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HUMAN MESENCHYMAL STEM CELL THERAPY IN THORACIC SPINAL CORD INJURY AND EVALUATION OF THE RESULTS-AN EXPERIMENTAL STUDY IN RATS

SIÇANLARDA TRAVMATİK ALT TORASİK OMURİLİK YARALANMASINDA İNSAN KAYNAKLI MEZENKİMAL KÖK HÜCRE TEDAVİSİ VE SONUÇLARININ DEĞERLENDİRİLMESİ

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SUMMARY:

Objective: The aim of this study is to investigate the hyper-acute and acute effects of mesenchymal stem cell (MSC) therapy on traumatic spinal cord injury (SCI) in an experimental animal model.

Materials and Methods: The study was carried out on 60 male Sprague-Dawley rats weighing 400- 500 grams. The subjects were separated into six groups. In-group 1, only thoracic 10 laminectomy was performed. In-group 2, trauma was applied to the spinal cord by using modified Allen trauma model after T10 laminectomy. In group 3, T 10 laminectomy and spinal cord injury was immediately followed by injection of 0.9 % 10 NaCl. In group-4 "1.1- Dioctadecyl-3.3.3´.3´-tetramethyllindocarbocyanine" labeled MSC derived from male human bone marrow was implanted to injury site immediately after spinal injury. In group-5, the MSC was implanted nine hours after spinal injury. In the last group 0.9 % NaCl was administered nine hours after T 10 laminectomy and spinal injury at this segment. All groups were sacrificed at the end of four weeks. The neurologic status was checked at Days 1,7,14,21 and 28 using BBB (Basso-Beattie, Bresnahan) locomotor rating scale and inclined plane values. Hematoxylin eosin, and Masson trichome staining were used to assess the degree of inflammation and fibrosis. In order to confirm the Y chromosome signal content of the cells, Y-18 chromosome specific centromere probe was used.

Results: Our results showed that MSC therapy has the potential of reducing the degree of inflammation and contributing to functional improvement following SCI. There was statistically significant difference between the hyperacute and nine hours delayed treatment groups.

Conclusion: The results of this research is in agreement with the findings of previously published studies indicating the neuroprotective nature of MSC.

Key Words: Mesenchymal stem cell, Thoracic spinal cord injury, treatment

Level of evidence: Experimental study, Level I

ÖZET

Travmatik omurilik yaralanmasında yüksek morbidite ve mortaliteye sebep olan önemli bir sağlık sorunudur. Travma sonrası ortaya çıkan fonksiyon kaybı, hem birincil yaralanmaya hem de birincil yaralanmanın tetiklediği ikincil yaralanma mekanizmalarına bağlıdır. son yıllarda TOY'ında kök hücre kullanımının umut verici sonuçlar vermiştir. TOIY'nda farklı kaynağı olan nöronal progenitör hücreler, nöronal kök hücreler, embriyonik kök hücreler veya mezenkimal kök hücreler kullanılamkatadır. Biz çalışmamızda ratlarda travmatik alt torasik yaralanmasında insan kaynaklı MKH tedavisi ve sonuçlarını inceledik.

Anahtar Sözcükler: mezenkimal kök hücre, torasik spinal kord yaralanması, tedavi.

Kanıt Düzeyi: Deneysel çalışma, Düzey I

INTRODUCTION

Spinal cord injury is a major cause of morbidity and mortality. The level and extent of injury are the main factors determining the outcome. Traumatic SCI occurs mostly at low cervical and thoracolumbar junction segments. Males are more frequently affected by traumatic SCI than females. In USA estimated annual incidence of SCI, not including those who die at the scene of accident, is nearly 40 cases per million population as of June 2009 or approximately 12000 new cases each year. Motor vehicle accidents account for 42.1 % of SCI, followed by falls (26.7 %), act of violence (15.1 %) and sports injuries (7.6 %). Only 11.5 % of traumatic SCI patients have been able to return to their regular jobs one year after trauma. The life-long therapeutic expenses of a 25 years old tetraplegic patient is approximately 1,800,000 $ [13].

In Turkey 18,000 new cases are added each year and a total of 54,000 neurologically disabled individuals survive as a result of SCI. Surgery seems to have little beneficial effect in SCI and in most cases neurologic improvement is limited. Decompression and instrumentation procedures aim to stabilize the spinal column thus allowing early mobilization and rehabilitation.

Fehlings and associates have conducted an international cohort study and have found that early decompression reduced secondary injury in SCI. However, with surgical therapy only 19.8 % of the 313 patients included into the study showed two or more grades of improvement in American Spinal Injury Association status [10].

In spite of all efforts, neuroprotection and spinal regeneration are not successful so far. The three properties of effective adjuvant therapy for SCI were mentioned as immunomodulation, neurotrophic properties to stimulate axonal growth and ability to replace injured cells [29]. Stem cell therapy seems to be a potentially beneficial mode of therapy in the management of traumatic SCI.

MATERIALS AND METHODS

The study was approved by the Ethic Committee of the Başkent University (January 17, 2011, DA 11/24) and was conducted at the Animal Breeding and Experimental Research Laboratory of Başkent University. A total of 60 Sprague-Dawley male rats weighing 400-450 grams were used. The general health of the subjects was checked prior to study. Each animal was marked according to its group. The subjects were kept in cages at room temperature of 25 º C on a 12 h light-12 h dark cycle. There was no food or water restriction. Bladders were emptied regularly. Subjects were subjected to overnight fasting before anesthesia. Anesthesia was induced by intraperitoneal injection of 60 mg/kg Ketamin (Ketalar® Pfizer) and 10 mg/kg Xylazine (Rompon®- 2 % Bayer). Anesthesia provided unresponsiveness to pain while spontaneous respiration was maintained. Additional doses were administered when necessary. The body temperatures were controlled by rectal temperature probes and was kept constant at 37° C. During the procedure, O2 at a rate of 1.5 lt./min was administered by mask. After the study the subjects were kept at room temperature of 23-25º C. After recovery from anesthesia the rats were shaved on the back and were placed on the operating table.

Povidine-iodine (Betadine®) was used for local antisepsis. A median skin incision has been made. Subperiostal blunt dissection of paravertebral muscles was followed by exposure of T 9 and T 10 laminae. T 10 laminectomy was performed with the help of rongeur. The spinal cord was exposed. Care was taken not to injure the spinal cord during surgical manipulations. Unintentionally injured animals were excluded from the study. Dura was left intact in group 1. In groups 2-6 spinal cord injury was induced by using the modified Allen method.

This method required 5 gr. steel weights specially designed for rats which were dropped perpendicular to the spinal cord through a 10 cm. long glass tubes. In groups IV and V " 1.1-Dioctadecyl-3.3.3´.3´-tetramethyllindocarbocyanine" labeled MSC derived from male human bone marrow was implanted. In groups III and VI % 0.9 SF was injected. Behavioral analysis (BBB) was checked on day 1, 7, 14, 21 and 28 in the late sacrificed subjects.

Sacrifice was done under deep anesthesia by drawing blood from the heart and intra-cardiac perfusion-fixation method.

Summary of groups:

Group I control group: only T 10 laminectomy.

Group II trauma group: T 10 laminectomy and SCI.

Group III hyperacute trauma group: T 10 laminectomy, SCI and 3x2 µL % 0.9 SF injection at injury site immediately after trauma.

Group IV hyperacute trauma and MSC group: T 10 laminectomy, SCI and male human bone marrow derived 3x2 µL MSC implantation immediately after trauma.

Group V acute trauma and MSC group: T 10 laminectomy, SCI and 3x2 µL MSC implantation 9 h after trauma.

Group VI acute trauma group: T 10 laminectomy, SCI and 3x2 µL % 0.9 SF injection 9 h after trauma.

Statistical analysis

Statistical analysis was performed with SPSS software (SPSS for Windows, Version 11.5, SPSS Inc, Chicago, IL, USA). Univariate parametric variance analysis was used for groups which did not fulfill the requirements of parametric test (ANOVA). Nonparametric data were analyzed with Kruskall

Wallis test. In order to investigate differences between the groups, Tukey test was used for parametric and Dunn test was used for nonparametric data. Statistical significance was defined by $p < 0.05$. The ratios were compared with Z test. $Z < 1.96$ values were regarded as statistically significant.

RESULTS

Tissue inflammation levels: Inflammatory cell levels were measured. Inflammation levels were addressed as none, minimal, moderate and severe. No inflammation was observed in 66.7 % of the subjects in "only laminectomy" group. In both hyperacute trauma group followed by stem cell therapy and acute trauma group followed by injection of SF, 16.67 % inflammation was observed. Severe inflammation was noted in 28.6 % of the subjects in acute and hyperacute trauma groups followed by stem cell therapy . No significant difference was found between the degree of inflammatory cell levels ($p > 0.05$).

Results of tissue inflammation level is shown in Table-1.

Tissue fibrosis levels: Fibrosis was graded as none, minimal, moderate and severe. Fibrosis was absent in 31.6 % of the " only laminectomy" group. In the other groups the rate was 26.3 %, 15.8 %, 10.5 %, 0 % and 15.8 % respectively. In the "acute trauma" plus MSC therapy group 100 % fibrosis was noted. In the "hyperacute trauma" plus MSC therapy group severe fibrosis was 0 %. There was statistically significant difference between the fibrosis levels ($p<0.05$). Tissue fibrosis results are shown in Table-2.

Tissue signal levels: Y chromosome signal was assessed as present or absent in the "acute" and "hyperacute plus MSC therapy" groups. In 88.9 % of the "acute trauma plus MSC therapy" group Y chromosome was present and the rate was 11.1 % in the" hyperacute trauma plus MSC therapy" group. There was statistically significant difference between the Y chromosome signal levels ($p< 0.05$). Tissue signal level results are shown in Table 3.

Light microscopy results: Normal findings were observed in the control group. Moderate amount of inflammatory cells and minimal fibrosis were seen in "only laminectomy" and trauma groups. In the "hyperacute trauma plus SF injection" group, moderate amount of inflammatory cells and minimal fibrosis were observed. In the "hyperacute trauma plus MSC therapy" group inflammatory cells and fibrosis were minimal. In the group with MCS therapy 8 hours after trauma, moderate amount of inflammatory cells and severe fibrosis were observed. In the acute trauma group with SF injection after trauma, moderate amount of inflammatory cells and minimal fibrosis were observed.

Table-1. Tissue inflammatory cell analysis results

	None	Minimal	Moderate	Marked
Control	66.7	14.3	0	0
Trauma	0	14.3	23.8	14.3
Trauma+hyperacute SF	0	14.3	23.8	14.3
Trauma+hyperacute MSC	16.7	19	14.3	28.6
Trauma+acute MSC	0	19	19	28.6
Trauma+acute SF	16.7	19	19	14.3

Table-2. Tissue fibrosis analysis results

	None	Minimal	Moderate	Marked
Control	31.6	3.7	0	0
Trauma	26.3	14.8	0	0
Trauma+ hyperacute SF	15.8	22.2	0	0
Trauma+ hyperacute MSC	10.5	25.9	16.7	0
Trauma+ acute MSC	0	14.8	50	100
Trauma+ acute SF	30	50	20	0

Table-3. Tissue signal level results

	Present	Absnet
Trauma+hyperacute MSC	11.1	81.8
Trauma+acute MSC	88.9	18.2

FISH results: In the "hyperacute trauma" group in which MSC was implanted immediately after trauma, only in one subject X and Y signals were observed. In the "trauma group" in which MSC was implanted 8 h after trauma, X and Y signals were observed in 8 subjects.

Basso-Beattie-Bresnahan(BBB) Locomotor Rating Scale: In the first post-trauma day all subjects experienced marked paresis in their back limbs. In the following weeks, partial recovery was seen. The group receiving MSC therapy 9 hours after trauma showed statistically significant improvement compared to the group receiving MSC therapy immediately after trauma. The improvement has begun on the seventh day and was maintained until the end of the study. Behavioral analysis (BBB) was checked on Day 1, 7, 14, 21 and 28. The BBB results are shown in Table-4.

Inclined plane rating: Coordinated motor functions of the subjects were tested up and down slopes. Front limbs were evaluated during climbing and back limbs were evaluated during down slope. There was no statistically significant difference between the "MSC therapy groups" and "SF injected group" while climbing ($p>0.05$). There was statistically significant difference between the "MSC therapy" groups and "SF injected" group while down slope ($p< 0.05$). This difference started on the first day of the study and maintained until the end. The differences among the MSC therapy groups were not statistically significant ($p>0.05$). Based on behavioral analysis, the positive effects of stem cells on the disturbed motor functions of back limbs due to spinal cord injury were observed.

Inclined plane climbing values and inclined plane down slope values are illustrated on Tables-5 and 6 respectively.

Table-4. The BBB values Av: Average, SE: Standard error of mean, Min: Minimum, Max: Maximum

	Day 1	Day 7	Day 14	Day 21	Day 28
TRAUMA					
Av.±SE	0.69±0.285	3.5±0.377	6.68,00±0.308	8.35±0.340	10,55±0.340
Median	1.00	4.00	7.00	9.00	11.00
Min-Max	0.00-2.00	3.00-5.00	6.00-8.00	8.00-10.00	10.00-12.00
MSC (hyperacute)					
Av.±SE	1.37±0.202	6.25±0.340	10.14±0.340	11.42±0.297	13.71±0.184
Median	2.00	7.00	11.00	14.00	16.00
Min-Max	1.00-2.00	6.00-8.00	10.00-12.00	12.00-14.00	15.00-16.00
MSC acute					
Av.±SE	1.00±0.218	6.14±0.340	10.14±0.260	12.71±0.359	14.85±0.340
Median	1.00	6.00	10.00	12.00	15.00
Min-Max	0.00-2.00	5.00-7.00	9.00-11.00	12.00-14.00	14.00-16.00

Table-5. Inclined plane climbing values Av: Average, SE: Standard error of mean, Min: Minimum, Max: Maximum

	Day 1	Day 7	Day 14	Day 21	Day 28
TRAUMA					
Av.±SE	45.57±2.608	53.28±2.542	55.7.±2.369	60.14±2.142	63.28±2.542
Median	50.00	55.00	60.00	65.00	65.00
Min-Max	35.00-55.00	40.00-60.00	45.00-65.00	50.00-65.00	50.00-70
MSC (hyperacute)					
Av.±SE	51.57±0.922	57.28±0.714	60.85±1.010	64.42±0.922	66.97±0.922
Median	55.00	60.00	65.00	65.00	65.00
Min-Max	50.00-55.00	55.00-60.00	60.00-65.00	65.00-70.00	65.00-70.00
MSC acute					
Av.±SE	51.12±2.608	56.14±1.010	61.12±0.922	63.71±2.020	65.85±1.010
Median	50.00	55.00	60.00	70.00	70.00
Min-Max	40.00-60.00	55.00-60.00	60.00-65.00	60.00-70.00	65.00-70.00

Table-6. Inclined plane down slope values Av: Average, SE: Standard error of mean, Min: Minimum, Max: Maximum

	Day 1	Day 7	Day 14	Day 21	Day 28
TRAUMA					
Av.±SE	11.35±1.844	16.04±2.640	22.32±2.102	23.42±2.102	31.52±2.608
Median	10.00	15.00	20.00	25.00	30.00
Min-Max	10.00-20.00	10.00-30.00	15.00-30.00	20.00-35.00	25.00-40.00
MSC (hyperacute)					
Av.±SE	40.42±0.922	45.62±0.922	51.32±0.922	56.14±1.010	62.65±1.010
Median	40.00	45.00	50.00	55.00	65.00
Min-Max	40.00-45.00	45.00-50.00	50.00-55.00	55.00-60.00	60.00-65.00
MSC acute					
Av.±SE	40.00±2.182	43.28±1.700	47.28±2.020	53.28±2.020	57.28±2.020
Median	40.00	45.00	50.00	55.00	60.00
Min-Max	30.00-50.00	35.00-50.00	40.00-55.00	45.00-60.00	50.00-65.00

DISCUSSION

Traumatic spinal cord injury is an event with serious consequences and there is no definite medical treatment yet. Surgical decompression and instrumentation have little impact in neurologic recovery and in most of the cases he aim of surgical intervention is early mobilization and rehabilitation.

Stem cells have been tried in the treatment of many central nervous system diseases like amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, stroke and multiple sclerosis.

More specifically MSCs have been transplanted in human subjects with multiple sclerosis [7,8,37] and Parkinson's disease [4]. There are many researches on effects of MSCs in animal model of Parkinson's disease [28,31].

Human adipose-derived stem cells have been noted in a wide variety of central nervous system disorders including spinal cord injury [5]. The recent studies on the mechanisms of cellular damage following SCI has emphasized the reliability of Allen's original description [1] of two phases of injury in 1911. The primary phase is the insult immediately after trauma and includes compression, contusion and/or laceration of the spinal cord. This is associated with the impact of the trauma. Damage to neurons, glial cells, and demyelination of the spinal tracts lead to anatomical discontinuity [35]. In practice, there is no definitive treatment for the first stage of insult and only preventive measures can be taken to minimize the damages of spinal trauma. No mode of therapy has succeeded in neuronal regeneration or marked clinical improvement of injured spinal cord tissue so far [2,11,14-18,22,32-34].

The attention has been focused on cellular therapy. This includes MSCs, pluripotent stem cells, embryonic stem cells, Schwann cells, olfactory cells, inhibitory molecules and gene therapies [13,23,30].

MSCs are multipotent adult progenitor cells which can differentiate into different types of mesodermal tissues including bone, cartilage, muscle and blood vessels. Bone marrow and umbilical cord blood are the richest sources of MSCs, however they can also be found in adipose tissue, skeletal muscle, trabecular bone and teeth [9]. It is generally believed that MSCs can be induced to secrete neurotrophic factors which may play a role in promoting axon growth, angiogenesis and anti-inflammatory actions [24]. MSCs have the disadvantage of causing increased incidence of hematological and other malignancies and tumor metastases [36].

Adult bone marrow MSC and neural crest stem cells have been found capable of inducing motor recovery in mice after SCI. They also may modify the inflammatory reaction in the lesion site. MSCs were able to secrete chemokines and attract macrophages in vitro. The authors concluded that both cell types have beneficial effects in experimental SCI [25].

Kim and associates have conducted a research regarding the effects of early IV injection of adipose-derived MSC in acute spinal cord injury in dogs. Their results revealed that adipose-derived MSC after acute SCI may prevent further damage through enhancement of antioxidative and anti-inflammatory

mechanisms and the authors have suggested that this treatment could be used as an alternative IV treatment modality for acute SCI [19]. 188

Bone-marrow derived MSCs were injected via intratechal route in a patient with a chronic (54 months) incomplete spinal cord injury in the form of atlanto-axial subluxation. The patient was followed by magnetic resonance (MR). Immediate MR after transplantation showed hypointense signal of paramagnetic substance tagged stem cells in the lumbar subarachnoid space. The same finding was observed at the surface around the cervical spinal cord at 48 hours, but it faded after two weeks and disappeared after one month. There was no neurologic improvement. There were some procedure related complications [6].

A phase III trial was performed in 16 patients with chronic ASIA B level who had experienced cervical trauma more than one year ago and showed no neurologic improvement during the last 3 months in spite of intense rehabilitation. Autologous MSCs were injected into the intramedullary compartment at the injured segment and MSCs were also injected into the subdural space. Outcome was evaluated at 6 months with neurologic examination, magnetic resonance imaging, diffusion tensor imaging MR (DTI-MR), and with electrophysiological analyses. Two patients showed neurologic improvement. The DTI -MR scans of these patients have revealed an appearance of continuity in the spinal cord tract. No complications were observed associated with MSCs injection. The author's remark was that the single MSCs application to intramedullary and intradural space is safe with very weak therapeutic effect compared to multiple MSCs injections [26]. Although the results of MSC therapy in experimental animal models of SCI are encouraging, clinical trials with MSC are few and the neurologic improvement especially in chronic SCI patients is not so satisfactory [3,20,27,37-38].

Cellular therapy for SCI may be successful by decreasing cell death, stimulating axonal growth or myelinated existing axons and replacing injured cells. Induced pluripotent stem cells may be help achieving this goal. It has been shown that mature adult cells can be reprogrammed to become immature stem cells [30]. More clinical studies with better outcome are needed to pave the way for effective MSC treatment in SCI.

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