Intraspinal Transplantation of Autologous Neurogenically-Induced Bone Marrow-Derived Mesenchymal Stem Cells in the Treatment of Paraplegic Dogs without Deep Pain Perception Secondary to Intervertebral Disk Disease

Öz

AMAC: Intervertebral disk hastalığı (IVDH) sonucunda paraplejik kalan ve derin ağrı duysusu olmayan köpeklerde, nörona yönlendirilmiş kemik iliği kökenli otolog mezenkimal kök hücrelerin etkisini araştırmaktır.

YÖNTEM ve GEREÇLER: Bilinen tedavi yöntemleriyile nörolojik yönünden herhangi bir gelişme kaydedilmeyen 7 köpek çalışmaya dahil edildi. Tüm köpeklerde manyetik rezonans görüntüleme (MRG) ile teşhis kondu ve operatif tedavi uygulandı. Her köpek 21 gün arayla toplam 2 kez olmak üzere, hemilaminetomi defektinden perkutan olarak omurilik: CNPase, MAP-2, GFAP ve beta III tubulin olarak pozitif olan 5,0x10⁶ nörona yönlendirilmiş kemik iliği kökenli otolog mezenkimal kök hücre süpsansiyonu enjekte edildi.

BULGULAR: Hürce transplantasyonundan 2 ay sonra sadece 1 olguda yürüyüş sağ kalan 1 skor gelişme kaydedildi. Dördüncü ayda, 5 olguda yürüyüş sağ kalan 1 skor gelişme, 1 olguda ise nosisepsiyon ve proprioepsiyon değerlendirmelerinde 1 skor gelişme kaydedildi. Sekiz ay süreye takip edilen 4 köpek; yürüyüş sağ kalan 3 olguda ilerleme izlenirken, proprioepsiyonu 2, nosisepsiyonu ise 3 olguda ilerleme kaydedildi.

SONUC: Bulgularımız, prognozu kötü olan IVDH' nda nörona yönlendirilmiş kemik iliği kökenli otolog mezenkimal kök hücre kullanımının umut verici bir yaklaşım yöntemi olduğunu önermektedir.

ANAHTAR SÖZCÜKLER: Omurilik hasan, Köpek, Mezenkimal kök hücre, Intervertebral disk hastalık

ABSTRACT

AIM: To investigate the effects of neurogenically-induced autologous bone marrow-derived mesenchymal stem cells (NIBM-MSCs) in paraplegic dogs without deep pain perception (DPP) secondary to intervertebral disk disease (IVDD).

MATERIAL and METHODS: Seven dogs which could not be improved neurologically with conventional treatment modalities were included in the study. All dogs were diagnosed by magnetic resonance imaging and surgically treated. Each dog received two times a suspension of autologous 5.0x10⁶ NIBM-MSCs, which were positive to CNPase and MAP-2, as well as to GFAP and beta III tubulin into the spinal cord through the hemilaminetomy defect percutaneously, with a 21-day interval.

RESULTS: Two months after cell transplantation, there were no changes except for 1 gait score improvement for 1 of the cases. At the 4th month, gait score had improved 1 score in 5 cases, and one score progress was recorded in proprioception and nociception in 1 case. In eight months-followed up 4 cases were evaluated by the same parameter; gait score had improved in 3 cases, and proprioception improved in 2 cases, and nociception improved in 3 cases.

CONCLUSION: Our findings suggest that utility of autologous NIBM-MSCs for cases with poor prognosis after IVDD can be a promising approach.

KEYWORDS: Spinal cord injury, Dog, Mesenchymal stem cells (MSCs), Intervertebral disk disease
INTRODUCTION

Spinal Cord Injury (SCI) is a common problem for humans and also companion animals. Even though the epidemiological data concerning the global prevalence of SCI is inaccessible, it is appraised that more than 130,000 humans suffer from SCI per year. Up to now, there is no cure available for these individuals, and unfortunately SCI usually leads to important life-long disability (1, 14).

Regenerative therapies based on stem cells have aroused considerable interest, and has led to promising results in a number of pre-clinical and clinical applications (11, 13). Numerous experimental studies have been reported with different success rates for SCI, however they have not been fully translated into the benefit of patients, both human and also companion animal (14, 40). This can be related to many differences between models and clinical cases, including the ones encountered after naturally occurring spinal cord injury (14).

An experimental study has revealed that repair of the whole injured spinal cord area for functional recovery may not be necessary to allow walking; just restoring 10-15% of connection in the spinal cord can be sufficient (7, 23). A better understanding of the pathophysiology of SCI has been attained in the early 1980s with neuroprotection, and has proceeded in the 1990s, and eventually the regenerative approach has become the main focus of investigators (40). Progress in understanding pathophysiology, acute trauma care, interventional surgery, spinal instrumentation, rehabilitation and regenerative medicine have come into a crucial level for spinal cord injury. This compromise encourages the investigators to search appropriate ways to cure this devastating disease which has impact on the emotional and physical well-being of affected individuals and the society (24).

The prognosis for functional recovery of thoracolumbar intervertebral disk disease is highly related to severity of internal injury to the spinal cord. Acute high velocity disk extrusions generally have a more guarded prognosis than the slow disk extrusions (5, 6). Poor clinical outcome is also associated with severe neurological deficits, especially the loss of deep pain perception (DPP) and the duration of its absence in hind limbs (28, 34). The naturally occurring disease is a crucial disease for translational medicine to human.

The purpose of this study is to report the results of the use of neurogenically-induced autologous bone marrow-derived mesenchymal stem cells (NIBM-MSCs) for naturally occurred neurogenically-induced autologous bone marrow-derived mesenchymal stem cells (NIBM-MSCs) for naturally occurred spinal cord injury secondary to thoracolumbar disk extrusion causing paraplegia without DPP.

MATERIAL and METHODS

Client owned companion dogs with spinal cord injury located between T10- L4 vertebrae (the canine spinal cord has seven lumbar vertebra and terminates at the L5 vertebra) secondary to IVD extrusion and paraplegia without DPP which is equivalent to thoracic ASIA (American Spinal Injury Association) ‘A’ for human patients were included in this study prospectively. The study protocol was approved by Ankara University ethical committee (2011-15-395), and informed consents were signed by the owners of each dog. In all cases, IVDD were diagnosed by MRI and were evaluated by electrophysiology. Dogs were underwent surgical treatment (hemilaminectomy, or hemilaminectomy and discectomy) following the diagnosis. Hemilaminectomy was carried out to do sequestrectomy (retrieving extruded disk materials from epidural space) and discectomy was carried out to remove degenerated nucleus pulposus from the intervertebral disc space. Bone marrow specimens were collected during operation in 6 dogs and before starting to physical therapy in one dog to produce stem cells. All dogs were treated by physical therapy following the surgery. All dogs were examined neurologically, no improvement detected dogs for at least 42 days after the initial injury (which is equivalent to about 6 months after injury in human patients) (12, 28) were included. This time period required for producing NIBM-MSCs was 42 days. In addition to aforementioned criteria, the cases followed at least 6 months were included in this study.

Neurological Evaluation

Texas spinal cord injury score (TSCIS) was conducted to evaluate the neurologic score at the admitted time and also outcomes. The gait score: no voluntary movement seen when the dog is supported score “0”, intact limb protraction with no ground clearance “1”, intact limb protraction with inconsistent ground clearance “2”, intact protraction with ground clearance >75% of steps “3”, ambulatory with consistent ground clearance and significant paresis-ataxia that results in occasional falling “4”, ambulatory with consistent ground clearance and mild paresis-ataxia that does not result in falling “5” and normal gait “6”. Pelvic limb proprioception score: absent “0”, delay “1” (correction occurred >1 second after positioning), and present “2”. Pelvic nociception score: absent “0”, deep nociception only present “1”, or both deep and superficial nociception present “2” (21).

Magnetic Resonance Imaging

All of the MRI and electrophysiological procedures were carried out under medetomidine hydrochloride (40 µg/kg i.m., Pfizer, USA) and ketamine hydrochloride (5 mg/kg, Interhas, Turkey). The images of all cases were obtained with an MRI unit (Superconducting magnet, field strength of 1.5 tesla, Siemens AG, Germany), using spinal coil. T1 weighted images (TR: 370-700 ms, TE: 15-20 ms) and T2 weighted images (TR: 2000-4000 ms, TE: 90-110 ms) were adjusted as 2 mm slice thickness. Gadolinium diethylene-triaminepentaacetic acid (Magnevist, Bayer®, Istanbul, Turkey) was used as the paramagnetic contrast medium, and was intravenously administered at a dose of 0.2 mmole/kg.

Electrophysiological Examination

Somatosensory evoked potentials were studied under the same general anesthesia protocol. Briefly, tibial nerve was
suspension was plated in tissue culture flasks which were disaggregated by accutase (Gibco, NY, USA) and the cell At the second step, the acquired neurospheres were stained to confirm positivity to Nestin (Santa Cruz). The resulting neurospheres were histologically and 10 ng/ml basic fibroblast growth factor (bFGF) (Millipore, MA, USA). The resulting neurospheres were histologically and 10 ng/ml basic fibroblast growth factor (bFGF) (Millipore) for further induction into the neurogenic lineage. At ~80% confluence, the cells were removed from the surface by 0.05% Trypsin/0.53 mM EDTA solution (Sigma), washed thrice with PBS, and 1 ml of cell suspension was transferred into a syringe and delivered to the surgery room within ~30 minutes. Immunohistochemistry revealed that these cells were positive to CNPase and MAP-2, as well as to GFAP and beta III tubulin (Abcam).

**Isolation and Culture of BM-MSCs**

Bone marrow was collected from the iliac crest and transported to the laboratory within an hour. BM-MSCs were cultured according to standard methods (4, 27). Under sterile conditions, ~5-10 ml of marrow suspended in α-MEM (Hyclone, Uppsala, Sweden) containing 1% penicillin–streptomycin (Pen/Strep) and 2 mM L-glutamine (L-Glu; Sigma, St. Louis, MO, USA) was washed thrice in 0.1 M PBS with sequential centrifugation (at 25°C and 1000 rpm). Subsequently, the cells were seeded into tissue culture flasks containing α-MEM supplemented with Pen/Strep, L-Glu, and 10% fetal bovine serum (Lonza, Basel, Switzerland) at 37°C with humidified 5% CO₂. The non-adherent cells were removed by replacing the medium on the second day of subculture. The cells were cultured up to passage 2 under the same conditions, with medium changes every other day. At this point, ~5.0 x10⁶ BM-MSCs were separated and induced into the neurogenic lineage. The remaining BM-MSCs were cultured likewise for surgical treatments revealed disk extrusion at different locations (Figure 1, 2), as seen in Table I and it was also accompanied with type 2 disk disease in four cases. One case which had been operated before for type one disk extrusion and failed to improve had epidural fibrosis at the

**In Vitro Neurogenic Differentiation of BM-MSCs**

BM-MSCs were induced into the neurogenic lineage by a two-step differentiation protocol (22). Briefly, cells were placed in tissue culture flasks coated with poly(hydroxyethyl methacrylate) (Sigma) and cultured in the B-27 and N-2 containing neurobasal medium (MesenPro™ with 10% MesenPro™ supplement, Pen/Strep, and L-Glu; Invitrogen), supplemented with 100 ng/ml epidermal growth factor (EGF) and 10 ng/ml basic fibroblast growth factor (bFGF) (Millipore, MA, USA). The resulting neurospheres were histologically stained to confirm positivity to Nestin (Santa Cruz).

At the second step, the acquired neurospheres were disaggregated by accutase (Gibco, NY, USA) and the cell suspension was plated in tissue culture flasks which were coated with poly(ornithine) (Sigma). The neurosphere-derived cell suspension was then cultured for six days in the neurobasal medium supplemented with 100 ng/ml nerve growth factor (NGF) and 10 ng/ml brain-derived neurotrophic factor (BDNF) (Millipore) for further induction into the neurogenic lineage. Under general anesthesia as provided for taking MRI, about 5.0x10⁶ NIBM-MSCs suspended in 1.0 ml of PBS were injected through the hemilaminectomy defect intraspinally at three different points. The right location of the needle tip was verified by the appearance of the cerebrospinal fluid coming from the needle, and directed into the spinal cord parenchyma. The cell transplantation procedure (~5.0x10⁶ NIBM-MSCs) was repeated after 21-days.

**Physical Therapy**

Active range of motion, massage to the hindlimbs, warming thoracolumbar spine, forcing the dog to stand and walk its hindlimbs with bellyband was proposed to the owners up to the processing collected bone marrow and also after transplantation.

**RESULTS**

Seven cases were matched with case selection criteria. Six cases were admitted for the first time (1-2 days), and one case that was operated 4 months before, but neurological improvement not attained were included in this study. The overall data are presented in Table I. The mean age at presentation was 5.79 years (range, 2.5-13 years). Breeds included poodle (n=2), pekingese (n=2), French bulldog (n=2) and mixed breed (n=1). Sex dispersion was 5 males and 2 females. The time from the beginning of clinical signs up to the admission was about 3 days in 5 cases, progressive paraparesis for 6 months and resulted in paraplegia for 1 week in one case, no progression in neurological status in one case which underwent surgery 4 months previously (case no:3 in Table I). MRI were taken from all dogs at the day of clinical examination, and they were operated the day after the admission in 6 dogs. One dog was not operated because of any signs of compression at the operated area or adjacent sites, and he was treated by physical therapy after bone marrow collection.

The MRI of the dogs which were presented at the first time for surgical treatments revealed disk extrusion at different locations (Figure 1, 2), as seen in Table I and it was also accompanied with type 2 disk disease in four cases. One case which had been operated before for type one disk extrusion and failed to improve had epidural fibrosis at the
**Table I:** Detailed Data of the Cases

<table>
<thead>
<tr>
<th>Case No</th>
<th>Signalment of the Dog</th>
<th>TSCIS at the Admission Time</th>
<th>X-Ray</th>
<th>MRI</th>
<th>Conventional Treatments</th>
<th>TSCIS &amp; SEP/MEP Before Cell Therapy</th>
<th>TSCIS 2nd Month</th>
<th>TSCIS 4th Month</th>
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<tr>
<td></td>
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<tr>
<td>1</td>
<td>13 y MI mixed breed progressive paraparesis for 6 months and paraplegia for 1 week</td>
<td>G0, P0, N0; Sd (4) Sp (0); Mp (-) Md (+)</td>
<td>T13-L1 radiopacity at the intervertebral foramen</td>
<td>T13-L1Lt</td>
<td>L1-L2</td>
<td>+</td>
<td>Hemilam + discectomy at the T13-L1 and L1-2</td>
<td>G0, P0, N0; Sd (4) Sp (0); Mp (-) Md (+)</td>
</tr>
<tr>
<td>2</td>
<td>6 y MI Pekingese, Acute onset paraplegia for 1 day</td>
<td>G0, P0, N0; Sd (4) Sp (0); Mp (-) Md (+)</td>
<td>unremarkable</td>
<td>T12-13Lt</td>
<td>L1-2Lt</td>
<td>+</td>
<td>+</td>
<td>Hemilam at both levels</td>
</tr>
<tr>
<td>3</td>
<td>10 y MI French Bulldog operated 4 months before for IVDD</td>
<td>G2, P0, N0; Sd (4) Sp (0); Mp (-) Md (+)</td>
<td>unremarkable</td>
<td>T13-L1Lt</td>
<td></td>
<td>+</td>
<td>T13-L1 fibrosis at the Lt hemilam defect</td>
<td>Physical therapy</td>
</tr>
<tr>
<td>4</td>
<td>3.5 y FI Poodle, Acute onset Paraplegia for 2 days</td>
<td>G0, P0, N0; Sd (4) Sp (0); Mp (-) Md (+)</td>
<td>narrowing T13-L1 IVD space</td>
<td>T13-L1Rt.</td>
<td>L1-L2</td>
<td></td>
<td></td>
<td>Rt Hemilam</td>
</tr>
<tr>
<td>5</td>
<td>3 y MI Pekingese, Acute onset paraplegia for 3 days</td>
<td>G1, P0, N0; Sd (5) Sp (1); Mp (-) Md (+)</td>
<td>unremarkable</td>
<td>T13-1Lt</td>
<td>T12-13Lt</td>
<td>L1-L2</td>
<td></td>
<td>Hemilam; discectomy at the 3rd IVD space</td>
</tr>
<tr>
<td>6</td>
<td>2.5 y FI French bulldog: Acute onset paraplegia for 4 days</td>
<td>G0, P0, N0; Sd (4) Sp (1); Mp (-) Md (+)</td>
<td>unremarkable</td>
<td>L2-3Lt</td>
<td>L1-2Lt</td>
<td>L3-4Lt</td>
<td>+</td>
<td>Mini hemilam + discectomy</td>
</tr>
<tr>
<td>7</td>
<td>2.5 y MI Poodle Acute onset paraplegia for 5 days</td>
<td>G0, P0, N0; Sd (2) Sp (0); Mp (-) Md (+)</td>
<td>unremarkable</td>
<td>L3-4Rt</td>
<td>L1-2T12-13</td>
<td></td>
<td>+</td>
<td>Hemilam, discectomy</td>
</tr>
</tbody>
</table>

**Abbreviations:** G: Gait score, P: Proprioception, N: Nociception, Sd: SEP distal, Sp: SEP proximal, Mp: MEP proximal, Md: MEP distal, Rt: Right, Lt: Left, MI: Male intact, FI: Female intact, Hemilam: Hemilaminectomy, y: years, Type 1: disk extrusion, Type 2: Disk protrusion or bulging.
hemilaminectomy defect. All cases had hyperintense lesions in T2 w images in the spinal cord.

The gait score before treatment was 0 in 5 cases, 2 in 1 case and 1 in one case. Proprioception and nociception score were 0 in all cases. After the surgical and physical treatment (n=6), and just physical treatments (n=1) for 42 days, which is the time period for cell processing; gait score had improved in 1 case as 3 (0 to 3), and 2 (0 to 2) scores in 1 case, and the rest were at the same score. Proprioception score was not changed in any case, however nociception had improved one score in 2 of the cases.

Two months after cell transplantation, there were no changes except for 1 gait score improvement for 1 of the cases. At the 4th month, gait score had improved one score in 5 cases, and 1 score progress was recorded in proprioception and nociception in one case. In eight months-followed up 4 cases were evaluated by the same parameter; gait score had improved in 3 cases (one score in 1, two scores in 1, and three score in 1), and proprioception improved 1 and 2 scores for each in 2 cases, and nociception improved in 3 cases as one score in 2 cases and two scores in 1 case.

SEP and MEP: The details of the results are introduced in Table I. One case showed improvement in both SEP and MEP as normal, and one case improved in MEP after cell transplantation.

**DISCUSSION**

Overall, the results of this study show some beneficial effect of intraspinal injection of autologous NIBM-MSCs in dogs with paraplegia without DPP secondary to thoracolumbar IVD extrusion. The prognosis of the dogs in this grade is poorest and they are equivalent to thoracal ASIA “A” in humans (23). Even though spontaneous improvement in this grade has been reported, they are at the lowest level among the spinal cord injuries in canine practice, and the hopeless cases have been selected for this study consciously. Small gains are acceptable for those kind of dogs as very crucial. To the best of our knowledge, this is the first study evaluating the beneficial effects of autologous NIBM-MSCs in chronic and unfavorable prognosis spinal cord injury after IVDD in dogs. The results of this study may also have potential contribution for translation to human thoracic spinal cord injury.

Bone marrow-derived stem cells can differentiate into glial cells or neurons that may improve the effects of regenerative therapy in spinal cord injury (26, 33). The best therapeutic outcomes can be achieved with optimal dosage, timing and their administration route (36). As MSCs can be expanded and differentiated into the neurogenic lineage through neurospheres *in vitro*, before being transplanted back, they may prove to be more beneficial for treating SCI (10). Because of the hopeless results of spinal cord injury, the best method possible as inferred from the experimental studies has been applied to this cohort of clinical cases.

Usage of MSCs has been examined in many studies for acute or chronic injury models of the spinal cord. In both models, MSCs...
have been shown to promote significant functional recovery (15, 37, 39). The percutaneous transplantation technique (19) preferred in this study, is minimally invasive, reliable, and easily applicable and does not require advanced level of experience. Especially in case of non-obesity, the hemilaminectomy defect can be felt by palpation as absence of articular processes. However in obese cases, the operated area can be guessed by the presence of scar in skin. Percutaneous insertion of the spinal needle into the subarachnoid space can be confirmed by the leakage of cerebrospinal fluid, and can be progressed into the spinal cord parenchyma. It was found easier than collecting CSF from L5-6 spinal tapping. Intraspinal injection as the cell delivery method was used in all cases presented in this study.

The use of different stem cell types including MSCs from the umbilical cord blood, Wharton’s jelly, adipose and bone marrow (20, 32) as well as embryonic stem cells (24) and oligodendrocytes, Schwann cells (29) and olfactory glial cells (16) have shown distinct rates of success. The spinal cord ependymal layer and periependymal region have become focus of interest in SCI research because of the potential for this region to serve as an endogenous source of neural stem cells to aid in tissue regeneration and repair (26). Many attempts have been made by researchers to clarify the side effects of autologous stem cell transplantation. Even though autologous MSCs transplantation has shown more beneficial effects than that of allogeneic MSCs transplantation, allogeneic MSCs transplantation has also been effective in improving functional recovery following SCI (17, 38). In this study, we have used autologous NIBM-MSCs for their known safety, following collecting, processing and neurogenic differentiation. Main limitation of using autologous NIBM-MSCs was found to be the required period of time and the high cost.

A recent study (31) reports the use of autologous BM-MSCs by administration (after exposing the operated area) into the spinal cord of 4 dogs with chronic spinal cord injury secondary to IVDD, and improvement in 3 dogs after 18 months follow up was noted. Neurological score of those cases are not so different from ours; while we have used the minimal invasive delivery method of autologous NIBM-MSCs, and have obtained some hopeful improvements.

In an experimental canine model of spinal cord injury, it was indicated that the use of neural-induced allogeneic MSC transplantation together with Matrigel revealed functional improvement 1 week after SCI. Findings were explained by neurotrophic effects, including neurotrophin production and decreased inflammation and astrogliosis, as well as increased neuronal extension and regeneration (30). The presented cases in this study had also failed with conventional treatments, and there was no improvement until about 42 days after hemilaminectomy and retrieving extruded disk materials from the epidural space. Because of early beneficial effect of cell therapy which is confirmed in experimental studies, we preferred to transplant the cells 2 times with 21 days interval to sustain this possible effect for a longer period, and follow up longer time for the outcomes. When the clinical and electrophysiological results of these cases recorded before cell transplantation with the 2 months results, there were no remarkable changes. The beneficial effects of cell therapy at the early period in these clinical cases that had chronic spinal cord injuries (more than 45 days) have not been seen. However, the use of BM-MSCs for SCI injury has revealed remyelination and neuroprotection from releasing cytokines in experimentally induced spinal cord injuries in rat models (2, 9). The time required for preparing autologous NIBM-MSCs is relatively long and it is not possible to deliver these cells at the acute stage of spinal cord injury. Solving the time limitation and some additional treatment modalities should be considered to provide a better environment for cells to assure better outcomes in chronic spinal cord injury.

The success rate of thoracolumbar disk disease, absent of DPP cases admitted longer than 24h, was found 10% in a case series (18). In another study for the cases at the same neurologic status; sixty-four of 70 dogs with intervertebral disk herniation had underwent surgery; 9(14%) were euthanized within 3 weeks after surgery (7 related to ascending myelomalacia), 37(58%) regained DPP and the ability to walk, 7(11%) regained the ability to walk without regaining DPP, and 11(17%) remained paraplegic without DPP (28). The previous report’s success rate ranges from 0% to 76%(3, 8, 5, 34).

Comparing recovery rates of different studies is complicated, because of many variables as case selection criteria, the duration of DPP absence, loss of motor function, and the applied treatment modality. When the results of this study are compared with the literature, the rate of success is not dramatically different. However the improvement in nociception and proprioception can be accepted as crucial for carrying out further investigation in this area, in higher number of cases with certain criteria.

In conclusion, percutaneous intraspinal injection of autologous NIBM-MSCs in dogs with paraplegia, lacking DPP after IVDD which has poor prognosis, and failed with conventional therapy provide some clinical benefits. The minimal invasive administration technique applied in this study was found to be simple and a reliable method for cell therapy without any complication.

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