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

Justin Benjamin William1, Rajamanickam Prabakaran1, Subbu Ayyappan1, Haridass Pushkhiraj1, Dhananjaya Rao2, Sadananda Rao Manjunath2, Paramasivam Thamaraikannan1, Vidyasagar Devaprasad Dedepiya1, Satoshi Kuroda3, Hiroshi Yoshioka4, Yuichi Morii4, Senthilkumar Preethy2,5 and Samuel JK Abraham2,6*

1Madras Veterinary College, Chennai, India
2Nichi-In Centre for Regenerative Medicine, Chennai, India
3Department of Neurosurgery, Hokkaido University- Graduate School of Medicine, Sapporo, Japan
4Waseda University, Tokyo, Japan
5Hope Foundation (Trust), Chennai, India
6Yamanashi University- Faculty of Medicine, Chuo, Japan

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 intralesional 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 -80°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 intralesional 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 Mononuclear 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
(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, agaroze, and alginites) and synthetic (e.g., Poly (lactic-co-glycolic acid), Poly (ethylene glycol), poly (N-isopropylacrylamido-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-dependent visco elastic synthetic scaffold has been reported to promote in-vitro 3D culture of cells and tissues in hydrogel state at 37ºC 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 intraslesional 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 intraslesionaly 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 mictiruition 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 right side and absence on the left side at T11 vertebra and caudal to it. 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 (4º to 8º C) to a Central cell processing facility.

**Processing of BMMNCs & Preparation of TGP Construct:** The aspirate was processed under cGMP SOP’s Class 10000 clean room and class 100 bio-safety hood. BMMNCs were isolated using Ficoll gradient method and were counted using Neubaur’s haemocytometer. 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 thermoreponsive polymer block [poly(N-isopropylacrylamide-co-n-butyl methacrylate)] and the

---

**Figure 1:** Caption: Pre-operative, Intra and Post-operative Images of the Canine with spinal cord injury treated with autologous Bone marrow stem cells. Legend: (A) Pre-operative Grade IV Paraplegia, (B) Pre-operative Myelogram in which the 12th vertebal compression can be visualized (See arrow), (C) C-Arm image of Bone Marrow Harvesting;the arrow indicates tip of the Jamshidi needle inside the Bone Marrow, (D) intra-operative image showing exposed spinal cord after hemilaminectomy in which the spinal cord is observed to be odematous and bulged out with intact duramater, (E) Post-operative Day 53 on which transient unassisted standing on the hind limbs by the canine was observed (F) Post-operative Day 98. The Canine could move around with incoordinated left hind limb movements.
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 fluorescent 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 10⁶ 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 10⁸ 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. Towl 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. Neurological 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

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

| Postoperative period in days | Neurological status | Olby Score Score | Stage |
|-----------------------------|---------------------|-----------------|-------|
| 0                           | Absence of deep pain and pelvic limb movement | 0 | 1 |
| 14                          | Presence of tail wagging and absence of pelvic limb movement | 2 | 1 |
| 28                          | Non weight bearing and involuntary movement of the right hind limb | 3 | 2 |
| 42                          | Non weight bearing and involuntary movement of both the hind limbs | 4 | 2 |
| 53                          | Transient unassisted standing | 6 | 3 |
| 79                          | Frequent attempts on unassisted standing | 7 | 3 |
| 98                          | Ambulation with incoordinated left hind limb movement | 10 | 4 |
| 133                         | Ataxic pelvic limb gait with normal strength | 13 | 5 |
| 180                         | Normal pelvic limb gait | 14 | 5 |
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 BMMNCs is more successful than methods which use sub fractionated cell preparations [33]. In a study when transplantation of human BMSCs and BMMNCs into rats with SCI was compared, it was observed that BMMNCs did not give rise to mature immune cells after transplantation which is a common issue concerned with allogeneic BMNMC 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 BMMNCs 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 pathologic of the spinal cord, which is a limitation. As per the evidences pointed out earlier, the application of BMMNCs is justified as they are relatively safe, easy to obtain and have proven efficacy in treating SCI. The temperature-dependant solid and liquid phase properties of TGP scaffold material helped to engraf 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 BMMNCs. Post-operatively, during recovery period, slight dorsal elevation of the thoracic vertebrae secondary to malalignment 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 intraslesional transplantation of BMMNCs 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 intraslesional implantation of autologous BMMNCs 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 BMMNCs combined with the decompressive procedures as they might have helped to hasten neuroregenerative 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 BMMNC 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.

Acknowledgements

The authors acknowledge M/S Hope Foundation (Trust), Chennai, India, for funding the study and Mr. V. Sampathkumar for technical assistance.

References

1. Schultz SS (2005) Adult stem cell application in spinal cord injury. Curr Drug Targets 6: 63-73.
2. Horky LL, Galimi F, Gage FH, Horner PJ (2006) Fate of endogenous stem/ progenitor cells following spinal cord injury. J Comp Neurol 498: 525-538.
3. Song HJ, Stevens CF, Gage FH (2002) Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. Nat Neurosci 5: 438-445.
4. Jones LS, Margolis RU, Tuszyński MH (2003) The chondroitin sulfate proteoglycans neurocan, brevican, phosphacan, and versican are differentially regulated following spinal cord injury. Exp Neurol 182: 399-411.
5. Okano H (2003) Making and repairing the mammalian brain: Introduction. Semin Cell Dev Biol 14: 159.
6. Saito F, Nakatani T, Iwase M, Maeda Y, Hirakawa A, et al . (2008) Spinal cord injury treatment with intrathecal autologous bone marrow stromal cell transplantation: the first clinical trial case report. J Trauma 64: 53-59.
7. Enomoto M, Wakabayashi Y, Qi ML, Shinomiya K (2004) Present situation and future aspects of spinal cord regeneration. J Orthop Sci 9: 108-112.
8. Pluchino S, Zanotti L, Deleidi M, Martino G (2005) Neural stem cells and their use as therapeutic tool in neurological disorders. Brain Res Brain Res Rev 48: 211-219
9. Parr AM, Kulbitski I, Zahir T, Wang X, Yue C, et al. (2008) Transplanted adult spinal cord-derived neural stem/progenitor cells promote early functional recovery after spinal cord injury. Neuroscience 155: 760-770.
10. Ohta M, Suzuki Y, Noda T, Ejiri Y, Dezawa M, et al. (2004) Bone marrow stromal cells infused into the cerebrospinal fluid promote functional recovery of the injured rat spinal cord with reduced cavity formation. Exp Neurol 187: 266-278.
11. Samdani AF, Paul C, Betz RR, Fischer I, Neuhaber B (2009) Transplantation of human marrow stromal cells and mono-nuclear bone marrow cells into the injured spinal cord: a comparative study. Spine (Phila Pa 1976) 34: 2605-2612.
12. Teng YD, Lavik EB, Qu X, Park KI, Övednick J, et al. (2002) Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. Proc Natl Acad Sci U S A 99: 3024-3029.
13. Jain A, Kim YT, McKeon RJ, Bellamkonda RV (2006) In situ gelling hydrogels for conformal repair of spinal cord defects, and local delivery of BDNF after spinal cord injury. Biomaterials 27: 497-504.
channels stimulate and guide linear axonal growth following spinal cord injury.

Stokols S, Tuszynski MH (2006) Freeze-dried agarose scaffolds with uniaxial surgery. (4th edn), Blackwell Science.

Injuries. Am J Vet Res 62: 1624-1628.

Development of a functional scoring system in dogs with acute spinal cord disorders. Diagnosis and surgery. London: Mosby-Wolfe.

Stromal Cell Transplantation for Central Nervous System Disorders – Recent progress and perspective for clinical application. J Stem Cell Regen Med 6: 10-14.

Biomaterials 27: 443-451.

Therapeutic efficiency of treatments for injuries to the spinal cord in animals. Vet Rec 155: 225-230.

Irradiation of Intralesional Bone Marrow Mononuclear Cells Embedded in Polymer Scaffold – Two Year Follow-up in a Canine. J Stem Cell Res Ther 1: 110. doi:10.4172/2157-7633.1000110

Bai H, Suzuki Y, Noda T, Wu S, Kataoka K, et al. (2003) Dissemination and proliferation of neural stem cells on the spinal cord by injection into the fourth ventricle of the rat: a method for cell transplantation. J Neurosci Methods 124: 181-187.

Sitalakshmi S, Kataoka K, Medina R, Muñana K, Sharp N, et al. (2004) Recovery of pelvic limb function in dogs following acute intervertebral disc herniations. J Neurotrauma 21: 49-59.

Peterson-Hansen AW, Howard MJ (2002) Repairing the damaged spinal cord: a summary of our early success with embryonic stem cell transplantation and remyelination. Prog Brain Res 137: 299-309.

Okita M, Suzuki Y, Noda T, Ejiri Y, Dezawa M, et al. (2004) Bone marrow stromal cells infused into the cerebrospinal fluid promote functional recovery of the injured rat spinal cord with reduced cavity formation. Exp Neurol 187: 266-278.

Lawall H, Bramlage P, Arnann B (2010) Stem cell and progenitor cell therapy in peripheral artery disease. A critical appraisal. Thromb Haemost 103: 696-709.

Olby NJ, Harris T, Burr J, Muñana K, Sharp N, et al. (2004) Recovery of pelvic limb function in dogs following acute intervertebral disc herniations. J Neurotrauma 21: 49-59.

Webb AA, Jeffery ND, Olby NJ, Muir GD (2004) Behavioural analysis of the efficacy of treatments for injuries to the spinal cord in animals. Vet Rec 155: 225-230.