Functional Recovery of Spinal Cord Injury Following Application of Intralesional Bone Marrow Mononuclear Cells Embedded in Polymer Scaffold – Two Year Follow-up in a Canine

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Abstract

Background: Bone marrow derived pluripotent stem cells hold a great promise for therapeutic repair of injured central nervous system. This report is on a six-month old paraplegic Boxer breed canine with traumatic spinal cord injury at the level of T12, which functionally recovered following intrallesional transplantation of autologous Bone Marrow Mono Nuclear Cells (BMMNCs) seeded on a Thermoreversible gelation polymer (TGP) combined with intravenous Cell Transplantation.

Materials and Methods: Thirty ml of Bone Marrow was aspirated and BMMNCs were isolated. From the total BMMNCs isolated, 20 x 10⁶ cells were seeded in 1.5 ml of TGP and implanted at the site of injured spinal cord. A fraction of BMMNCs isolated were stored at -80deg C from which 4.16 x 10⁶ BMMNCs were thawed and transfused intravenously by suspending in 2ml saline on the 19th post-operative day. The animal was followed up by assessment every two weeks for a period of two years.

Results: Recovery of motor and sensory functions were noticed on the 53rd day, attempt for standing on the 79th day and ambulation on the 98th day after the initial cell transplantation. The animal had satisfactory ambulation on the 133rd day and thereafter the life style of the animal was gradually restored to normalcy. Status quo of this recovery has been maintained for the past two years.

Conclusion: The outcome proves the safety of intrallesional transplantation of autologous BMMNCs embedded in TGP in spinal cord injury and makes us recommend the same for more number of similar cases.

Keywords: Bone Marrow Stem cells; Canine Diseases; Cell Transplantation; Spinal Cord Injuries; Thermoreversible Gelation Polymer (TGP)

Abbreviations: TGP- Thermoreversible Gelation Polymer; SCI- Spinal Cord Injury; BMSC- Bone Marrow Stromal Cells; BMMNCs- Bone Marrow Mono Nuclear Cells; LAL- Limulus Amebocyte Lysate; FITC- Fluorescein Isothiocyanate; SEP- Somatosensory Evoked Potential; HSC- Hematopoietic Stem Cells; MSC- Mesenchymal Stem Cells

Introduction

Regenerative potential of central nervous system is limited [1] and treatment of traumatic Spinal Cord Injury (SCI) in canines continues to be a challenging task. SCI leads to severe functional impairment like paraplegia, quadriplegia and tetraplegia with upper or lower motor neuronal deficits and causes severe distress, devastating changes in quality and life expectancy of the animal, with a frustrating situation to the pet owners. Functional deficits following SCI result from interruption in axonal tracts or damage to axons, loss of neurons, oligodendrocytes, astrocytes, endothelial cells, precursor cells and demyelination [2]. With the initial mechanical insult to the spinal cord, SCI also leads to a series of secondary cascades like ischemia, anoxia and free-radical formation, which impede regeneration of axons due to the release of myelin associated inhibitory proteins, extracellular matrix-derived inhibitory cues and glial scar formation [3,4]. The key elements of repair of SCI require not only the neural cell proliferation and survival, but also the promotion of axonal growth, remyelination and neosynaptogenesis [5]. The treatment attempts on spinal stabilization by surgical procedures mainly aid to restore the anatomical integrity of the damaged vertebrae and prevent the secondary cascade of events without any therapeutic potential for spinal cord regeneration [6]. Stem cell-based transplantation therapies are being attempted as the current regenerative pathway for the treatment of spinal cord lesions and several animal experiments as well as clinical trials are being reported to promote neuronal regeneration and improve spinal cord function [7-9]. Bone Marrow Stromal Cells...
Traumatic SCI in canines. Intralesional and intravenously can aid in functional recovery of transplantation of autologous BMMNC seeded with TGP applied two-year follow-up that along with decompressive surgical procedure, traumatic SCI in canines is limited. Here, we report our results after a seeded in TGP and intravenous administration of BMMNCs for injury was classified as per Denny and Butterworth [23] as Grade 5 localized between T10-13 vertebrae. The clinical severity of the neural spinal reflexes was indicative of upper motor neuron lesion and was Anal sphincter reflex was intact. Neurological examination of the above right side and absence on the left side at T11 vertebra and caudal to it. reflex was normal up to the level of T10 vertebra with a decrease on the deep pain reflex, conscious proprioception of mictiruition and defecation was noticed and the vital signs were on palpation at caudal thoracic region. Distended bladder with absence be comfortable in sternal recumbency posture and showed severe pain when it was on a loose-leash walk on the road. The animal was found to function. The canine was brought four days after an automobile accident to our hospital for treatment of paraplegia with total loss of motor and (BMSCs) and Bone Marrow Mononuclear Cells (BMMNCs) in animal model studies of SCI have been found to replace white and grey matter, neuronal and axonal regeneration, astrocyte proliferation, myelination, neovascularisation and functional improvement which presents an encouraging scope for clinical translation [10,11]. Further engraftment of stem cells with biomaterial scaffolds provides a promising strategy for engineering diseased tissues and cellular delivery. Numerous previous studies have used a variety of natural (e.g., collagen, fibrin, chitosan, agarose, and alginates) and synthetic (e.g., Poly (lactic-co-glycolic acid), Poly (ethylene glycol), poly (N-isopropylacrylamide-co-n-butyl methacrylate), copper capillary alginate gel polymers for repair of damaged spinal cord or brain [12-16]. Thermo reversible gelation polymer (TGP), a temperature-dependant visco elastic synthetic scaffold has been reported to promote in vitro 3D culture of cells and tissues in hydrogel state at 37°C and also aid the tissue regeneration process by activation of stem cells and prevention of the inflammatory process [17]. Several in vitro and animal model studies also demonstrate that TGP promoted regeneration of damaged tissues like pancreas [18], liver [19], cornea [20], and neural tissues [21]. Very recent studies have reported that surgical transplantation of TGP constructed bone marrow-derived stem cells enhance the engraftment of donor cells onto the cerebral infarct of mouse neocortex [21,22].

However, knowledge on intralesional application of BMMNC seeded in TGP and intravenous administration of BMMNCs for traumatic SCI in canines is limited. Here, we report our results after a two-year follow-up that along with decompressive surgical procedure, transplantation of autologous BMMNC seeded with TGP applied intralesionally and intravenously can aid in functional recovery of traumatic SCI in canines.

The case report

Patient history: A six month old, congenitally deaf, intact male Boxer cross-bred canine with body weight of 15 kilograms was brought to our hospital for treatment of paraplegia with total loss of motor and sensory functions of the hind limbs and that of bladder and bowel function. The canine was brought four days after an automobile accident when it was on a loose-leash walk on the road. The animal was found to be comfortable in sternal recumbency posture and showed severe pain on palpation at caudal thoracic region. Distended bladder with absence of micturition and defecation was noticed and the vital signs were within the clinical limits. Deep pain reflex, conscious proprioception reflex, patellar reflex of the hind limbs were absent and the panniculus reflex was normal up to the level of T10 vertebra with a decrease on the right side and absence on the left side at T11 vertebra and caudal to it. Anal sphincter reflex was intact. Neurological examination of the above spinal reflexes was indicative of upper motor neuron lesion and was localized between T10-13 vertebrae. The clinical severity of the neural injury was classified as per Denny and Butterworth [23] as Grade 5 paraplegia, and the pelvic limb function, according to Olby et al. [24] scoring system, was scored as stage 1 and point 0 condition (Figure 1A). The owner was counseled and an informed consent was obtained to perform the decompressive surgery with autologous bone marrow stem cell therapy. Approval for the study was obtained from the ethics committee of the Madras Veterinary College in which the study was conducted. All procedures were accomplished in accordance with the national and international Guidelines for Care and Use of animals in scientific research. The canine was induced anaesthesia with propofol at the dose of 4.5 mg/kg B.W. and maintained under isoflurane as inhalant agent throughout the procedures which were performed on the same day.

Localization of lesion: Plain radiography followed by myelography using Iohexol (Omnipaque, 350 mgI/ml @ 0.3 ml/kg body weight intracisternally) revealed compression fracture of the 12th thoracic vertebra and abrupt stoppage of the contrast column cranial to 12th thoracic vertebra. (Figure 1B)

Bone marrow Aspiration: Right femur was prepared and using Jamshidi needle, 30 ml of bone marrow was aspirated under C-arm guidance (Figure 1C). The bone marrow was preserved in a bag containing citrate dextrose anticoagulant and transported in cold chain storage (4° to 8° C) to a Central cell processing facility.

Processing of BMMNCs & Preparation of TGP Construct: The aspirate was processed under cGMP SOP’s Class 100 clean room and class 100 bio-safety hood. BMMNCs were isolated using Ficoll gradient method and were counted using Neubaur’s hemocytometer. From the total quantity of BMMNCs isolated, 20 x 106 cells were seeded in 1.5 ml of thermoreversible gelation polymer(TGP) which is a copolymer composed of thermoresponsive polymer block [poly(N-isopropylacrylamide-co-n-butyl methacrylate)](poly(NIPAAm-co-BMA)) and the
hydrophilic polymer block (polyethylene glycol [PEG]). A fraction of the cells was preserved in – 80°C.

**Quality control testing:** Before seeding the cells in the TGP, the cells were subjected to Flowcytometry analysis for quantifying the CD34+/CD45- cells by appropriate fluorescein isothiocyanate (FITC) antibodies (Becton Dickinson, Jan Jose, USA) and analyzing data’s using BD Cell quest pro software. The Endotoxin test was also carried out using Limulus Amebocyte Lysate (LAL) Kit method for confirming the sterility before cell transplantation.

**Hemilaminectomy and Intralesional Engraftment:** Left hemilaminectomy was performed as per the technique described by Wheeler and Sharp [25] and durotomy at T12 vertebra and the injured spinal cord was exposed. The injured site was oedematous and durotomy revealed blood clots (Figure 1D). The construct of 1.5 ml TGP seeded with 20 x 10^6 BMMNCs was engrafted in liquid phase at the site of injured spinal cord and within a few minutes the construct became solidified. The laminectomy defect was overlaid with fat graft harvested from the subcutaneous tissue and the surgical site was closed in a routine fashion. No stainless steel metallic implants were used for internal fixation of the vertebral fracture due to the concern on post-operative MRI evaluation.

**Intravenous Transfusion:** On the 19th postoperative day, 4.16 x 10^6 BMMNCs were thawed from previously stored BMMNCs and suspended in 2 ml of normal saline and transfused intravenously.

Post-operative management included antibiotics, analgesics, bladder management, nursing care to prevent decubital ulcers, cage rest for six weeks and passive range of motion- physiotherapy. Motor and sensory functions were evaluated every two weeks post-operatively and Olby scoring system [24] was used for quantitative evaluation of functional outcome in this study.

**Results**

Post-operatively after intralesional BMMNC transplantation no toxic and adverse effects were noticed. The canine was monitored continuously for the first 72 hours and no alterations in vital sign parameters were recorded. The animal was discharged from the hospital on the fifth day and the owners were explained thoroughly on the follow-up care and management strategies. On the 14th day, seroma formation at the surgical site was noticed and the serous fluid was tapped out. Also, slight deep pain sensation and tail wagging were noticed. Towel sling exercise was advised to be followed until the recovery of unassisted standing and on the 16th day, a slight dorsal elevation of the vertebra at the surgical site was noticed. It indicated a slight disruption in the vertebral alignment. In order to potentiate the efficacy of cell transplantation as reported earlier [26] a second dose was administered on the 19th day by intravenous route and no adverse reactions were noticed following the same. Neuromagnetic examination on the 28th day revealed a return of deep pain sensation in its right hind limb and a slight sluggish movement in the left hind limb. Involuntary movement of the right hind limb and absence of movement in the left hind limb was noticed. Defecation was reported to be normal and bladder function was maintained by manual evacuation by the owner. On the 42nd day, improvement in deep pain, patellar reflex, involuntary movement and conscious proprioception reflex was noticed in both the hind limbs and panniculus reflex was noticed caudal to T12 vertebrae. Transient unassisted standing on the hind limbs was noticed on the 53rd day (Figure 1E). The animal was encouraged to move freely and it made transient self attempts to stand by itself on the 79th day. Thereafter, the animal made attempts to stand and walk with in-coordinated hind limb movements. It made ambulation with in-coordinated left hind limb movement on the 98th day (Figure 1F) and on the 133rd day unassisted standing, normal ambulation and ability for prolonged walking resumed and a normal life style of the animal was fully restored. Long-term follow up on the 180th day confirmed that the animal continued its normal life style with normal pelvic gait movements with no recurrence of neurological disorders. Subsequently, the animal was followed up periodically and the last follow up was at the end of two years since the first cell transplantation, which confirmed the status quo of all the improvements up to the restoration of normalcy. The Olby scores of pelvic limb status from the postoperative period to the recovery time are listed in Table 1.

**Discussion**

In canines, the treatment strategies for complete recovery from traumatic spinal cord injury are still under research. Till date, irrespective of the type of strategy followed, treatment for severe SCI remains to be unresolved. The current clinical and animal studies on treatment of SCI aim to inhibit the secondary inflammatory and degenerative changes and to augment the neural regeneration. Reports reveal that stem cells transplanted into the injured lesion differentiate into oligodendrocytes and astrocytes, integrate into axonal pathways and regenerate and remyelinate the injured axons [27-30]. BMMNCs of autologous origin offer advantages of multi-potency with definitive in- vivo and in- vitro neuronal differentiation, avoidance of immunological and ethical issues. In murine model studies, BMSCs after transplantation are demonstrated to migrate and attach with the injured neural tissue and the cells finally disappeared within three weeks indicating the release of some trophic factors from BMSCs to rescue neurons and glial cells from degeneration and to stimulate differentiation of neural stem cells in the injured spinal cord tissue [6,31,32] Similar mechanisms are expected on intralesional and intravenous route of administration of BMMNCs where the cells could possibly migrate to injured spinal cord tissue and repair the damaged tissue as reported earlier [26]. Though BMSCs present an attractive strategy, their purification and expansion is quite a cumbersome process. In contrast, BMMNC isolation is relatively easy. Importantly, BMMNC contains different cell fractions.

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Table 1: Assessment of functional outcome based on pelvic limb function by Olby score.

<table>
<thead>
<tr>
<th>Neurological status</th>
<th>Olby Score</th>
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<tbody>
<tr>
<td>Postoperative period in days</td>
<td>Stage</td>
</tr>
<tr>
<td>0</td>
<td>Absence of deep pain and pelvic limb movement</td>
</tr>
<tr>
<td>14</td>
<td>Presence of tail wagging and absence of pelvic limb movement</td>
</tr>
<tr>
<td>28</td>
<td>Non weight bearing and involuntary movement of the right hind limb</td>
</tr>
<tr>
<td>42</td>
<td>Non weight bearing and involuntary movement of both hind limbs</td>
</tr>
<tr>
<td>53</td>
<td>Transient unassisted standing</td>
</tr>
<tr>
<td>79</td>
<td>Frequent attempts on unassisted standing</td>
</tr>
<tr>
<td>98</td>
<td>Ambulation with incoordinated left hind limb movement</td>
</tr>
<tr>
<td>133</td>
<td>Ataxic pelvic limb gait with normal strength</td>
</tr>
<tr>
<td>180</td>
<td>Normal pelvic limb gait</td>
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including CD34+ hematopoietic stem cells (HSC), mesenchymal stem cells (MSCs) and endothelial progenitors. In principle, organogenesis or tissue regeneration by any type of cell therapy should go hand in hand with angiogenesis, where the tissue building process as it progresses should be supported by blood supply for successful regeneration of the damaged or dysfunctional organ. Studies have proven that the application of whole BM-MNCs is more successful than methods which use subfractionated cell preparations [33]. In a study when transplantation of human BM-MSCs and BM-MNCs into rats with SCI was compared, it was observed that BM-MNCs did not give rise to mature immune cells after transplantation which is a common issue concerned with allogeneic BM-MNC transplantation. There was no increased host immune response or tissue loss when compared with BMSC-transplanted animals. In contrast there was an increased host macrophage/microglia response after BMSC transplantation which the authors attributed to exposure of cells to serum-containing media. The efficacy of BMSCs and BM-MNCs were found to be similar in that study [11]. In our study since the animal is alive, we are unable to show the post-transplantation pathology of the spinal cord, which is a limitation. As per the evidences pointed out earlier, the application of BM-MNCs is justified as they are relatively safe, easy to obtain and have proven efficacy in treating SCI. The temperature-dependent solid and liquid phase properties of TGP scaffold material helped to engraff the stem cells in gel phase of TGP over the injured spinal cord area exposed through the laminectomy defect and after the solidification, the TGP – stem cell construct was observed to be intact without loss of structural stability. Further, TGP could have favoured neuronal and oligodendrocyte differentiation of residual neural stem cells and the BM-MNCs. Post-operatively, during recovery period, slight dorsal elevation of the thoracic vertebral secondary to malignment of the injured vertebrae was noticed. This could be due to the lack of adequate epaxial muscle support following surgical trauma, inadequate muscle strength and support of the hind limbs to maintain the posture and absence of internal fixation at T12 vertebral segment and these reasons could also be attributed to the delayed recovery. In this case study, assessment of the severity of SCI by somatosensory evoked potential (SEP) values and its correlation with the functional outcome was not carried out and it is reported that the functional scoring system was found to be more sensitive than SEP measurements [34]. For the quantitative assessment of the functional outcome of the Canine SCI based on the pelvic limb function, Olby scoring system [24] was used and the reliability of the same has been confirmed by previous reports [35,36]. During the post-operative follow up period, when the canine was left on Marble and Granite floors which were slippery, the animal had difficulty in initiating attempts to stand by itself which could also be attributed to the delay in recovery of pelvic limb functions. Later, covering the floor with carpet or non-slippery floor conditions helped the canine to make more progressive attempts to stand on its own. In canines, interestingly, few cases have been reported to be able to walk following severe spinal injury, but they fail to regain deep pain and continence due to higher input from higher center mediated through a few intact axons surviving across the lesion and is termed as spinal reflex walk. These canines always remained incontinent and the recovery of walking ability occurred only after four months [36]. In this case study, a full functional recovery was noticed after three months and the recovery has been sustaining for more than two years. These results indicate that a combination of surgical decompression with intrathecal transplantation of BM-MNCs seeded in TGP followed by an intravenous injection could produce a functional recovery of injured spinal cord in canines. However, the fate of the transplanted cells remains to be investigated in the regeneration of the spinal cord after an injury.

Conclusion

Our clinical study revealed that the intrathecal implantation of autologous BM-MNCs seeded in a TGP followed by an intravenous injection into a canine was safe without any complications following the treatment for two years. The functional recovery could be due to the beneficial effects of BM-MNCs combined with the decompressive procedures as they might have helped to hasten neurological recovery, which otherwise could not have been expected in this short-span, going by the earlier reports. The factors determining the outcome could be the age of the canine, severity of the injury, time interval between the injury and the cell transplantation, mode of transplantation, role and utility of TGP scaffold and the dosage of the stem cells transplanted, all of which need to be evaluated elaborately. Further in-vitro and in-vivo studies are needed to clarify the mechanisms of action of BM-MNC on neuronal regeneration to confirm the above results. Though safety of the procedure has been proven in this case, a larger study is warranted to ascertain the efficacy.

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References


