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Clinical, Histopathological and Electrophysiological Evaluation of the Effects of Allogeneic Mesenchymal Stem Cell Transplantation in Experimental Induced Spinal Cord Injury in Rats *

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The aim of this study was to investigate the effects of allogeneic bone marrow-derived MSCs (BM-MSCs) transplantation in acute stage of experimentally generated severe spinal cord injury (SCI) in rats at different intervals. Seventy seven Sprague-Dawley rats were used in this study. The bone marrow was harvested from femurs and tibias of the one day-old rats, then processed using the standard methods. SCI was generated with an aneurysm clip after T9-T10 laminectomy. Intraparenchymal transplantation of the stem cell or stem cell diluent was perfomed at 6th hour after injury. The effects of the BM-MSCs were evaluated by clinical, electrophysiological and histopathological examinations on the 1st, 3rd, 7th and 21st day postoperatively. In clinical examination, hematuria rate was significantly low in treatment comparing to control (P=0.003). The survival rate of the 21th day treatment group was significantly higher than the control group. Significant improvement in somatosensory evoked potentials (SEP) was observed on the 3rd and 7th day in treatment group. The histopathological changes were similar in the control and treatment groups at all intervals. In conclusion, intraparenchymal transplantation of BM-MSCs in the acute phase of the SCI provided some benefical effects regarding to hematuria rate, SEP scores and survival rate. The results of this study found as promising for doing further, more detailed studies on the treatments of spinal cord injury by the MSCs.

Key Words: *Spinal cord injury, rat, allogeneic, mesenchymal stem cell*

Ratlarda Deneysel Omurilik Yaralanmalarında Allojenik Mezenkimal Kök Hücre Transplantasyonunun Etkilerinin Klinik, Histopatolojik ve Elektrofizyolojik Değerlendirmesi

Bu çalışmanın amacı, allojenik kemik iliği kaynaklı MKH'lerin (KK-MKH) transplantasyonunun sıçanlarda deneysel olarak oluşturulan şiddetli omurilik hasarının (OH) akut evresindeki etkilerini farklı aralıklarla araştırmaktır. Bu çalışmada yetmiş yedi adet Sprague-Dawley sıçan kullanıldı. Kemik iliği, bir günlük sıçanların femur ve tibialarından elde edildi, daha sonra standart yöntemlerde olduğu gibi işlendi. OH, T9-T10 laminektomiden sonra bir anevrizma klipsiyle oluşturuldu. Kök hücre veya kök hücre sulandırıcısının intraparenkimal transplantasyonu hasar oluşturulduktan 6 saat sonra yapıldı. KK-MKH'lerin etkileri postoperatif 1, 3, 7 ve 21. günlerde klinik, elektrofizyolojik ve histopatolojik incelemelerle değerlendirildi. Klinik muayenede, hematüri oranı tedavi grubunda kontrol grubuna göre anlamlı derecede düşüktü (P= 0.003). 21. gün tedavi grubunun sağkalım oranı kontrol grubundan anlamlı olarak yüksekti. Tedavi grubunda 3. ve 7. günlerde somatosensoriyel uyarılmış potansiyellerde (SUP) belirgin bir ilerleme görüldü. Histopatolojik değişiklikler kontrol ve tedavi gruplarında tüm aralıklarda benzerdi. Sonuç olarak, omurilik hasarının akut fazında KK-MKH'lerin intraparenkimal transplantasyonunun hematüri oranı, SUP skorları ve sağkalım oranı ile ilgili bazı umut verici etkiler sağladığı görülmüştür.

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Introduction

Clinical and experimental SCI studies currently focus on surgical intervention, pharmacological and cell-based therapy, rehabilitation, or a combination of several treatment protocols (1, 2) Despite the exceptional efforts in human and veterinary medicine and some promising developments according to the results of current studies, spinal cord injury (SCI) is still a devastating disease (3-5). The main objectivity of these therapeutic approaches is to protect surviving functional neural tissues, minimizing secondary injury, repair the injured spinal cord and increase the neuronal plasticity (1, 5). Although many agents including neuroprotective drugs have been experimentally investigated for the limitation of secondary injury just a limited agent like methyl prednisolone and polyethylen glicol (6) were studied in clinical cases. However, there is no consensus between authors in using drugs against harmful effect of the secondary injury (7, 8).

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Cell-based therapy for SCI has begun to attract great interest in recent years. Schwann cells, olfactory ensheating cells, neural precursor cells, activated macrophages and stem cells from different tissue sources have been investigated in many different experimental and clinical studies for the treatment of SCI (9-15). Some characteristics of the stem cells such as their differentiation abilities, secretion of some cytokines and growth factors have made them a promising candidate for the treatment of SCI, in terms of their neuroregenerative, neuroprotective and immunomodulatory potentials (16). Human-umbilical cord blood stem cells, human embryonic stem cells, placenta-derived mesenchymal stem cells, adipose or bone marrow derived mesenchymal stem cells (BM-MSCs) have been used in a numerous experimental and clinical studies (13, 14, 17-25). Some characteristics of mesenchymal stem cells (MSCs) have made them the focus of SCI studies. MSCs may exhibit adipogenic, osteogenic, chondrogenic and neuronal differentiation, also they synthesize cytokines, chemokines, enzymes and extracellular matrix proteins. MSCs are capable of rapid replication and cause minimal immunoreactions even in the case of allogeneic use (26, 27).

The aim of this study was to investigate the effects of BM-MSCs transplantation in acute stage of experimentally generated severe spinal cord injury in rats on different time points.

Materials and Methods

Seventy seven Sprague-Dawley rats were used in this study. The study protocol was approved by the Animal Experimentations Local Ethic Committee (no: 2013/22). All rats were housed under the same conditions (standard individual cages, 18-21°C, 12 hours light/12 hours dark, and food and water ad-libitum).

Isolation and Culture of the BM-MSCs: One day old five rats were used for bone marrow collection. Rats were euthanized by decapitation. Briefly femurs and tibias of the rats were harvested and medullary cavity of femur and tibias were flushed with 10 mL DMEM-LG to harvest bone marrow. Harvested bone marrow was processed as described in the standard methods. Bone marrow suspension with DMEM-LG was spread in 10 ml Ficoll solution and mononuclear cells were separated by using density gradient method (at 25 °C and 900 rpm, 30 minutes centrifuged) (28). Subsequently, the cells were seeded into tissue culture flasks containing DMEM supplemented with Pen/Strep, L-Glu, and 10% fetal bovine serum (Lonza, Basel, Switzerland) at 37°C with humidified 5% CO2. Medium was changed every day for the first 3 days then changed every third day. On the 14th day, CFU-F was observed under invert microscope, 1.5 ml Tripsin-EDTA-C was added to culture flasks and following the 5 minutes incubation at 37 ºC cells were passaged 5 times. Derived cells were analyzed by flow cytometry using hematopoietic and mesenchymal stem cell surface markers [CD11b/c(-), CD45 (-), CD90 (+), CD73(+), CD105 (+)].

Induction of spinal cord injury and grouping: Anesthesia was achieved by intraperitoneal injection of 10 mg/kg xylazine hydrochloride (Basilazin 2%, BaVET, Turkey) and 60 mg/kg ketamine hydrochloride (Ketasol 10%, Interhas, Turkey) for surgery. The operation area was prepared aseptically. The skin incision was made along the T8 - T11 vertebrae. The paraspinal muscles were dissected and the processus spinosuses were removed. T9-T10 laminectomy was performed with a care to ensure that the dura mater was intact. An aneurysm-clip was placed vertically to spinal cord and a 70 g compression was applied for 1 minute (Figure 1). After surgery, urinary bladder of the rats was emptied manually twice a day and intraperitoneal cefazolin (25 mg/kg) was used for antibiotherapy for 3 to 7 days. All animals were injected subcutaneously with 2 mL of saline after surgery to prevent dehydration. Rats were randomly allocated to treatment and control groups.

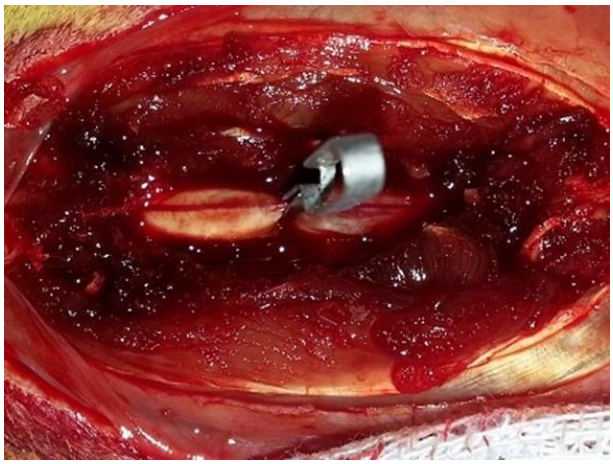

Figure 1. Placement of the aneurysm-clip

On 1st, 3rd, 7th and 21st day postoperatively 5 rats were euthanized in G1 (control group). G1 group was divided sub-groups according to the day of euthanasia (1st, 3rd, 7th and 21st days) as G1-1, G1-3, G1-7 and G1-21, respectively. G2, G3, G4 and G5 were the treatment groups and respectively on 1st, 3rd, 7th and 21st day postoperatively all rats in the groups were euthanized. In G1-21, 17 rats were used, because at the first trial only 1 rat survived until 21th day and the experiment was repeated 2 times again with each consisted 6 rats.

Transplantation of MSCs: Transplantation of the stem cell or stem cell diluent was performed after 6 hours post-operatively. Anesthesia was provided by 1-2% isoflurane (Forane Liquid, Abbot) using a gas anesthesia device (HNG-6, GEWO Feinmechanik GmbH, Germany) manufactured for rodents. About $2x10^6$ stem cells suspended in 20 µL of diluent was injected by a 0.5 ml insulin syringe into the spinal cord about 0.5 cm rostral and caudal to the trauma center in equal volume. The rate of injection was about 10 µL/min. Control groups received 20 µL PBS with the same method as treatment groups.

Clinical and Behavioral Assessment: The animals were checked daily and the results were recorded by monitoring the presence or absence of hematuria during manual expression of the bladder. Locomotor function was scored between 0-21 according to BBB (Basso, Beatie, Bresnahan) spinal cord injury scale (29). Firstly, the rats were expected to calm down, then independent movements were observed by two individuals, and scores were recorded before the transplantation and at the end of the experiment.

Electrophysiological Examinations: SEP were studied under the general anesthesia protocol which was used for the surgical procedures in all groups. Briefly, tibial nerve was stimulated from the popliteal fossa and the potentials were recorded from two segments caudal and two segments cranial of the lesion, by a monopolar stainless steel needle. The recording needles were placed as active electrode inserted near to the arcuate ligament and the reference electrode inserted sub-fascially about 2 cm laterally over the paraspinal muscles on the ipsilateral side. The ground electrode was inserted subcutaneously between the stimulating and recording electrodes and 250 responses were averaged. The injury potential recorded from both caudal and cranial site of the injury were evaluated qualitatively according to Sirin et al. (30)'s modified scale.

Histological Examination: The T9-T10 spinal cord segments which includes the injury center was excised at the end of the experiment for each case after the euthanasia. The harvested tissue specimens were fixed in 10% buffered formaldehyde. Routine tissue processing for histological and immunohistochemical staining were obtained by the automated tissue processor (Leica, Germany). Tissues were then embedded in the paraffin and sections of 5 µm thickness were taken for all staining from each block. Tissue sections were stained with hematoxylin-eosin (HE) and toluidine blue after deparafinization and dehydration for assessing the severity of the spinal cord injury. Axonal degeneration, hemorrhage, inflammatory cell infiltration, neuron degeneration and demyelination were examined in ten different reas in microscope. The severity of the lesion was graded as slight, moderate, and severe.

Statistical Analyzes: All statistical analyzes were performed using the SPSS version 14.0 package program (license no: 9869264). Pearson Chi square was used to examine the frequency distribution of hematuria among control and treatment groups after combining control and treatment groups to avoid expected frequencies below 5. Mann-Whitney test was used to determine the significance of differences between the treatment and control groups according to SEP scores (G1-1 and G2, G1-3 and G3, G1-7 and G4 were compared). Kruskal-Wallis Test was used to analyze the time-dependent changes of the SEP scores in control (G1-1, G1-3 and G1-7 were compared. Only 1 rat survived in G1-21 so this group was not taken account) and treatment groups (G2, G3, G4 and G5 were compared). A comparison was made between the G1-21 and G5 groups because of a significant difference in survival rates and the Pearson Chi-square test was used.

Results

Clinical and Behavioral Test Outcomes: All groups were observed daily starting from stem cell transplantation until the euthanasia. Rats in the control groups were observed to be more quiescent and exhausted, and their sanitation and body conditions were worse than the treatment groups, but no scale was used for quantitative evaluation of body condition and/or sanitation. In the day-to-day examinations, none of the animals were found to have an infection or wound dehiscence at the surgical site.

Hematuria was the main problem for both control and treatment groups, but mean hematuria rate of control and treatment groups on the first day after transplantation were 80% and 37.5%, respectively, also difference between the groups was significant (Table 1).

Table 1. Statistical analyses of the hematuria rates of control and treatment groups

	Hematuria				
	Positive		Negative		
	n	n%	n	n%	P
Control	16	80.00	4	20.00	0.003
Treatment	15	37.50	25	62.50	

n: number of the rats, n%: percentage (Pearson Chi square test).

In G1-21, only one rat survived until the end of the experiment, so the experiment was repeated to reach a statistical number of rats for this group. Totally 17 rats were used, but the result did not change and only 1 rat survived. In the G5, the rate of the rat which could complete the experiment period was 60%, in the G1-21 group it was 5.88% and the difference was statistically significant.

All rats had a BBB score of 21 before generation of the SCI and the scores were decreased to 0–1 after the injury. According to BBB score no progress was observed in any of the rats, except three rats from G3 and two rats from G5. The difference between the control and treatment groups was not statistically significant.

Electrophysiological Examination Results: Rostral and caudal SEP scores were statistically compared between the control groups, treatment groups, and control-treatment groups by time. SEP scores at the rostral site of injury significantly higher at the 3rd and 7th day in treatment group (G2 and G4) than control groups (G1-3 and G1-7) (Table 2).

Table 2. Statistical analyses result of the rostral SEP scores of the groups

	G1-1 X±S	G2 X±S	G1-3 X±S	G3 X±S	G1-7 X±S	G4 X±S
Rostral SEP	3.8±0.447	4.1±0.316	1.8±0.837	3±0.667	2±0.816	2.5±0.527
P value			**0.028**		**0.036**	

SEP scores of the control and treatment groups of the same time interval were compared. The difference between the 1st day control and treatment groups (G1-1 and G2) was not statistically significant, but it was statistically significant in 3rd and 7th day groups. The SEP scores of the treatment group G3 and G4 were higher than the control groups G1-3 and G1-7 respectively.

X: mean, S: Standard deviation, (Mann-Whitney test).

Histopathological Changes: The histopathological changes resulting from the trauma of the spinal cord were seen in both gray and white matter in all groups. The lesions formed in the gray matter were more intense in the dorsal horn than in the ventral horn. Lesions in the dorsal and lateral funiculi of the white matter were more severe than those of the ventral funiculi. The severity of the histopathological changes were similar in the control and treatment groups and consisted of hemorrhage in the gray and white matter, axonal dystrophy, demyelination, Gitter cell reaction, gliosis and axonal degeneration (Figure 2). It was also determined that the nucleus was not visible in some neurons. Glial cells (neuronophagy) were detected around some neurons. Nissl granules were quite explicit in some neurons and they were localized near the cell wall (chromatolysis).

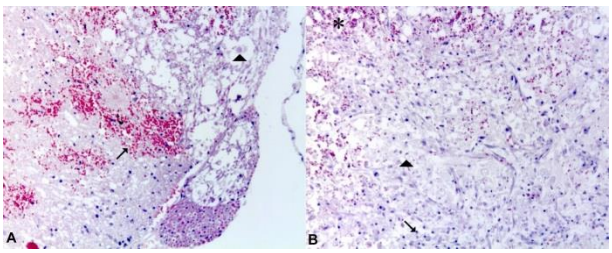

Figure 2. In A histopathologic changes in control group G1-1 are shown; axonal dystrophy (arrowhead), bleeding (arrow) and leukomyelomalasic changes. In B, histopathologic changes in treatment group G3 are shown; gliosis (arrow), Gitter cells (arrowhead), bleeding (asterisk) and leukomyelomalasic changes. HE, × 100.

Discussion

The effects of acute transplantation of allogeneic MSCs derived from bone marrow in severe spinal cord injury generated by clip compression model were evaluated by the clinical, electrophysiological, histopathological and immunohistochemical analysis in this study. Survival rate of the cases which had received MSCs were higher on the 21th day. The rate of hematuria and SEP score were promising for using MSCs in SCI.

The clip compression model was first developed by Rivlin and Tator (31) to create experimental spinal trauma in rats using an aneurysm clip, and it generates both contusion and compression injury in spinal cord, by this way the injury created by the clip compression can mimic the vertebral fracture/luxation and Hansen type-I disc disease. In this study, clinical examination on the first day after trauma showed that all subjects had a BBB score of 0 or 1, and this revealed that the created injury is a severe, reproducible and standard. The clinical unimprovement of the control cases represents the repeatable irreversible damage (physical transection) to the spinal cord.

Several methods have been used in the evaluation of functional improvement after experimental SCI in rats, but the most common method is BBB scale (19, 32). Small gain in spinal cord injury is appreciative by the patient, owner and also investigators because of its poor prognosis. In some studies, it was shown that progression of the locomotor function could have occurred in the mildly or moderately traumatized rats within 3 to 7 days after spinal cord trauma, even if no treatment is applied (18, 19, 21). Absence of any clue for the clinical improvement of control cases presented here on the 1, 3 and 7 th day can be explained by the severity of injury and ascendens and/or descendens degeneration.

There are many studies showing that MSCs therapy improves the BBB score when compared with control groups (18, 19, 21, 33, 34). In contrast, Park et al. (20) found that intraparenchymal injection of human MSCs nine days after the moderate spinal cord injury created via a weight dropping method can reduce inflammation but not enough to improve locomotor function and neurogenic bladder. In the present study, the post-injury BBB score did not improve both in MSCs transplanted and not transplanted rats, except 5 rats in the transplantation groups (3 in G3 and 2 in G5). This may show that the injury created with an aneurysm clip with 70 g closure force is a very severe injury and BM-MSCs transplantation in the early acute stage of the very severe SCI cannot provide a functional improvement, even though some improvement was recorded in some individuals.

Even though there was no study investigating the direct effect of MSC transplantation on survival rate of the subjected animals after SCI. Yazdani et al. (13) reported lesser mortality rate, better hygiene and health conditions in the rats treated with neuron-induced BM-MSCs or OECs than the control groups. In an another study about the effects of intraparenchymal transplantation of OCT, MVD, and OCT-MCT seven days after spinal cord injury were investigated, highest weight loss in the control groups was reported (33). In this study, the rate of the rat which could complete the experiment period in the G5 was 60%, in the G1-21 group it was 5.88% and the difference was statistically significant. In our study, same person (PC) applied all stages of the experiment; there was no change in the

environmental factors of animals such as housing, feeding and water, and that the only difference between the two groups was MSCs transplantation, so it was thought that this is not an incidental finding and MSCs may provide a positive effect on survival. In addition, this positive effect may be related with MSCs, based on the observations that the rats in the treatment groups had better food-water intake, better hygiene and less hematuria. Although it was not investigated in detail by the present study, it was thought BM-MSCs has a systemic effect via the secreted cytokines and neuroprotective factors, and immunomodulation, thus positively affects general health status and survival. More detailed experimental studies which focused on the effects of MSCs on survival rate after spinal cord injury are needed to clarify if there is a positive correlation between the MSCs transplantation and longer survival rate of the subjects.

The neurogenic bladder after spinal cord injury is a major problem in human beings and animals; lower urinary tract inflammation or infection can lead to hematuria and ascendants nephritis. Park et al. (20) reported that there was no difference in terms of bladder volume and urodynamic measurements between the groups treated with stem cells and those who did not after spinal cord trauma, but no data on hematuria ratios were reported. Herein, hematuria rates were lower in stem cell-treated groups compared to control groups, and this was thought to be associated with the anti-inflammatory effect of the MSCs.

Somatosensory evoked potentials can also be used as ancillary test for determining functional integrity of spinal cord after injury (5, 18, 30, 32, 33, 35). Sirin et al.'s (30) modified scale was used in the presented study for the qualitative evaluation of the injury potentials in post-traumatic SEP recordings. When the control groups were compared with the treatment groups, the rostral SEP scores were found higher in the treatment groups and the difference was statistically significant, while the difference in the caudal SEP score was not significant. In a study, the useful effect of the MSCs on the SEP latencies was reported, but in the same study authors showed no positive effect on the SEP amplitudes (18). Although the latency and amplitude of SEP was not measured in this study, better results according to Sirin et al.'s modified scale (30) were recorded in the treatment groups compared to control groups. Our study results showed that intraparenchymal transplantation of BM-MSCs in the acute phase of the SCI has a positive effect on SEP.

Histopathologic examinations revealed extensive necrotic areas and accompanying inflammatory cell infiltrations in both groups. There was no difference between the control and treatment groups according to histopathologic examination, and the severity of the inflammation, necrosis or other histopathologic changes were similar. It should be noted here that the injury created in this study was severe and extensive necrotic areas were prominent in the sampled spinal cord segments (T9-T10).

In conclusion, even though the intraparenchymal transplantation of BM-MSCs in the acute phase of the SCI can provide some useful effects to hematuria rate, SEP scores and survival time, we have not shown the functional recovery after severe spinal cord injury in this study. The results of this study found initiative to plan further studies for clarifying the role of MSCs in different degree of SCI.

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