Received: 08.12.2011 / **Accepted:** 17.04.2012
DOI: 10.5137/1019-5149.JTN.5582-11.1

Combined Treatment with Progesterone and Magnesium Sulfate Positively Affects Traumatic Brain Injury in Immature Rats

Progesteron ve Magnezyum Sülfatın Kombine Kullanımı İmmatür Sıçanlarda Travmatik Beyin Hasarını Olumlu Etkilemektedir

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ABSTRACT

AIM: It is well known that head trauma results in damage in hippocampal and cortical areas of the brain and impairs cognitive functions. The aim of this study is to explore the neuroprotective effect of combination therapy with magnesium sulphate (MgSO4) and progesterone in the 7-days-old rat pups subjected to contusion injury.

MATERIAL and METHODS: Progesterone (8 mg/kg) and MgSO4 (150 mg/kg) were injected intraperitoneally immediately after induction of traumatic brain injury. Half of groups were evaluated 24 hours later, the remaining animals 3 weeks after trauma or sham surgery. Anxiety levels were assessed with open field activity and elevated plus maze; learning and memory performance were evaluated with Morris Water maze in postnatal 27 days.

RESULTS: Combined therapy with progesterone and magnesium sulfate significantly attenuated trauma-induced neuronal death, increased brain VEGF levels and improved spatial memory deficits that appear later in life. Brain VEGF levels were higher in rats that received combined therapy compared to rats that received either medication alone. Moreover, rats that received combined therapy had reduced hipocampus and prefrontal cortex apoptosis in the acute period.

CONCLUSION: These results demonstrate that combination of drugs with different mechanisms of action may be preferred in the treatment of head trauma.

KEYWORDS: Traumatic brain injury, Immature rat, Spatial learning and memory, Apoptosis, VEGF, Magnesium sulfate, Progesterone

ÖZ

AMAÇ: Kafa travmasının hipokampal ve kortikal hasar ile kognitif fonksiyonlarda bozulmaya neden olduğu bilinmektedir. Bu çalışmanın amacı, magnezyum sülfat (MgSO4) ve progesteronun combine kullanımının kontüzyon hasarı oluşturulan 7 günlük sıçan beynindeki nöroprotektif etkilerini araştırmaktır.

YÖNTEM ve GEREÇLER: Travmadan hemen sonra 8 mg/kg dozunda progesteron, 150 mg/kg dozunda MgSO4 intraperitoneal tek doz olarak verildi. Deney gruplarının yarısı travmadan 24 saat sonra, kalan yarısı ise 27 günlük olduklarında değerlendirildi. Anksiyete deneyleri açık alan ve yükseltilmiş + labirent test düzenekleri kullanılarak; öğrenme ve bellek performansı ise Morris su tankı aracılığı ile 27 günlükken değerlendirildi.

BULGULAR: Kombine tedavinin beyin dokusunda VEGF'yi arttırarak travma ile oluşan nöronal ölümü azalttığı ve ileriki dönemde spasyal bellek bozukluğunu iyileştirdiği görüldü. VEGF seviyesinin kombine tedavide ilaçların tek başına kullanımına göre daha fazla arttığı bulundu. Ayrıca ilaç tedavisinin akut dönemde de VEGF seviyesini arttırarak apoptozis seviyesini azalttığı görüldü.

SONUÇ: Bu sonuçlar, farklı etki mekanizmasına sahip ilaçların kombine kullanımının kafa travması tedavisinde tercih edilebileceğini göstermektedir.

ANAHTAR SÖZCÜKLER: Travmatik beyin hasarı, İmmatüre sıçan, Spasyal öğrenme ve bellek, Apoptosis, VEGF, Magnesium sülfat, Progesteron

INTRODUCTION

Traumatic brain injury (TBI) is the leading cause of death and disability during childhood (1). Children have a higher survival rates compared to adults with head trauma; but, the long-term sequelae are often more devastating in children depending on the age and developmental period (1). The cognitive dysfunction is a common sequela of head trauma, which is not limited to severe injury but it can be seen in mild and moderate injury (4, 17,31,32).

Primary damage, which is seen immediately after trauma, and secondary damage, which is seen several hours or days later, occur after TBI (16). Two pathogenic mechanisms, excitotoxicity and apoptotic, have been described during the secondary damage. Apoptosis may be induced by extracellular or intracellular events, such as oxidative stress or excess calcium (22).

Glutamate, which is released following head trauma, plays a critical role in excitotoxicity (7). Glutamate can cause neuronal damage in 2 phases. First, neuronal edema is due to sodium, calcium-related neuronal degeneration that follows. The activation of glutamate receptors (NMDA-, AMPA and kainate-receptors) is associated with increase in the level of free intracellular calcium. The cytosolic increase in free calcium activates proteases, lipases and endonucleases which in turn initiate processes leading to neuronal degeneration and cell death following TBI (9).

The immature brain is extremely vulnerable to TBI-induced apoptotic neurodegeneration (12,31,32). This developmental vulnerability period corresponds to the brain growth spurt period, the period that coincides with the first two postnatal weeks in the rats and the first 2 years in humans (5). Immature neurons are exceedingly vulnerable to excitotoxic damage from mature neurons, because NMDA-mediated Ca influx is much more prevalent in the immature brain (28). However, calcium accumulation may be more and last longer in mature compared to immature brains (30). These different results demonstrate that age and the severity of the insult play important roles in excitotoxicity.

The activity of NMDA receptor can be modulated by magnesium as a noncompetitive NMDA antagonist (36). In the cascade of events following TBI, there is a depletion of magnesium because of increase in urinary excretion; as a result its homeostatic control of the NMDA receptors is lost (41). This leads to a massive influx of calcium, resulting in neuronal degeneration and cell death. Recently, it has been demonstrated that magnesium has a beneficial influence on learning and memory by increasing synaptic plasticity in hippocampus (37). Moreover, magnesium treatment following TBI improved learning and memory that was adversely affected by trauma (13).

Neuronal repair mechanisms are activated after brain damage. After traumatic brain injury, neurotrophic factors such as VEGF, BDNF are up-regulated (26, 46). Hippocampal VEGF begins to increase after 12 hours of trauma and peaks at 7 days after trauma. It was shown that VEGF infusion protected neurons in acute and chronic injuries (39). Hippocampal neurogenesis increases in parallel with this increase in VEGF (26).

Progesterone, used in traumatic brain injury, is a neurohormone that has neuroprotective effects (45). Progesterone increases neurotrophic factors such as VEGF (11). In addition, progesterone inhibits voltage-gated calcium channels and reduces excitotoxicity (27).

The cortex and hippocampus are predominantly affected in head trauma (42). Hippocampal neurons normally play a crucial role in the processing of spatial memory and learning (29). The loss of hippocampal neuron is frequently seen after head trauma and thought to be related to learning and memory deficits (38).

The aim of this study was to explore the neuroprotective effect of MgSO4 and progesterone combination therapy during acute and chronic phases following contusion injury in 7 days old rat pups.

MATERIAL and METHODS

Animals and Experimental Design

All experiments were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the Dokuz Eylul University, School of Medicine. Wistar Albino rats with dated pregnancies were maintained at the same center and housed in individual cages with free access to water and laboratory chow. Forty-two litters delivered spontaneously were grown with their dams until the time of experimentation at 7 days of postnatal age. All rats were maintained on a continuous 12-h-light/dark cycle at constant room temperature (21°C), and humidity (60%). Rats were divided into 6 groups: (1) Control (n=12), (2) Sham (n=12), (3) TBI (n=12), (4) TBI + 150 mg/kg magnesium sulfate (n=14), (5) TBI + 8 mg/kg Progesterone (n=12), (6) TBI + 150 mg/kg magnesium sulfate + 8mg/kg progesterone (n=12).

We used a modification of percussion trauma model in immature rats that imitates the head trauma of infant and early childhood (5). The trauma device consists of a hollow tube 40 cm long, perforated at 1-cm intervals to prevent air compression. The device was kept vertical to the surface of the skull and guided a falling weight onto a circular footplate resting upon the surface of the parietal bone. The trauma was performed to the right hemisphere of parietal cortex, the dominant hemisphere (23). The center of the footplate was stereotaxically positioned 3mm anterior and 2mm lateral to the lambda and was fixed in place under ether anesthesia. A force of 160 g cm produced by a 10-g weight was selected to create brain contusion. Experiments were performed between 9.00 and 11.00 am in a sound-attenuated and air-regulated experimental room. All pups were kept on a heating pad until returned to their mothers at 4 h after the trauma or sham surgery. Progesterone (8 mg/kg) and Magnesium sulfate (150 mg/kg) were injected intraperitoneally immediately after TBI.

In order to compare acute and long-term alterations, half of the animals in each group (*n*=7/group) were sacrificed 24 hours later, and the remaining animals (*n*=7/group) 3 weeks after trauma or sham surgery.

Learning and Memory Test

The spatial memory performance was evaluated 3 weeks after trauma or sham surgery, using the Morris Water Maze (29). The Morris Water Maze device's diameter is 140 cm and its height is 75 cm. The water level was 75 cm and there was an invisible platform in the pool, which was 1.0 cm above the surface. The pool was filled with opaque water to prevent visibility of the platform. The animals were tested for 5 days. On each day, rats were placed in the water (22±1°C) and allowed to swim (maximum swim time 60 s) until they found a hidden, but fixed escape platform, using extra maze cues. The escape platform was placed in the middle of one of the random quadrants of the pool. If the animal failed to locate the platform in 60 s, the experimenter placed the rat on the platform and left it there for 20 s. Each rat was tested with five consecutive trials per day, with an inter-trial interval of 60 s. Each rat was exposed to the task for four consecutive days (a total of 20 trials), and on day 5, the platform was removed from the pool, and the rats were placed in the pool and swam for 60 s (probe trial). The outcome measures were latency to find the platform and time spent in the correct quadrant. Morris water maze training was recorded using the HVS image tracking system.

Histopathological Procedures

For histological examination, half of the animals in each group (*n* = 7/group) were perfused by 10% formalin under ether anesthesia 24 h after trauma or sham surgery. Brain tissues were removed and fixed in 10% formalin in phosphate buffer for 24 h. The brains were sectioned coronally into sequential 6 µm slices using a rat brain slicer. Each sample was subjected to the estimation of neuron number by taking three coronal sections through the hippocampus and parietal cortex that corresponded approximately to plates 21, 23, 25, and the prefrontal cortex that corresponded approximately to plates 9, 11 in the rat atlas of Paxinos and Watson. All sections were stained by cresyl violet. The images were analyzed by using a computer-assisted image analyzer system consisting of a microscope (Olympus BH-2 Tokyo, Japan) equipped with a high-resolution video camera (JVC TK-890E, Japan). The number of neurons in the hippocampal CA1, CA2, CA3 and gyrus dentatus regions were counted with the help of a 6000 µm2 counting frame viewed through a 20×Nikon lens at the monitor. The counting frame was placed randomly five times on the image analyzer system monitor and the number of neurons was counted (UTHSCA Image Tool for windows version 3.0 software) and the average was calculated.

To detect DNA fragmentation in cell nuclei, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) reaction was applied to the paraffin sections. The DeadEnd Colorimetric TUNEL system kit (Roche, Germany) was used for apoptotic cell detection. Serial 5 µm thick paraffin-embedded sections were deparaffinized, rehydrated in graded alcohol, and pretreated in proteinase K for 15 min. After washing in phosphate-buffered saline (PBS), the specimens were incubated with fluorescein-labeled deoxy-UTP and TdT at 37°C for 60 min. Then, the converter POD solution was applied to the slides. Sections were stained with DAB and counter-stained with mayer hematoxylin and analyzed using a light microscope.

For quantitative measurement of the number of apoptotic cells, 1000 cells were randomly counted in these different areas and the percentage of the apoptotic cells was calculated.

VEGF expression was detected by avidin-biotin-complex method using Santa-Cruz biotechnology (SC-7629)(R&D-Systems) according to the manufacturer's protocol. Immuno-reactivity was graded as follows: More than 10% of the cells staining was graded as positive. No detectable staining or <10% of cells staining was graded as negative. The qualitative intensity of staining for VEGF was assessed using a scale between 0 and +++, with 0 representing no detectable stain and +++ representing the strongest stain.

Statistical Analysis

Differences between the learning days in learning test were analyzed using GLM-repeated measure. Differences between the groups were analyzed using two-way-ANOVA, with Bonferroni as the post-hoc test. Correlations among groups were calculated using Pearson correlation analysis. Results are presented as mean+_SEM. DA *p value* < 0.05 was considered statistically significant.

RESULTS

Acute Effects of Treatment

At 7-days after trauma or sham surgery, the density of the ipsi- and contra-lateral hippocampal dentate gyrus, parietal and prefrontal cortex neurons were significantly less in all traumatic-rats compared to sham and control groups ($p<0.05$). Magnesium sulfate and progesterone treatments did not increase with the decreasing number of neurons in the acute period (Figure 1A, B). TUNEL + staining was performed in the brain regions of decreased cell numbers. TUNEL-positive neurons were detected in both ipsi- and contra-lateral hippocampal regions of all injured animals, but there were markedly fewer TUNEL-positive cells of ipsi and contra-lateral dentate gyrus of hippocampus and prefrontal cortex in the rats treated with magnesium sulfate and progesterone ($P < 0.01$). However, there was no statistically significant difference between treatment groups (Figure 2A, B). Immune staining of VEGF was more intense in treated groups, but combined treatment with MgSO4 and progesterone group was associated with the most intense staining in acute and chronic period of TBI (Figure 3A, B) (Table I).

Chronic Effects of Treatment

As shown in Figure 4A, the mean latency to find the platform declined progressively in all animals. However, the vehicle-

treated TBI rats had longer escape latencies throughout the training days than sham and control animals ($P < 0.001$). Treatment with MgSO4 and progesterone significantly shortened this prolongation of mean latency at the second, third and fourth days of training as compared with the saline-treated group ($P < 0.01$) (Figure 4A). In probe trials, time spent in the target quadrant was used to evaluate long-term memory. Vehicle-treated TBI rats spent significantly less time in the target quadrant and more time in the opposite quadrant than other groups ($P < 0.001$) (Figure 4B).

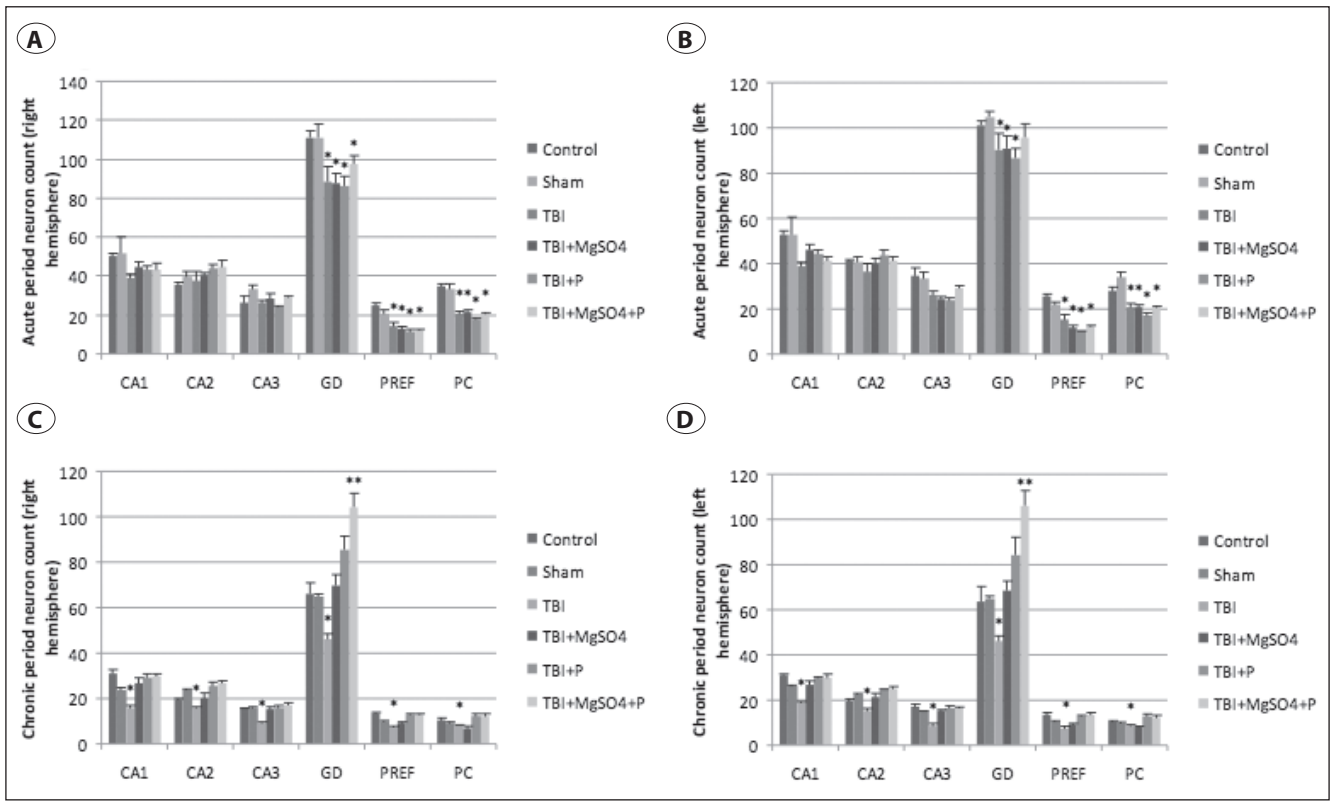

Figure 1: The results of quantitative evaluation of the number of neurons. **(A)** The ipsi-lateral neuron number of groups in acute period of trauma. **(B)** The contra-lateral neuron number of groups in acute period of trauma. **(C)** The ipsi-lateral neuron number of groups in chronic period of trauma. **(D)** The contra-lateral neuron number of groups in chronic period of trauma. * p< 0.05 compared with control and sham. ** p<0.05 compared with TBI group. **GD;** Gyrus Dentatus, **Pref;** prefrontal cortex, **PC;** parietal cortex, **TBI;** Traumatic brain injury, **MgSO4;** magnesium sulfate, **P;** progesterone.

Magnesium sulfate and progesterone treatment increased the time spent in the target quadrant and decreased the time spent in the opposite quadrant in probe trial. There was no statistically significant difference between treatment groups in Morris water maze task results. The density of hippocampal neurons was significantly decreased in the contra and ipsi-lateral hippocampal CA1, CA2, CA3, dentate gyrus, prefrontal cortex and parietal cortex of vehicle-treated TBI rats in comparison with sham and control groups at 27 days. Magnesium sulfate and progesterone treatment significantly increased the ipsi-lateral neurons of hippocampal CA1 region, contra and ipsi-lateral prefrontal and parietal cortices affected against TBI as compared to the vehicle-treated TBI group ($P<0.01$). Combined treatment with MgSO4 and progesterone increased the number of neurons in ipsi and contra-lateral gyrus dentatus of hippocampus ($p<0.05$) (Figure 1C, D).

DISCUSSION

The present study demonstrated that traumatic injury in the immature rats caused spatial memory deficits in later developmental period, and treatment with MgSO4 and progesterone improved these functional deficits and increased brain tissue VEGF levels, which was parallel to protection against acute neuronal loss.

Apoptosis has a major role in the mechanism of traumatic injury in the immature brain and is very severe in the brain of 7-day-old rats (3). Apoptotic cell death is highest 24 hours after trauma and continues for 7 more days (5). The amount of apoptosis necessary for physiological brain development is determined by the degree of myelinization and the water content of the brain (5). In this study, we demonstrated that apoptosis increased with head trauma in all ipsi- and contra-lateral hippocampal regions. Neuron numbers are regulated

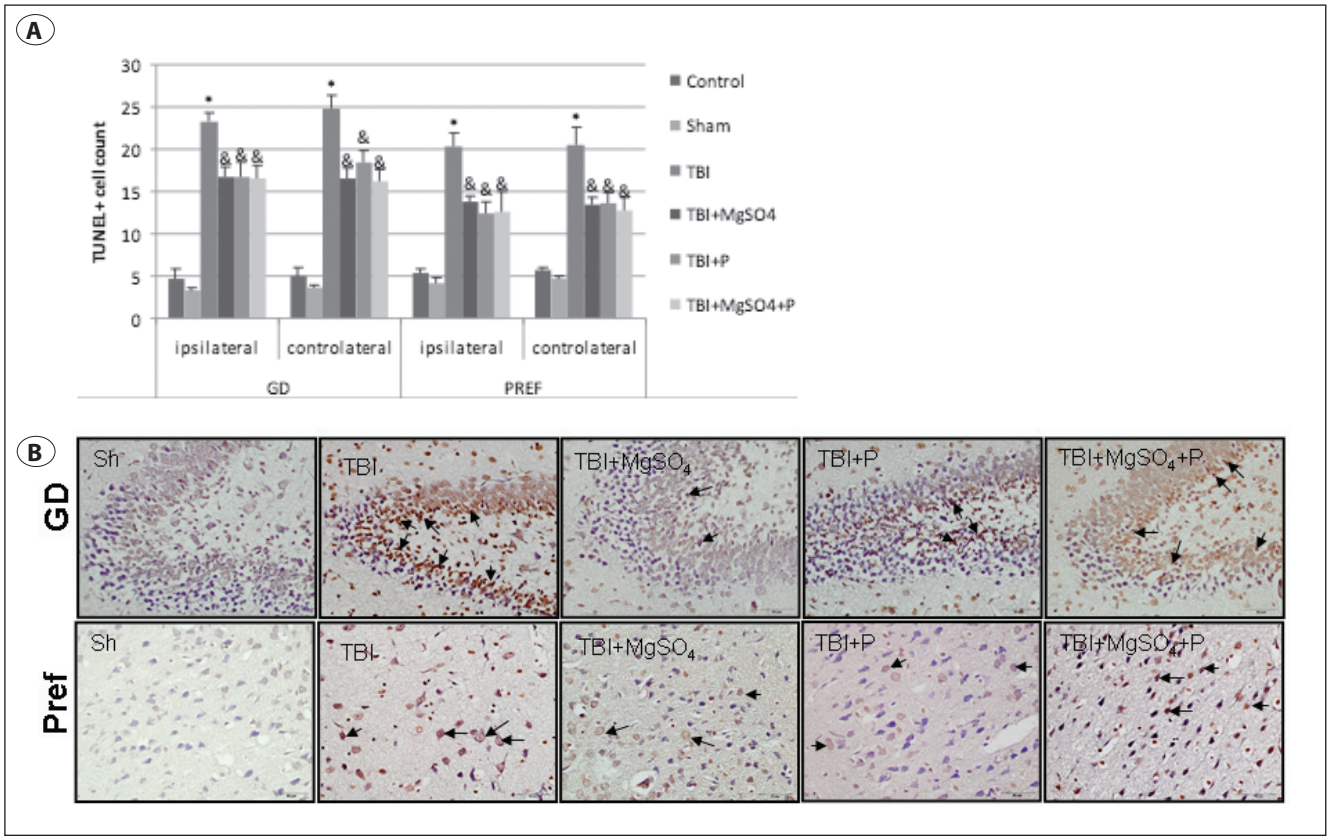

Figure 2: The extent of apoptosis following TBI. **(A)** The percentage of TUNEL + cells. * p< 0.05 compared with control and sham. p<0.05 compared with TBI group. **(B)** Representative photomicrographs of (TUNEL)—stained sections in the gyrus dentatus and prefrontal cortex. The number of TUNEL-labeled neurons was increased in TBI rats. Treatment with MgSO4 and progesterone decreased trauma induced apoptosis. Bar = 50 µm. **GD;** Gyrus Dentatus, **Pref;** prefrontal cortex, **TBI;** Traumatic brain injury, **MgSO4;** magnesium sulfate, **P;** progesterone.

by cell proliferation and death. In this study, we showed that following head trauma, neuron numbers are decreased in gyrus dentatus and prefrontal and parietal cortex due to apoptosis in the acute period.

The release and up-regulation of endogenous neurotrophic factors is the most important mechanism in neuronal repair after brain damage (8). VEGF is up regulated in parallel with hippocampal neurogenesis after traumatic injury (24). In our study, VEGF immunostaining decreased in the TBI group. It is well established that TBI results in reactive astrogliosis and angiogenesis (24, 33). Therefore, our results give only VEGF-marked neurons. Also, in our study, VEGF immunostaining decreased in parallel with decreased neurogenesis in TBI group.

In the later period, the hippocampus, prefrontal cortex and parietal cortex neuron numbers decreased in the traumatic group. Also, VEGF immunostaining decreased in TBI group. Moreover, head trauma during maturation of rat brain resulted in neuronal loss in areas of the brain associated with learning and led to learning-memory dysfunctions. During the training learning test period, the time spent to find the platform decreased steadily until it reached a plateau for all rats in the study. However, the escape latency was longer in TBI rats compared to other groups at second, third and fourth days of Morris water maze test. There was no difference in the swimming speed among groups. During the probe trial, rats with head trauma spent less time on the target quadrant and more time on the opposite quadrant. It is well known that hippocampus-related learning and memory are affected with head trauma (38). In our previous studies, we demonstrated that learning and memory were impaired with head trauma in immature rats (31). Several previous studies have shown that traumatic insult causes neuronal injury in the hippocampus (31, 38). In the water maze tank experiment with an invisible platform, the experimental animals learn the place of the platform by the help of clues that are located outside the tank (44). During this learning and remembering process, the hippocampus must be healthy and functional (6). A decrease in gyrus dentatus regions of hippocampal neuron numbers was also seen in our study. During the process of spatial learning and remembering, the hippocampal areas function together with entorhinal cortex, PFC, parietal cortex, anterior cingulate cortex, striatum and amygdale (14). The DG is characterized by the convergence of sensory inputs to create a metric spatial representation and hence is important for

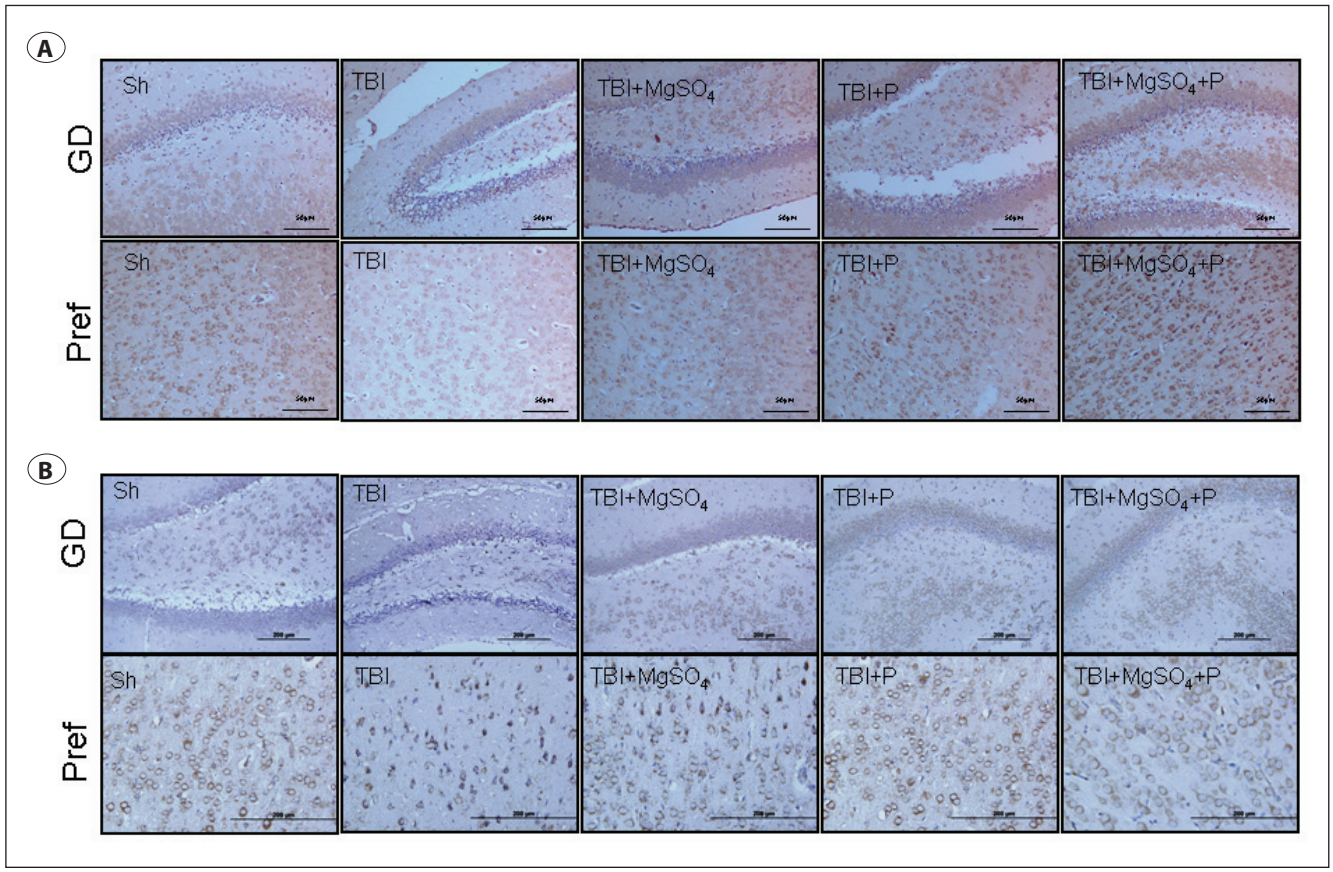

Figure 3: VEGF+ immune staining cells. **(A)** Representative photomicrographs of VEGF+ immune staining cells in acute period of TBI. **(B)** Representative photomicrographs of VEGF+ immune staining cells in chronic period of TBI. Bar = 50 µm. **GD;** Gyrus Dentatus, **Pref;** prefrontal cortex, **TBI;** Traumatic brain injury, **MgSO4;** magnesium sulfate, **P;** progesterone.

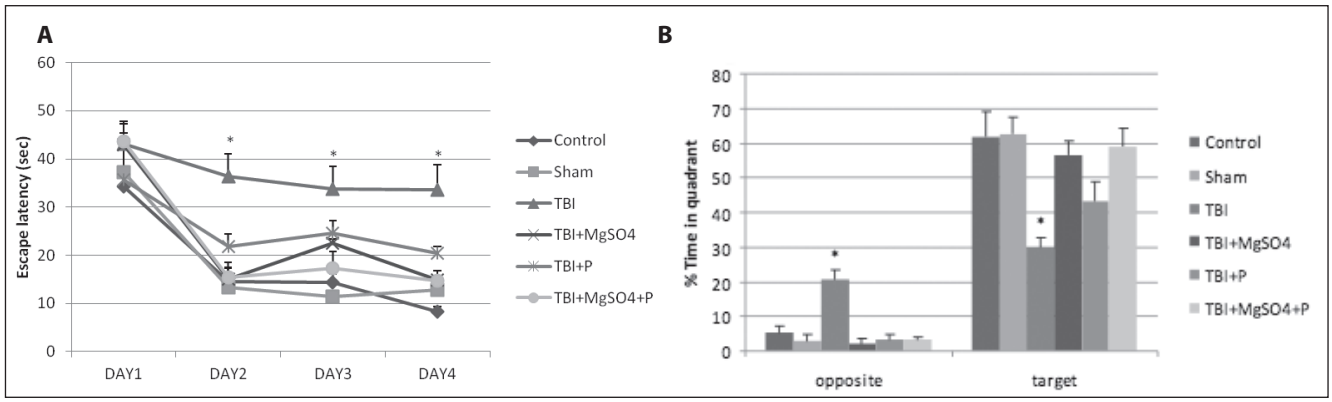

Figure 4: Effects of TBI on Morris water maze performance. **(A)** Mean daily latencies to escape from the start point onto the hidden platform. * p<0.05, compared with the other groups. **(B)** The time spent in the target quadrant in the probe trial on the fifth day. * p < 0.05 compared with the other groups.

spatial pattern separation. During the learning process, the CA3 and gyrus dentatus region neurons of the hippocampus first process the information and then integrate with the information that comes from the PFC (14). The prefrontal cortex is one of the most important areas in the completion of spatial learning model. Hippocampus and prefrontal cortex compensate each other during spatial learning and memory functions (25). A healthy GD, CA3 and PFC are prerequisites to find the correct path and the target quadrant during the probe trial (29). In this study, we found that the neuron numbers in the GD, CA3 and prefrontal cortical areas are decreased in rats with head trauma. These rats had also worse scores in water tank experiment in learning and remembering, compared to other groups.

Table I: VEGF Immunostaining Results

		Sham	TBI	TBI+MgSO4	TBI+P	TBI+MgSO4+P
CA1	Acute	+++	+	++	++	++
	Chronic	+++	-	++	+	++
GD	Acute	+++	+	+	++	++
	Chronic	+++	-	++	++	++
Pref	Acute	+++	+	+	++	++
	Chronic	+++	+	+	++	++

The qualitative intensity of staining for VEGF was assessed using a scale between 0 and +++. With 0 representing no detectable stain and +++ representing strongest stain. ***GD;*** *Gyrus Dentatus,* ***Pref;*** *prefrontal cortex,* ***TBI;*** *Traumatic brain injury,* ***MgSO4;*** *magnesium sulphate,* ***P;*** *progesterone.*

Magnesium plays a major role in normal function, integrity and stability of cell membrane by antagonizing NMDA receptor and preventing calcium entry into the cell (15). In patients with TBI, the correlation between decrease in magnesium ion levels and the severity of the injury and the neurological deficits led to the hypothesis that magnesium might be responsible for the secondary injury (43). Decrease in magnesium results in increased lipid peroxidation that is a secondary injury mechanism of TBI (2). In rats, following brain injury, decrease in magnesium levels is seen in parallel with neurological findings and behavioral disturbances (18). In a rat head trauma model, a dose dependent neuroprotective effect of magnesium is seen against motor and behavioral changes (19, 20). However, magnesium treatment following head trauma does not always improve morbidity and mortality. In head trauma models where subdural hematoma is created in rats, magnesium treatment that is administered following injury did not improve the motor activity. Magnesium treatment restored the magnesium concentrations in the group without hematoma; however, it did not affect the magnesium concentrations in the group with hematoma (35). Head trauma causes disturbances in magnesium hemostasis in humans as well. Increased magnesium excretion in urine is seen following head trauma (21). In patients with head trauma, the serum magnesium ion concentration is a marker of irreversible brain injury. Early diagnosis and replacement of magnesium ion loss decreased the late sequelae related to head trauma (34). However, some studies have shown that magnesium increased apoptotic cell death (10). These conflicting results may be explained by the differences in the severity of the trauma, age of the subjects, and more importantly, magnesium dosage and administration time. In our study, we demonstrated that magnesium treatment administered immediately after head trauma decreased apoptosis, and increased neuron numbers in the cortex and the hippocampal CA1 regions. Concurrent with these findings, it also improved learning and memory.

Progesterone was shown to be a neuroprotective neurohormone, to decrease edema and infarct/lesion volume, to regulate the inflammatory response, and to enhance neurological recovery in animal TBI models (10, 40). Progesterone also increases neurotrophic factors such as VEGF (11). In addition, progesterone inhibits voltage-gated calcium channels and reduces excitotoxicity in head trauma (27). In this study, progesterone increased hippocampal and cortical VEGF in TBI rat model. It is known that, the lower doses (8–16/mg/kg) of progesterone increase cognitive performance (40). In our study, progesterone (8 mg/kg) improved spatial learning and memory.

In the current study, we did not evaluate VEGF levels in different brain regions. One of the limitations of our study is the detection of VEGF levels by the immunohistochemistry method. VEGF levels and VEGF receptor levels could also be investigated by ELISA, and thus VEGF levels could be examined in different brain regions. Combined treatment with MgSO4 and progesterone treatment prevented trauma-induced apoptosis. It also increased VEGF levels and neuron numbers. In this study, there was no difference between the number of neurons, apoptosis, learning and memory performance among the groups that received the drugs alone or in combination. But, VEGF immunostaining was more intense in combined treatment with magnesium sulfate and progesterone. Further studies are needed to elucidate the VEGF levels of different brain regions following TBI.

CONCLUSION

This is the first study reporting on the effects of combined treatment with progesterone and MgSO4 in immature TBI rat model. Use of drugs with different mechanisms of action in the treatment of head trauma has led to better treatment outcomes. Combined treatment with progesterone that is neuroprotective and MgSO4 that is an NMDA receptor blocker ameliorated the effects of TBI in immature rats. Further research is needed to investigate whether different doses of these medications would result in even better outcomes following TBI.

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