Evaluation of transplantation of autologous bone marrow stromal cells into the cerebrospinal fluid for treatment of chronic spinal cord injury in dogs

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Objective—To evaluate effects of transplantation of bone marrow stromal cells (BMSCs) into the CSF for the treatment of chronic spinal cord injury in dogs that had not responded by 1 month after decompressive surgery.

Animals—23 dogs.

Procedures—Dogs with paraplegia and loss of nociception in the pelvic limbs for at least 1 month after decompressive surgery were assigned to transplantation or control groups. Dogs in the transplantation group received BMSCs injected into the CSF 1 to 3 months after decompressive surgery. Dogs in the control group did not receive additional treatments. Improvements in gait, proprioceptive positioning, and nociception were evaluated by use of the Texas Spinal Cord Injury Scale for ≥ 6 months after BMSC transplantation.

Results—6 of 10 dogs in the transplantation group regained the ability to walk, whereas only 2 of 13 dogs in the control group regained the ability to walk. Scores for the Texas Spinal Cord Injury Scale in the transplantation group were significantly higher than scores in the control group at the endpoint of the study (6 months after BMSC transplantation or after decompressive surgery for the transplantation and control groups, respectively). Only 1 dog (transplantation group) recovered nociception. All dogs from both groups had fecal and urinary incontinence. No complications were observed in relation to BMSC transplantation.

Conclusions and Clinical Relevance—Injection of BMSCs into the CSF caused no complications and could have beneficial effects on pelvic limb locomotion in dogs with chronic spinal cord injuries. (Am J Vet Res 2011;72:1118–1123)
Various kinds of cells have beneficial effects on the promotion of functional recovery and tissue repair in SCI. Cells examined include Schwann cells,12 neural stem cells,13 macrophages,13 olfactory ensheathing cells,14 and BMSCs.15 It has been reported that BMSCs, which comprise approximately 0.125% of the total marrow cells, can be isolated by exploiting their tendency to adhere to tissue culture plastics.16 Results of in vivo and in vitro experiments have indicated that BMSCs include a small number of mesenchymal stem cells.17

Several studies15,16–20 in rodents with experimentally induced contusions have revealed that BMSC transplantation improves functional recovery of animals with acute and chronic SCI. However, investigators in another study21 failed to detect similar benefits. In a study18 conducted by our research group, we found that BMSCs administered into the CSF promoted functional recovery in rats with SCI. Rats injected with BMSCs had much better gait performance than did control rats, with the Basso, Beattie, and Bresnahan score reaching a mean ± SEM of 13.9 ± 3.0 at 5 weeks after SCI in BMSC-injected rats; control rats had a mean score of 10.1 ± 2.1 at 5 weeks after SCI. The BMSCs that were transplanted into the CSF were conveyed via the CSF to the spinal cord (where most BMSCs attached to the spinal cord surface, with a few invading the lesion) and promoted tissue repair. These results indicated that infusion of BMSCs into the CSF could be applied in the murine species. The clinical administration of BMSCs via lumbar puncture to human patients with acute SCI has been reported by our research group.22 However, no studies have been conducted in dogs with regard to BMSC transplantation via lumbar puncture for treatment of paraplegia associated with SCI.

The objective of the study reported here was to determine whether injection of BMSCs into CSF could promote improvement of locomotor function with minimal complications in dogs with chronic SCI. Our hypothesis was that transplantation of autologous BMSCs into the CSF could promote locomotor and sensory function in dogs with chronic spinal cord injury.

Clinical procedures—The diagnosis of thoracolumbar IVDH was made on the basis of myelography and confirmed during decompressive surgery. Dogs were administered atropine (0.05 mg/kg, IV) and diazepam (0.2 mg/kg, IV). Anesthesia was induced with propofol (5.0 mg/kg, IV) and maintained by administration of isoflurane. Physical conditions were continuously monitored via an ECG and measurement of Paco2. Myelography was performed on anesthetized dogs by use of iotrolan (0.45 mL/kg) injected via lumbar puncture. Multiple myelographic views were obtained after the iotrolan injection for evaluation of compressive disk materials located circumferentially around the spinal cord in the affected area.

Decompressive surgery was performed immediately after myelography. Methylpredonisolone sodium succinate (30 mg/kg, IV), cefazolin (30 mg/kg, IV), and lantentyl (3 to 5 mg/kg/h, IV) were administered before surgery. Hemilaminectomy was performed on the side ipsilateral to the spinal cord compression, as determined by the results of myelography. Extruded disk material was removed. Postoperative management included the administration of antimicrobials and analgesics, management of urinary incontinence, and nursing care to prevent decubital ulcers. All dogs were managed in the same way with respect to rehabilitation during the 6-month postoperative period.

One month after decompressive surgery, dogs that were paraplegic with no improvement in locomotion and a lack of nociception in the pelvic limbs were assigned to transplantation (n = 10 dogs) or control (13) groups such that there would be no differences in age, body weight, and site of disk herniation. Power analysis indicated that a sample size of 17 dogs in each group (34 total) would be needed to detect differences of 45% in recovery of motor function with a power of 0.80 and α of 0.05.

Transplantation of BMSCs—Although all owners were informed about BMSC transplantation 1 month after decompressive surgery, the time of owner consent, and hence BMSC transplantation, varied. Therefore, dogs received BMSC transplants between 1 and 3 months after decompressive surgery.24 Control dogs did not receive sham lumbar punctures or injections because the clients did not agree to the sham treatments.

Isolation and culture of BMSCs—The bone marrow of the femur was aspirated by use of a perfusion method described in other studies.24,25 Briefly, 2 sterilized 13-gauge bone marrow biopsy needles23 were inserted into the proximal and distal ends of the femur. The needle in the proximal end of the femur was connected to a syringe that contained 50 mL of physiologic saline (0.9% NaCl) solution with heparin. The needle in the distal end of the femur was connected via an extension tube to a 50-mL syringe to collect bone marrow fluid. The physiologic saline solution was infused gently into the bone marrow cavity to flush bone marrow cells toward the other end of the femur for collection. Extracted bone marrow cells were collected in a syringe. This process was repeated twice for each dog.

Bone marrow perfusate was centrifuged, and pelleted cells were suspended in 15 mL of Dulbecco PBS.
solution.\textsuperscript{a} Cell suspensions were loaded onto a lymphocyte separation solution\textsuperscript{b} (15 mL) and centrifuged at 400 $\times$ g for 30 minutes at 20°C. Theuffy coat at the interface was collected, mixed with 20 mL of Dulbecco PBS solution, and centrifuged again at 300 $\times$ g for 5 minutes. Pelleted cells were washed twice with Dulbecco PBS solution. The bone marrow cells obtained were plated on three 25-cm$^2$ tissue culture flasks\textsuperscript{c} in Dulbecco modified Eagle medium\textsuperscript{d} that contained 10% fetal bovine serum\textsuperscript{e} and 1% antimicrobial-antimycotic solution.\textsuperscript{f} Nonadherent cells were removed by replacing the medium 48 hours after initial plating.

Injection of BMSCs—Autologous BMSCs were injected into the CSF 3 times at 1-week intervals, as described in another study.\textsuperscript{26} Briefly, BMSCs were obtained 1, 2, and 3 weeks after the start of culture; cells were removed from culture plates via incubation with 0.25% trypsin and 1 mM EDTA\textsuperscript{a} at 37°C. The number of cells was counted by use of a hemacytometer. Number of transplanted BMSCs ranged from $0.3 \times 10^6$ cells to $3.0 \times 10^6$ cells (median, $1.3 \times 10^6$ cells) at each time point. The total number of transplanted BMSCs ranged from $1.4 \times 10^6$ cells to $5.6 \times 10^6$ cells (median, $4.7 \times 10^6$ cells). Dogs were treated with atropine and diazepam, and anesthesia was induced with propofol and maintained by administration of isoflurane. A 23-gauge spinal needle was inserted by use of fluoroscopic monitoring into the subarachnoid space between L5 and L6. Intrathecal placement of the needle was ensured by outflow of CSF. The BMSCs were suspended in 0.5 mL of physiologic saline solution and injected into the CSF slowly over a 5-minute period, and then were theoretically conveyed through the CSF to the lesion.

Clinical evaluation and TSCIS—Physical and neurologic examinations of dogs were performed every month for $\geq$ 6 months; the endpoint of the study was 6 months. Time 0 for the control group was the day of the decompressive surgery, whereas time 0 for the transplantation group was the day of the last of the 3 BMSC injections. The referring veterinarians, who were unaware of the treatment group for each dog, video recorded the dogs and evaluated the behaviors and assessed proprioception and nociception. Dogs were identified as serial numbers for the investigators, who also were unaware of the treatment group for each dog. The investigators, who were not the personnel who cultured the BMSCs, also scored the dogs. Successful recovery was defined as a dog that could walk without assistance.

Investigators also evaluated improvement of function by use of the TSCIS.\textsuperscript{27} This system involves evaluation of each pelvic limb separately and has 3 components: gait, proprioceptive positioning, and nociception. All components were based on standard clinical neurologic examination techniques for dogs.\textsuperscript{27} Gait scores for pelvic limbs ranged from 0 to 6, as determined on the basis of clinically relevant movement of the limbs. Dogs were categorized as grade 0 (no voluntary movement), grade 1 (protraction with no ground clearance), grade 2 (protraction with inconsistent ground clearance), grade 3 (protraction with ground clearance in $> 75\%$ of steps), grade 4 (ambulatory with moderate paresis-ataxia), grade 5 (ambulatory with mild paresis-ataxia), or grade 6 (normal gait). Proprioceptive positioning (also referred to as knuckling) was used as a postural reaction test (score of 0 to 2). Lack of a response was assigned a score of 0. Dogs that corrected limb positions after a prolonged period (> 2 seconds) were referred to as a delayed response and assigned a score of 1. Dogs were judged to be clinically normal and assigned a score of 2 if they were able to correct the limb positions immediately. Nociception was assessed by applying a painful stimulus (clamping a hemostat on the distal aspect of a limb or on a nail bed of a digit) and observing the dog for physiologic (tachycardia, tachypnea, and mydriasis) or behavioral (orientation toward the stimulus, vocalization, and licking) responses. Dogs were scored as having normal nociception (score, 2) or no nociception (score, 0). Dogs were assigned scores of 1 to 10 for each pelvic limb; thus, the maximum combined limb score was 20.

Statistical analysis—Distributions of age and body weight of dogs were compared between the 2 groups by use of a Student t test. Recovery rate of locomotor function was analyzed by use of a Fisher exact test. Scores of the TSCIS were compared between the 2 groups by use of a Mann-Whitney U test. Values of $P < 0.05$ were considered significant.

Results

The transplantation group consisted of 10 dogs (3 males and 7 females) that were 4 to 7 years old (median, 4.5 years) and weighed 3.2 to 8.8 kg (median, 5.2 kg). The sites of disk herniation ranged from T10-11 to L1-2 (Figure 1). The control group consisted of 13 dogs (11 males and 2 females) that were 3 to 9 years old (median, 5 years) and weighed 3.3 to 10.0 kg (median, 5.5 kg). The sites of disk herniation ranged from T10-11 to L2-3. There was no significant difference in age ($P = 0.88$) or body weight ($P = 0.77$) between the transplantation and control groups.

![Figure 1—Sites of disk herniation in 23 dogs with thoracolumbar IVDH resulting in paraplegia and loss of pelvic limb nociception that persisted for at least 1 month after decompressive surgery. Dogs were assigned to 2 groups: 10 dogs received autologous bone marrow transplant (black bars) and 13 control dogs that received no additional treatment (white bars). Two dogs in the transplantation group and 3 dogs in the control group had herniated disks at 2 sites.](https://example.com/figure1.png)
Transplantation of BMSCs was performed between 1 and 3 months (mean, 51.5 days) after decompressive surgery. The BMSC group was evaluated for 6 to 35 months (median, 23 months) after BMSC transplantation. The control group was evaluated for 7 to 36 months (median, 24.5 months) after decompressive surgery.

Dogs were paraplegic with loss of nociception in pelvic limbs at the time of BMSC transplantation. Six of 10 dogs in the transplantation group regained the ability to walk, which was significantly (P = 0.037) higher than the proportion of dogs in the control group (2/13) that regained the ability to walk. The 6 dogs in the transplantation group that regained the ability to walk were 4 to 7 years old, whereas the 2 dogs in the control group that regained the ability to walk were 3 and 5 years old. There was no association between age and functional recovery. In the transplantation group, dogs regained the ability to walk 0.5 to 4 months (mean, 2.1 months) after BMSC transplantation. Two of those 6 dogs had further improvement of coordination between the thoracic and pelvic limbs. In contrast, the 2 dogs in the control group regained the ability to walk at 2.5 and 3 months after decompressive surgery; however, they did not have further improvements in coordination between the thoracic and pelvic limbs. There was a significant (P = 0.026) difference in TSCIS score between the transplantation and control groups at the endpoint of the study (6 months after BMSC transplantation or after decompressive surgery for the transplantation and control groups, respectively; Table 1).

All dogs had fecal and urinary incontinence. Only 1 dog (transplantation group) recovered nociception. No complications, including signs of pain, aggravation of neurologic function, or tumorsgenesis, were detected in association with injection of BMSCs for > 6 months after transplantation.

Table 1—Mean (range) TSCIS scores of 23 dogs with thoracolumbar IVDH resulting in paraplegia and loss of pelvic limb nociception that persisted for at least 1 month after decompressive surgery.

<table>
<thead>
<tr>
<th>Time (mo)</th>
<th>Control group*</th>
<th>Transplantation group*</th>
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</thead>
<tbody>
<tr>
<td>0†</td>
<td>0 (0)</td>
<td>0.8 (0–2)</td>
</tr>
<tr>
<td>1</td>
<td>0.5 (0–2)</td>
<td>3.2 (0–8)</td>
</tr>
<tr>
<td>2</td>
<td>1.2 (0–2)</td>
<td>5.0 (2–12)</td>
</tr>
<tr>
<td>3</td>
<td>2.3 (0–6)</td>
<td>5.4 (2–14)</td>
</tr>
<tr>
<td>4</td>
<td>2.3 (0–6)</td>
<td>5.6 (2–14)</td>
</tr>
<tr>
<td>5</td>
<td>2.3 (0–6)</td>
<td>5.6 (2–14)</td>
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<tr>
<td>6‡</td>
<td>2.3 (0–6)</td>
<td>5.6 (2–14)</td>
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</table>

*The control group comprised 13 dogs that received no additional treatment, whereas the transplantation group comprised 10 dogs that received BMSC transplantation 2.3 (0–6) 5.6 (2–14) times at 1-week intervals beginning 1 to 3 months after decompressive surgery. †Time 0 for the control group was the day of the decompressive surgery, whereas time 0 for the transplantation group was the day of the last of the 3 BMSC injections. ‡End point of the study.

To our knowledge, there have been no reports about BMSC transplantation for the treatment of paraplegic dogs with chronic SCI. The study reported here revealed that improvement of locomotor function can be achieved by use of BMSCs injected into the CSF of dogs with chronic SCI. No adverse effects of BMSC transplantation were detected during the study.

In the present study, 1 of 10 dogs that received BMSCs recovered nociception, whereas none of the 13 control dogs recovered nociception. This is consistent with the observation that BMSC transplantation in rats had no effect on sensory function. Although no significant differences in recovery of nociception were detected between the transplantation and control groups, it is possible that use of a larger population may have enhanced our ability to detect whether BMSCs would impact nociceptive recovery. Power analysis indicated that a sample size of 200 dogs in each group (400 dogs total) would be needed to detect differences of 10% in recovery of sensory function (power of 0.80 and α of 0.05). It is clear that a larger cohort would be needed.

It has been reported that the prognosis is poor when nociception does not return within 2 weeks after injury. Therefore, dogs that were paraplegic with no sign of improvement in pelvic limb locomotion and nociception for at least 1 month after decompressive surgery were used in the present study. Two of 13 dogs in the control group regained the ability to walk < 3 months after decompressive surgery. In another study with a population of 64 dogs with IVDH that initially lacked nociception, 9 were euthanatized within 3 weeks after surgery; 37 regained nociception and the ability to walk, and 18 survived but never regained nociception. Of those 18 dogs, 7 eventually regained the ability to walk after decompressive surgery. The interval until those 7 dogs regained the ability to walk ranged from 16 to 72 weeks (mean, 37.6 weeks). The recovery rate for dogs that lived and never regained nociception (7/18) in that study was higher than the recovery rate for the control group of the present study (2/13) and that for dogs that could walk (1/10) in another report. It is difficult to compare recovery rates among studies because of the numerous variables, such as inclusion criteria, type of surgery performed, assessment of nociception, euthanasia, and the endpoint used for the outcome. In the present study, no dogs were euthanatized after surgery. Monitoring the dogs for a longer period may have changed the outcomes in the present study. The most likely explanation for the conflicting findings is a small number of dogs in each study population.

In the present study, all dogs were managed in the same way with respect to rehabilitation. Training on a treadmill, which is a more aggressive form of rehabilitation, enhances the recovery of motor function in rodents with experimentally induced contusions. Cell transplantation plus training on a treadmill may promote more substantial beneficial effects of functional recovery in dogs.

Recovery of locomotor function in dogs without nociception may be a result of development of spinal reflex walking. Spinal reflex walking is believed to be attributable to the central pattern generator of the spinal cord. In one report, dogs walking apparently via spinal reflex became nonambulatory following transection of the spinal cord proximal to the site of the injury, which suggests that ambulation depends, at least in part, on intact axons crossing the region of the
original spinal cord lesion. In the study reported here, 2 dogs in the transplantation group were able to walk and had coordination of the thoracic and pelvic limbs, which suggested some contribution of axonal connections within the injured spinal cord. The preservation of as few as 5% to 10% of the descending axons is adequate to drive the local circuits responsible for basic locomotion.32

Various techniques, including evaluation of motor-evoked potentials and magnetic resonance imaging,33,34 have been used to objectivley assess SCI. Magnetic resonance imaging and evaluation of sensory-evoked potentials can be performed in lightly sedated dogs, but the obtained variables do not necessarily reflect actual functional improvement.34–36 Therefore, direct functional evaluation is considered more practical and useful. The TSCIS is a method for assessment of gait, proprioceptive positioning, and nociception. The TSCIS score has been associated with magnetic resonance signal characteristics within the spinal cord of dogs with disk herniation, and inter-raiter agreement is high.26 In the present study, the TSCIS was used for evaluation of functional improvement. However, a technique for computerized gait analysis in dogs has been reported.37 This method may be used to detect more subtle changes in gait than would the TSCIS and could provide a continuous, rather than an ordinal, measure.

Mesenchymal stem cells are readily obtained from the bone marrow by aspiration; they can then be cultured to large quantities and used as autologous transplants for the treatment of SCI. They have no harmful immunologic reactions. Currently, they are considered to be one of the cells most useful for the clinical treatment of SCI.16 Other cellular treatments are currently being investigated and may hold equal or greater promise.

Transplantation of BMSCs improves motor function in rats with acute and chronic paraplegia.15,19,20,38 Bone marrow stromal cells could differentiate into neurons and glial cells following transplantation and promote motor function.20,38 In another study18 conducted by our research group in rats with SCI, BMSCs administered into the CSF were conveyed through the CSF to the spinal cord (where most BMSCs attached to the spinal cord near the surface, with a few invading the lesion) and promoted tissue repair and functional recovery. However, BMSCs on the spinal cord as well as within the lesion disappeared by 3 weeks after injection.18 Bone marrow stromal cells did not differentiate into neural cells in the host tissue as determined by results of immunohistochemical analysis.19 Bone marrow stromal cells protect and repair damaged neurons in vitro via production of growth factors.39 These facts suggest that the effects of BMSCs may be caused, at least in part, by trophic factors released from BMSCs that rescue neuronal tissue from degeneration and promote axonal regeneration. Identifying compounds that rescue neuronal tissue from degeneration and promote motor function.20,38 In another study18 conducted by our research group in rats with SCI, BMSCs administered into the CSF were conveyed through the CSF to the spinal cord (where most BMSCs attached to the spinal cord near the surface, with a few invading the lesion) and promoted tissue repair and functional recovery. However, BMSCs on the spinal cord as well as within the lesion disappeared by 3 weeks after injection.18 Bone marrow stromal cells did not differentiate into neural cells in the host tissue as determined by results of immunohistochemical analysis.19 Bone marrow stromal cells protect and repair damaged neurons in vitro via production of growth factors.39 These facts suggest that the effects of BMSCs may be caused, at least in part, by trophic factors released from BMSCs that rescue neuronal tissue from degeneration and promote axonal regeneration. Identifying compounds that rescue neuronal tissue from degeneration and promote motor function.20,38 In another study18 conducted by our research group in rats with SCI, BMSCs administered into the CSF were conveyed through the CSF to the spinal cord (where most BMSCs attached to the spinal cord near the surface, with a few invading the lesion) and promoted tissue repair and functional recovery. However, BMSCs on the spinal cord as well as within the lesion disappeared by 3 weeks after injection.18 Bone marrow stromal cells did not differentiate into neural cells in the host tissue as determined by results of immunohistochemical analysis.19 Bone marrow stromal cells protect and repair damaged neurons in vitro via production of growth factors.39 These facts suggest that the effects of BMSCs may be caused, at least in part, by trophic factors released from BMSCs that rescue neuronal tissue from degeneration and promote axonal regeneration. Identifying compounds that rescue neuronal tissue from degeneration and promote motor function.

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