Evans blue was significantly reduced in all treatment groups with respect to the control group (p<0,05) (Figure 2). Intragroup

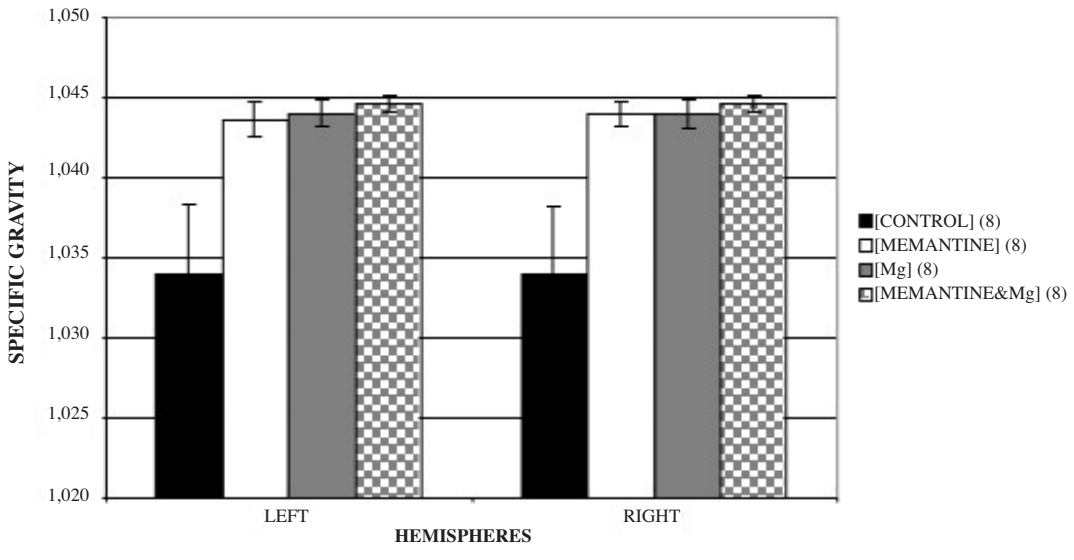

Figure 1. Brain tissue specific gravity of control and treatment groups.

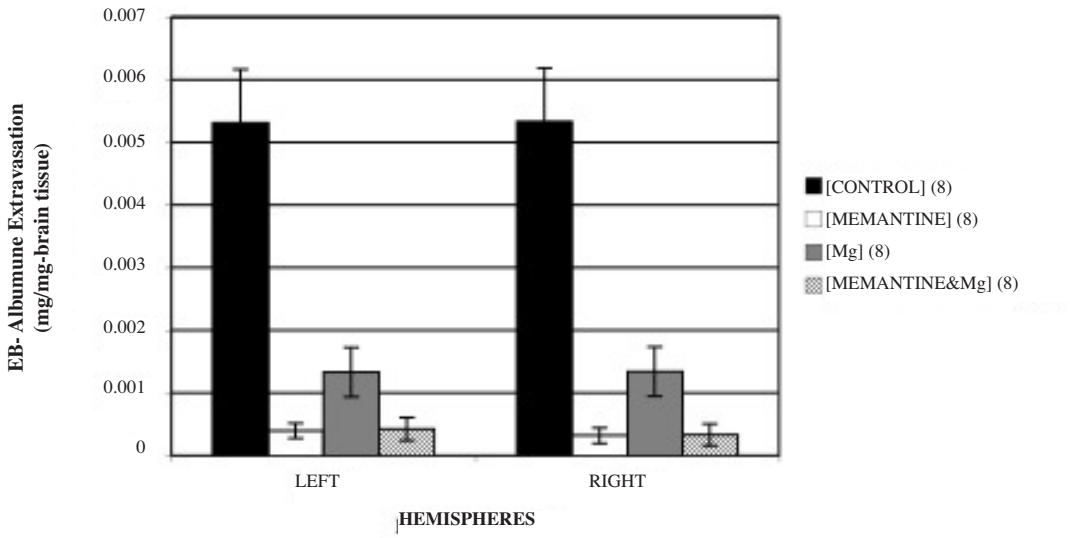

Figure 2. BBB permeability of control and treatment groups.

comparisons showed no significance in this parameter.

DISCUSSION

This study has shown that NMDA receptor antagonists; magnesium, memantine and combination of magnesium and memantine decrease diffuse cerebral oedema, however memantine and magnesium plus memantine administration restore blood brain barrier permeability after traumatic brain injury.

To design an experiment, we selected a standard closed head injury model described by Marmarou et al. [17]. This model was able to produce massive diffuse axonal injury that primarily involved the corpus callosum, internal capsule, the optic tracts, cerebral and cerebellar peduncles and the long tracts in the brainstem [10,19]. It is clinically evident that severe axonal injury is the most drastic and fatal consequence of TBI. Furthermore, brain oedema associated with axonal injury is almost always the accompanying feature of TBI and the severity of the brain oedema is directly proportional to the radiological and clinical severity of TBI [22]. Thus, we considered the parameters of brain oedema in order to evaluate the severity of TBI and therapeutic efficacy of NMDA antagonists.

The drugs we used were two NMDA antagonists. The first one, magnesium is an ion, which is essential for the stability and the normal functioning of the cell membranes and its depletion has been closely associated to both focal and diffuse axonal injuries. Mg also acts as an endogenous, non-competitive NMDA antagonist [1,9,13,23]. Exogenous Mg has been shown to be effective in improving neurological outcome in rats after diffuse axonal injury proceeds by the same method used in our experiment [12]. Magnesium was also shown to be effective in reducing brain oedema, and restoring BBB permeability after diffuse axonal injury [13,14]. We clearly demonstrated in this study that exogenously administered magnesium did reduce cerebral oedema effectively. These experimental findings were found to be in concordance with the previous studies in the literature [1,9,12,14,23]. Thus, it is our strong conviction that the routine clinical use of magnesium in emergency settings after TBI in human subjects should be considered.

The other drug we tested is memantine, which is a reversible open channel blocker of the NMDA receptors. There are both in vitro and in vivo

studies showing that memantine did effectively reduce neuronal excitotoxic damage caused by the excessive release of glutamate and NMDA [8,11,15,16,20,25]. We have also clearly shown the beneficial effects of memantine on decreasing brain oedema and improving BBB integrity after TBI. Furthermore, we also investigated the therapeutic utility of magnesium, memantine and the combination therapy, therapeutic efficacy of which was not tested experimentally before.

Analysis of the statistical outcomes have shown that magnesium, memantine and magnesium plus memantine were effective agents in reducing brain oedema, although all treatment groups were significantly effective with respect to the control group. In restoring blood brain barrier permeability, the combination of magnesium and memantine was the most effective group although again, all treatment groups were significantly effective with respect to the control group.

Our study justifies that memantine and magnesium combination therapy stands as a new therapeutic modality in preventing the harmful and fatal consequences of neurotrauma by restoring BBB permeability. Finally, the extent of actual therapeutic efficacy of these NMDA antagonists in clinical settings still needs to be proven on human subjects by large prospective studies.

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