Establishment and Use of Injectable Human Embryonic Stem Cells for Clinical Application

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Spinal cord injury (SCI) is a devastating and challenging neurological ailment that affects millions across the world[1]. Generally caused by sports, car accidents, tumors, falls, or infection, SCI leads to paralysis and loss of sensory and motor functions and sometimes is accompanied by urinary, cardiac, and respiratory dysfunctions. SCI is associated with permanent disability and decreased life expectancy. The poor regenerative capacity of the adult spinal cord results in severe sensory and motor deficits. Presently, there is no cure. It impacts the patient physically, psychologically, and socially and financially.

A systemic review estimated the incidence rate of traumatic SCI in Asia as ranging from 12.06 to 61.6 per million in people between the ages of 26 and 56. A recent review of SCI epidemiology in developing countries reported the incidence to be 25.5 million cases per year (Rahimi-Movaghar et al. 2013). In India alone, approximately 1.5 million

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live with SCI, and 10,000 new cases are added each year (Gupta et al. 2008). The neurological recovery in patients with traumatic SCI, as evaluated by the American Spinal Injury Association (ASIA) is low (6–13%), and only about 2.1% of these patients have been reported as gaining any functional strength (Kirshblum et al. 2004). According to ASIA, the severity of an injury is categorized as either complete or incomplete: a complete injury is the complete absence of sensory and motor function below the level of injury. If there are some preserved motor and sensory functions below the level of injury, the case is diagnosed as incomplete SCI (Grossman et al. 2012; Dalbayrak et al. 2015).

Human embryonic stem cells (hESCs) are self-renewing cells with a potential to differentiate into all types of human cells. hESCs are able to replicate indefinitely, differentiate into all three primary germ layers cell lines, and are karyotypically stable (Erceg et al. 2008; Keirstead et al. 2005; Ware et al. 2014; Shroff 2005).

These cells have the potential for cell replacement and regeneration therapies for human diseases. hESCs were derived and characterized as early as 1982 from fresh or frozen cleavage-stage donated human embryos produced by in vitro fertilization (IVF) (Shroff et al. 2014). The viable cell lines were obtained from the inner cell mass or blastocyst. hESCs have also been derived and established from single blastomeres of the four- or eight-celled embryo and 16-celled morula. Since then, a plethora of research has indicated that hESCs can be used for various diseases like diabetes; liver, autoimmune, and immune disorders; Parkinson's disease; Alzheimer's disease; age-related macular degeneration; and SCI.

Despite its great potential in treating clinical conditions such as SCI, hESCs have not been used extensively in humans. This is largely due to the technology which advocates hESC lines and a lack of knowledge about their use. Furthermore, hESC cell lines have shown chromosomal and genomic instabilities, with acquisition of loss of heterozygosity or copy-number variation in cancer-related genes. hESCs have also been associated with teratoma formation and fear of being immunologically rejected. There have been safety concerns and challenges in the use of hESCs. The use of animal feeder cells leads to cross contamination. There is a risk of xenogeneic pathogen cross-transfer and other unknown substances capable of eliciting a detrimental immune response

in transplanted hosts. There are ethical issues which center around the repeated need for blastocyst to create the cell lines.

In this chapter, I will describe the establishment and transplantation of injectable hESCs for chronic SCI. I also will illustrate the working of hESC through case-study data and discuss how this unique therapeutic platform has achieved significant success in treating chronic SCI.

Literature

Several studies in the past two decades have researched cell-based therapies for SCI. The replacement of damaged neural tissues and reestablishing connections between the central and peripheral nervous systems are vital for SCI treatment strategies, and cells with a potential of self-renewal and the ability to differentiate into multiple cell types would be best suited for SCI patients. Park et al. (2005) performed autologous bone marrow cell transplantation at the injury site in conjunction with the administration of granulocyte-macrophage colony-stimulating factor in five patients with complete SCI and followed up for 6–18 months. Overall, three patients improved from ASIA scale A to C, one improved from ASIA scale A to B, and one did not show any improvement. None of the patients, however, showed any serious complications. Lima et al. (2010) transplanted olfactory mucosa autografts in seven patients ranging from 18 to 32 years of age and with an ASIA scale of A. Every patient had improved ASIA scales, and two of the patients had moved to ASIA scale C by the end of treatment. In another study, a 37-year-old female SCI patient was transplanted with HLA-matched human cord blood cells at the site of injury. Investigators observed improved sensory perception and movement in the hips and thighs 41 days after the transplantation. Regeneration of the spinal cord at the injured site was observed in the computed tomography and MRI scan. Kang et al. (2005) recommended that hESCs' transplantation protocols should encourage the use of human material, as animal components carry a risk of xenogeneic pathogen cross transfer.

A Phase I hESC human clinical trial was approved by the FDA in 2009. Popularly referred to as the Geron trial, it was abandoned midway

due to financial constraints (Lukovic et al. 2014). The cells in the Geron trial also contained animal components such as the B27 supplement or Matrigel. Asterias Biotherapeutics have bought the rights to Geron to conduct a human clinical trial, approved by the FDA (Leuty 2014). Ocata is also developing hESC-based therapies for various disorders; initial results in patients with macular degeneration have been promising. A number of studies have been conducted in animal models to observe the capabilities of stem cells in improving the motor functions in SCI patients. In transplanted placenta-derived mesenchymal stem cells to rat models with SCI, these rats showed significant improvements in their motor and hind-limb functions after three weeks of study. The Basso, Beattie, and Bresnahan scale also shifted from 2 to 13 within three weeks of treatment (Sharp et al. 2010). Kerr et al. (2003) studied the human pluripotent stem cell derivatives transplanted to rat models to observe the improvement in the motor functions and observed significant improvements in hind-limb locomotion as compared with controlled animals in 12 weeks of study.

Sharp et al. used hESC-derived oligodendrocyte progenitors in adult cervical contusion rat models and observed that transplanted hESC-derived OPCs survived even after the nine-week study period. Rossi et al. (2010) observed that transplanted animals had an improved functional outcome with an early recovery rate of balance and coordination and skilled forelimb movement when human motor neuron progenitor cells derived from hESCs were transplanted in rats with SCI. In addition to the evaluation of stem cells in animal models, early-phase clinical trials regarding the efficacy of stem cells in SCI yielded mixed results. Yoon et al. (2007) conducted a non-randomized Phase I/II clinical trial to treat SCI with transplantation of bone marrow cells and observed a significant change in the ASIA scores of patients in the acute and sub-acute treatment groups although no improvement was observed in the chronic treatment group. In Mackay-Sim et al.'s (2008) three-year clinical trial of 12 paraplegic patients, olfactory ensheathing cells (OECs) were transplanted through multiple routes and no changes were made to ASIA scores, and no patients experienced improvements to neurological and functional levels. Lima et al. performed a pilot scale clinical study in seven patients with chronic SCI. They transplanted the OEC to the SCI

patients through surgical mode. The authors found significant changes in the MRI observations and ASIA score. The bowel and bladder movement of patients were also improved.

We have previously reported improvements in some of our SCI patients with SCI after undergoing hESC therapy (Shroff and Gupta 2015). In another study, we have reported improvement in bowel and bladder sensation and control (Shroff 2015c). Patients with acute SCI were not included, to rule out the natural recovery of disease. All the patients in our study had SCI for more than one year and had not benefitted from any other treatment. Bretzner and his colleagues (2011) state that chronic SCI patients are more acceptable for hESC-derived transplantations as compared to acute patients, as the former are 'less likely to suffer opportunity costs from study participation', an important ethical consideration when 'knowledge value', not 'therapeutic benefit', motivates research.

The standard treatment for chronic SCI includes high doses of steroids (methylprednisolone) and immunosuppressants. The mild therapeutic effect of methylprednisolone is associated with a number of other side effects (Willerth and Sakiyama-Elbert 2008; Bracken et al. 1990; Bracken 2012). Hugenholtz states there are no evidence-based standards regarding the use of high doses of methylprednisolone for SCI treatment (Kirshblum et al. 2011), and surgery for SCI patients does not show any improvement between treated and non-treated patients (Ronaghi et al. 2010). Thus, very few or negligible treatment options are available.

The Breakthrough

Since 2000, I have researched and developed a unique in-house patented technology to culture and maintain hESCs in our GMP-, GLP-, and GTP-compliant laboratory. In particular, I have studied very small stem cells (VSELSCs) of pre-blastomeric origin derived from a two-celled stage fertilized egg. These cells (0.7–1.5 μm) known as blastomeric-like express pluripotency genes and differentiate into cell types from all the germ layers.

These cells were taken from a fertilized ovum discarded during a regular IVF cycle, with full donor consent. The cell-culture technique pro-

duces an hESC line free from animal products, feeder layers, growth factors, leukemia inhibitory factor, supplementary mineral combinations, amino acid supplements, fibroblast growth factor, membrane-associated steel factor, soluble steel factor, and conditioned media (US Granted Patent No US 8592, 208, 52). The hESC line was characterized for its pluripotent nature, its differentiation into neuronal lineages, and its use for the treatment of neurological disorders. We have characterized the hESC at the molecular, cellular, and functional levels using scanning electron microscopy, transmission electron microscopy, confocal microscopy, reverse transcriptase polymerase chain reaction, and flow-cytometry analysis. The cell line has also been characterized based on its long-term proliferation and maintenance, karyotyping, and in vivo differentiation as teratoma formation assay. It has been chromosomally stable since the year 2000 and for >4000 passages. The study also provides a composition of injectable stem cells in a ready-to-inject form that is simple to prepare and safe, cost effective, efficient, easily transportable, scalable, and with a shelf life of greater than 6 months.

The safety and efficacy of the cell line has been established (Shroff et al. 2015b). We have used these cells to treat over 1500 patients with diverse ailments including diabetes, myocardial infarction, cerebral palsy, SCI, Lyme disease, spinocerebellar ataxia, Friedrich's ataxia, Alzheimer's disease, Parkinson's disease, autism, and cerebral palsy (Shroff et al. 2014, 2015, 2015d; Shroff 2015). No teratoma formation has been observed. The hESCs were obtained from a one-time harvest at the pre-blastomeric stage. The cell line thus developed was created from a single expendable fertilized ovum 24 hours after fertilization. With no animal products in the media. We have developed a simplified cell-culture system free of exogenous cells and supplements of animal origin for expansion of hESCs in a substantially undifferentiated state.

The study was approved by an independent ethics committee. The study was conducted in accordance with the Declaration of Helsinki in a GCP-compliant condition. Each patient provided verbal, written, and video consents; the ethics committee approved this process. In addition, the cells are cultured and maintained per our in-house patented technology (United States Granted Patent No US 8592, 208, 52) in our labora-

tory certified as being compliant with good manufacturing, good laboratory, and good tissue practices.

Patient data was validated by Moody's International (document number NH-heSC-10–1), GVK Biosciences (NM-Hesc-10–1, 18 November 2010), and Quality Austria Central Asia (document number QACA/OCT/2013/26). These companies were allowed to examine the medical and statistical data at the institute and were able to meet the patients.

Case Studies

Here, I present our data on paraplegic and quadriplegic patients treated with hESCs. All the patients were scored on the basis of a scale developed by ASIA before and after treatment (ASIA/IMSOP, 1996). After the treatment, all three patients showed significant improvement in their sitting balance, bowel and bladder control and sensation, and power and movement of lower and upper limbs. No adverse events were reported. The treatment strategy was divided into four phases. In the first, T1 (eight weeks for paraplegics, 12 for quadriplegics), 0.25 mL (<4 million cells) hESCs were administered intramuscularly twice daily to 'prime' the body and to prevent the recipient's immune system from rejecting the stem cells. Every 10 days, 1 mL hESCs (<16 million cells) were administered intravenously to 'home in on' the required area. Every 7 days, 1–5 ml were administered via any of supplemental routes (brachial plexus block, intrathecal, caudal, epidural, popliteal block, and/or deep spinal muscle and epidural catheter) to introduce the stem cells as closely to the injured site as possible (local action). After a gap period of 4–8 months, successive phases like T2 (four to six weeks) and T3 (four to six weeks) used the T1 dosage regime; treatment was repeated annually if needed. This protocol was developed on the basis of a pilot study conducted on 72 patients that found the extension of a treatment period more than eight weeks in paraplegics and more than 12 in quadriplegics do not lead to better results. A gap of 4 months between the subsequent treatment phases was determined to allow the injected hESCs to develop into mature cells and regenerate the affected parts. T2 and T3 treatment periods were incorporated to add more cells into the body, thus allowing more repair and

regeneration. Biochemical and radiological investigations were completed before the start of the treatment and at regular intervals. In-house physicians and nurses carefully observed patients for antigenic or anaphylatic responses.

Patient 1

An Australian patient with quadriplegia for 14 years was admitted to Nutech Mediworld on 8 March 2008. Patient history revealed he had suffered a major trauma to the neck while playing rugby, which resulted in injury to the spine at the C1 and later receded to the C2 level. His investigator assigned an ASIA score of an A.

At the time of admission, the patient was unable to move his upper and lower limbs and suffered from a complete loss of sensation except on his face. He was on ventilator support with tracheostomy at 17 breaths per minute, and speech was co-incident with the ventilator. He had no sitting balance, and the plantar and abdominal reflexes were absent with an exaggerated ankle jerk. His lower limb had clonus, and he had no deep sensation. In addition to having no bladder or bowel control, he needed three full-time caretakers and could not eat more than one meal a day. Magnetic resonance imaging tractography showed the visualization of nerve fibers/tracts in the upper cervical cord from the cervicomedullary junction caudally up to the C2; cord fibers were not discerned up to the D1 (Fig. 1).

The patient underwent four sessions of hESC therapy. After his treatments, the patient was weaned off his ventilator and was able to remain from it for up to 12 hours. He was able to freely move his neck, shrug his shoulders, and show movement of his arms and hands. His sitting balance improved significantly, and he could stand with a chest orthosis and Hip Knee Ankle Foot Orthosis. His deep sensation was increased up to the abdomen. His post-treatment ASIA score was a C, and his last follow-up was 8 November 2013 (Fig. 2).

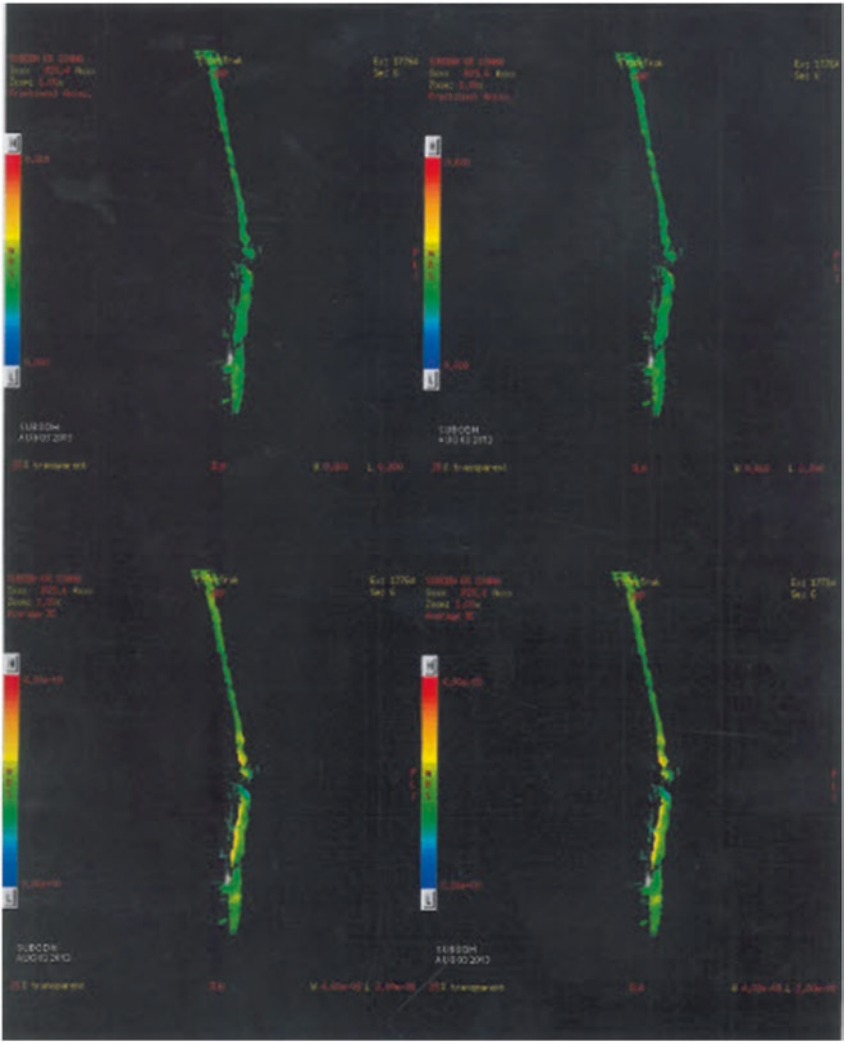

Fig. 1 Trachtography

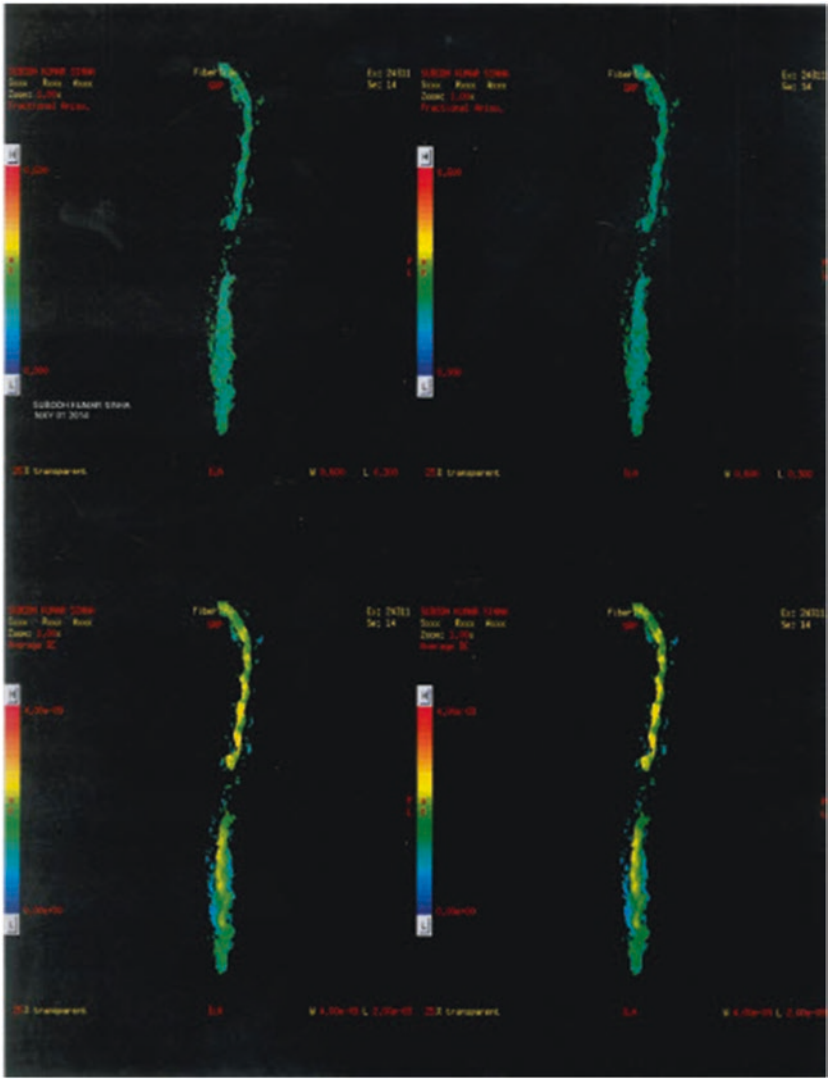

Fig. 2 Patient 1 after treatment

Patient 2

A 28-year-old Indian male was admitted to Nutech on 8 October 2011, with complaints of loss of bowel and bladder control, a left lower limb paralyzed with intact sensation, and right lower-limb movements with no sensation. The patient had had an accident in October 2010, sustaining D11 and D12 fractures and a right ulnar fracture. He underwent surgical procedure with instrumentation at the D9-L1 level and after surgery had regular sessions of physiotherapy and occupational therapy, which helped improve motor functions in the right lower limb and sensation in the left lower limb. He was able to lift and hold his right lower limb and could hop on crutches but was unable to walk. His MRI showed a fracture of the D11 vertebral body with anterior subluxation of D10 over D11, compression fracture of D12 vertebral body, decreased spinal size at D11 due to fracture, subluxation with indentation of the cord at D10-D11, and minimal extradural hematoma at D10–D11. Cord contusion was noted at the D10–D11 level, and a D11 and laminar fracture was seen on the right side. His ASIA score was an A (Fig. 3).

The patient underwent two sessions of hESC therapy. After the first treatment, he showed improvement in bladder and bowel sensation with partial control, improvement in sensation in his left and right lower limbs (he could bend his knees and hold that position). He showed a decrease in clonus and jerks in the lower limb and in calf pain. He was able to walk with Ankle Foot Orthosis calipers and walkers and could climb stairs with little help.

After a gap of more than two years, the patient had another treatment. During therapy, he was also given supplements, including calcium (Calcitriol 500 mg × OD), 15 units of B-complex, ferrous fumarate and folic acid OD, cholecalciferol sachets (one per week), and nectra powder. This treatment protocol resulted in improved lower-limb strength, improved left lower-limb sensation at the foot-planar and dorsal aspects, and improved calf and gait with a quadripod stick and bars. The right thigh and toe movements were stronger than before. His bowel and bladder control improved with full voiding and evacuation sensation. The patient's last follow-up was 30 October 2013.

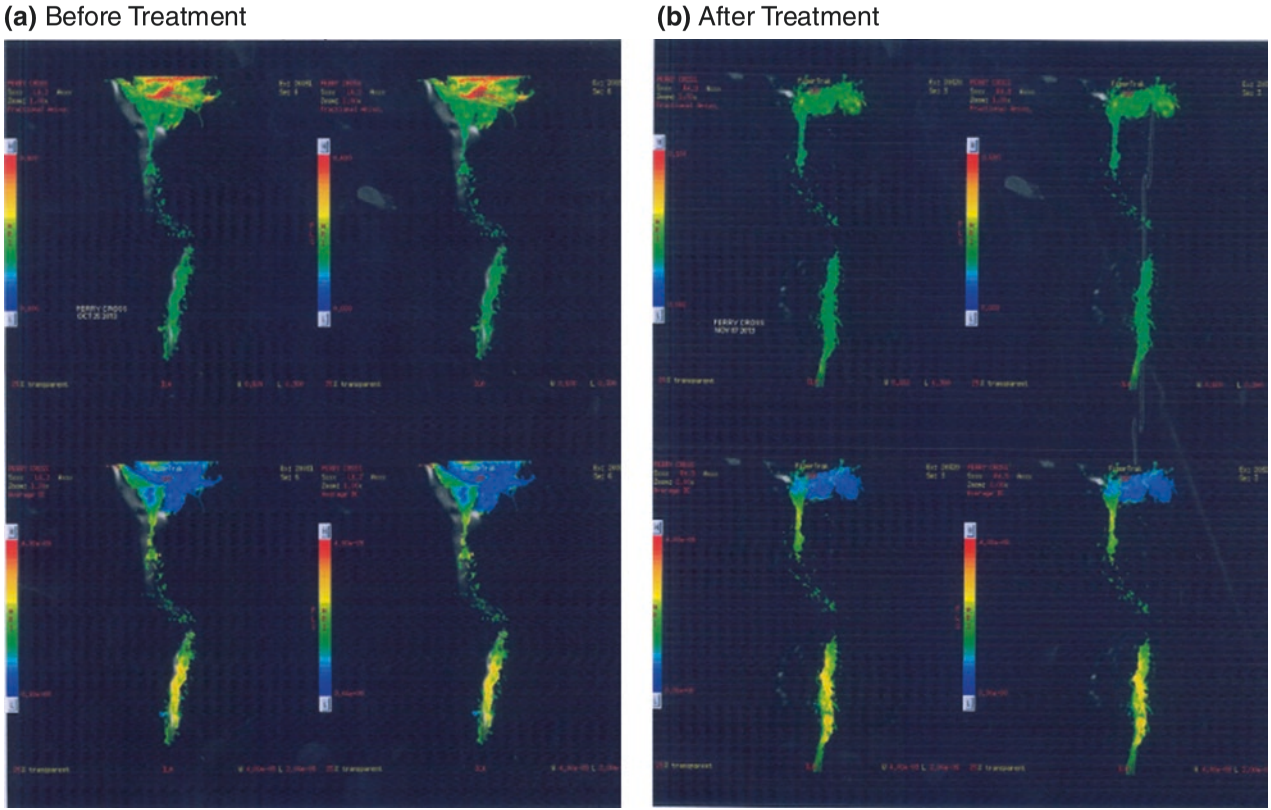

Fig. 3 Tractography images of Patient 2 before and after treatment

Patient 3

A 36-year-old female from the USA was admitted on 1 February 2010. She had been healthy until 2007, when she had an accident, sustaining injury at C6-C7. She underwent surgical fixation of C5-T1 with rods. She had been wheelchair bound on a quad support since that time, and her MRI revealed that a spiral fracture of the right humerus was surgically fixed with titanium plates and screws. She also had no movement of the B/L lower limb, slight movement in the B/L upper limb, absence of sensation below the sternum, no bowel or bladder sensation or control, pain from waist to toe, neuralgic pain below her waist, the inability to grasp any object, poor sitting balance, inability to walk, and dizziness on sitting up. On examination, she had claw hand, her thumb movement was absent from the right hand and weak in the left, and she had no hand function, a weak grip, and had poor pelvic control in the quadruped position. Her ASIA score was an A.

The patient was treated with hESC along with extensive physiotherapy and occupational therapy. She underwent six sessions of hESC therapy with a gap phase between sessions varying from 4–11 months, with her last follow-up on 23 December 2013. Following the treatment, she showed improvement in upper-limb muscle strength, contraction of B/L hips and knees, and an improvement in posture, with good sitting balance. The patient had developed a good standing balance with calipers, good grasping with her right hand, good thumb movement in both hands, and a good pinch and release. She was able to write, make a near-normal fist, had developed sensation until B/L knees and at the sole of the foot, and had appreciable extension in both knees; she also could stand for long durations and even take steps with the Knee Ankle Foot caliper and binder. She also showed improvement in bladder sensation (could hold the speed of voiding) and bowel sensation (pushed while passing motions). She was able to crawl independently and stand from a sitting position while holding onto bars. Her post-treatment ASIA score was a B.

We can assume the hESCs in our study may have followed the pattern of homing in at the injured sites and regenerating the affected regions, as discussed previously. We cannot rule out the possibility that in our study,

the hESCs may have differentiated into neurons and helped in tissue repair at the site of injury in the spinal cord and regenerated the cells for improvement in the functioning of the spinal cord.

Dramatic improvements in the health of all three patients we studied were observed following the treatment with hESC in our study. None of the patients developed teratomas and were not given any immunosuppressants. hESCs could be an effective and safe therapeutic option for treatment of patients with SCI. However, there is a need to conduct clinical trials on large number of patients with SCI to determine the safety and efficacy of this treatment.

A Retrospective Study

Nutech Mediworld has evaluated the efficacy and safety of hESC therapy in 226 patients with SCI (Shroff 2016). In the first treatment phase (T1), 0.25 mL hESCs were administered intramuscularly twice daily (1 mL every 10 days intravenously and 1–5 mL every 7 days). Of the 153 patients on the ASIA scale A at the beginning of T1, a significant number of patients (n = 80; 52.3%) had moved to lower scales at the end of T1 (p = 0.01). At the end of T2, of the 32 patients on ASIA scale A, 12 patients (37.5%) had moved to scale B (p = 0.01). Of 19 patients, three (37.5%) had moved to scale B at the end of T3 (p = 0.02). No serious adverse events were observed.

Study Design

The data of a single cohort of SCI patients treated with hESCs conducted from 24 May 2005 to 31 August 2012, at a single site in New Delhi, India, and were collected retrospectively. In the initial two years (2002–2004), the safety of hESC therapy was assessed in 33 patients (not included in this analysis) with various incurable diseases. Thereafter, efficacy of the therapy, dose schedule, and protocol for administration of hESCs and therapy schedules were established in a

pilot study conducted on 72 patients. After that, a study (validated by GVK Biosciences) was conducted on 108 SCI patients verifying the safety and efficacy of hESC in SCI patients (not included in the present analysis). The present study of 226 SCI patients was undertaken after these two studies. The same protocol was followed in the group of patients analyzed in this study.

The study was performed under the proper supervision of a team of physicians that included external consultants and was validated by an external clinical research organization. The patients were scored per ASIA scale30 by independent physicians before and after the treatment and by in-house physicians and the rehabilitation team. After confirmation of diagnosis, patients were tested for hypersensitivity reactions with hESCs (0.05 mL injected subcutaneously). The three treatment phases were separated by gap periods so the hESCs could grow, repair, and regenerate the affected parts.

Patients with a documented diagnosis of SCI elsewhere of fewer than 3 months before the start of therapy were included in this study. All these patients had undergone other treatment(s) such as physiotherapy, occupational therapy, and so forth before coming to our center. Patients with acute SCI were excluded, to rule out results of the body's natural healing abilities. Patients who were pregnant, lactating, or confirmed to have received other forms of cell therapies within the last 12 months of treatment were not accepted. All patients provided written and video informed consents before the treatment began.

Patients then entered the first treatment phase (T1): eight weeks for paraplegics and 12 for quadriplegics, wherein 0.25 mL (<4 million cells) hESCs were administered intramuscularly twice daily to 'prime' the body and prevent the recipient immune system from rejecting the stem cells. In addition, every 10 days 1 mL hESCs (<16 million cells) were administered intravenously to home in on the required area and for systemic reach. To introduce the stem cells as closely to the injured site as possible (local action), 1–5 mL hESCs (depending on the route of administration) were administered every 5 to 7 days by any of the supplemental routes (Fig. 4).

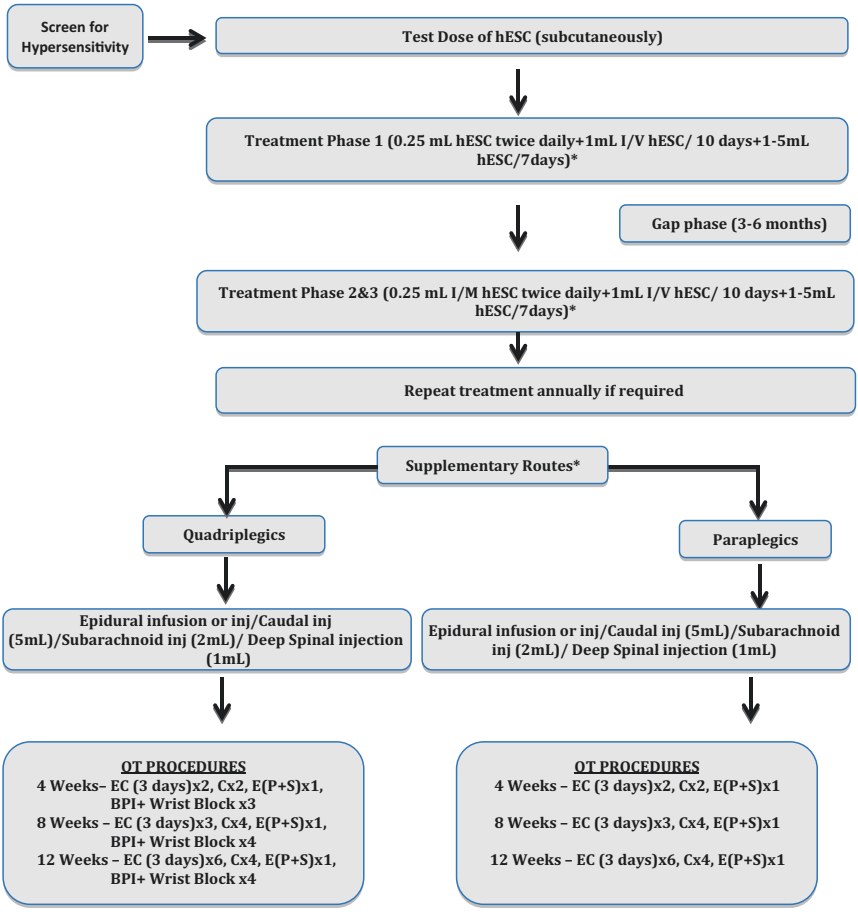

Fig. 4 Treatment plan for Spinal Cord Injury

The duration of treatment and gap phases varied in quadriplegic patients and paraplegic patients, as quadriplegics are generally more difficult to treat (Paralysis, Paraplegia, and Quadriplegia. MD guidelines 2015). After a gap period of 4–8 months, patients entered the subsequent treatment phases (T2 and T3), in which they were administered the same dosage regime as in T1. Each treatment phase lasted four to six weeks and was 4–8 months apart. In T2 and T3, an additional dose of hESCs was administered through any of the supplemental routes.

No immunosuppressants were given to the patients, who also received physiotherapy and/or occupational therapy. Rehabilitation focused on patients' overall improvement, and patient mobility was encouraged by using different ambulatory aids depending on the requirement (e.g., a patient with paraplegia was made to stand with full support on a hip-knee-ankle-foot orthosis, and as connectivity was regained, the support was reduced to a knee-ankle-foot orthosis, then knee brace and ankle support, and then ankle support). The walking aids were gradually reduced from manual support and walker to walker to crutches to walking stick to, finally, no aid.

Assessment

Each patient's pre-therapy status was assessed at admission. The percentage with changes or no changes were calculated after each session of the therapy and reported. Statistical tests or tests of significance were performed.

Data Validation

Patient data was validated by Moody's International (document number NH-hESC-10-1), GVK Biosciences (NM-Hesc-10-1, 18 November 2010), and Quality Austria Central Asia (document number QACA/OCT/2013/26). These companies examined the medical and statistical data present at the institute and met the patients.

Statistical Analysis

Descriptive statistics were performed to summarize data. SPSS software version 19.0 (IBM Corporation, Armonk, NY) was used for the data analysis. A chi-squared test was used to compare the AIS score at baseline and at the end of the therapy. A p value of < 0.05 was considered significant (Table 1).

Table 1 Change in American Spinal Injury Association scales of patients (overall) from admission to discharge at the end of each treatment period

	End of the treatment					
	ASIA scale					
Baseline characteristics	A; no. (%)	B; no. (%)	C; no. (%)	D; no. (%)	E; no. (%)	*p* value
T1 (*n* = 226)						0.02
A (*n* = 153)	73 (47.7)	23 (15)	57 (37.3)	–	–	
B (*n* = 32)	–	18 (56.3)	13 (40.6)	1 (3.1)	–	
C (*n* = 36)	–	–	31 (86.1)	5 (13.9)	–	
D (*n* = 5)	–	–	–	2 (40.0)	3 (60.0)	
T2 (*n* = 58)						0.01
A (*n* = 32)	20 (62.5)	12 (37.5)	–	–	–	
B (*n* = 9)	–	7 (77.8)	2 (22.2)	–	–	
C (*n* = 17)	–	–	17 (100)	–	–	
T3 (*n* = 19)						0.02
A (*n* = 8)	5 (62.5)	3 (37.5)	–	–	–	
B (*n* = 4)	–	4 (100)	–	–	–	
C (*n* = 7)	–	–	6 (85.7)	1 (14.3)	–	

ASIA American Spinal Injury Association

Results

A total of 226 SCI patients (paraplegic = 136, quadriplegic = 90) were included in the study. Overall, 203 patients had SCI due to trauma and 23 to miscellaneous causes like transverse myelitis (*n* = 4), Potts spine (*n* = 7), tumors (*n* = 3), and contusion (*n* = 9). The majority of these patients were men (167, 73.9%), and the mean age was 28 (range 20–34 years). Among paraplegic patients, 124 had complete injury, whereas among the quadriplegic patients, 71 had complete injury. The average days of treatment in T1 was 73 days for quadriplegic patients and 62 days for paraplegic patients, and the average gap period was 122 days for quadriplegic patients and 136 days for paraplegic patients.

All patients started intensive dosing, and 50 were present in all study periods. Overall, patients who discontinued the study for various reasons cited personal status and financial reasons (39%), satisfaction with their progress and cure (32%), dissatisfaction with their progress (5%), and

returning for treatment after a long gap (24%, i.e., after 31 August 2012, these patients were not part of this analysis).

Efficacy Evaluation

Changes in the ASIA impairment scale from admission to discharge at the end of each treatment period are presented in Table 1. Of the 153 patients in ASIA scale A at the beginning of T1, a significantly higher number of patients (n = 80, 52.3%) had moved to lower scales at the end of T1 (p = 0.02). At the beginning of T2, 32 patients were in ASIA scale A; of these, 20 patients remained in scale A and 12 (37.5%) had moved to lower scales by the end of T2 (p = 0.01). Of the 19 patients at the start of T3, eight patients were in ASIA scale A. At the end of T3, three of these patients (37.5%) had moved to scale B (p = 0.02). The improvement in scales at the end of T1 and T2 is shown in Fig. 5. At the end of T1, 45% of the patients had improved by at least one ASIA grade. At the end of T2, 58% of the patients had improved by at least one grade, and at the end of T3, 70% of the patients had improved by at least one grade (Table 2).

MRI scans for 65 of patients and tractographies for 25 patients were conducted before and after therapy. Improvements were observed in the MRI tractography images of these patients before and after the therapy (Fig. 1 and 2).

Table 2 Change from baseline to last period in total American Spinal Injury Association scores by extent and level of injury

Study period	Results	ASIA grades No. of patients (%)
End of T1 (n = 226)	Improved by 1 ASIA scale	102 (45)
	Stationary	124 (55)
	Not improved	–
End of T2 (n = 58)	Improved by 1 ASIA scale	62 (58)
	Stationary	44 (42)
	Not improved	–
End of T3 (n = 19)	Improved by 1 ASIA scale	35 (70)
	Stationary	15 (30)
	Not improved	–

ASIA American Spinal Injury Association

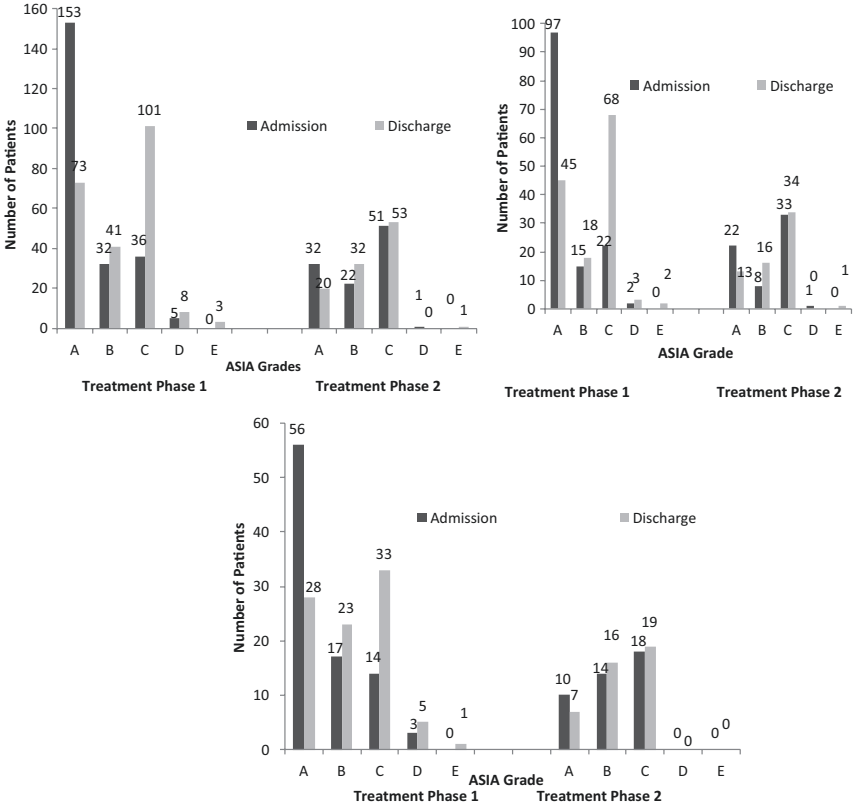

Fig. 5 Overall change in American Spinal Injury Association (ASIA) scale at the end of treatment phase 1 and 2

Paraplegics and Quadriplegics

Among the 136 patients with paraplegia, 97 were on ASIA scale A at the beginning of T1. Of these, a significant number of patients (52, 53.6%) had moved to lower scales by the end of T1 ($p < 0.05$). Of the 64 with paraplegia at the beginning of T2, 22 were on scale A. At the end of T2, nine patients (40.9%) had moved to scale B.

Among quadriplegic patients, 28 (50%) had shifted from scale A to the lower scales at the end of T1 ($p > 0.05$). Of the ten patients in scale A at the beginning of T2, three (30%) had improved and moved to lower scales. Of the three patients on ASIA scale A before T3, one (33.3%) had moved to scale B at the end of T3.

Gender Analyses

Of the 59 women in the study, 32 were on ASIA scale A at the beginning of the study. At the end of T1, 13 were on ASIA scale A and the rest had moved to lower ones. Among the 167 men, 121 were on ASIA scale A at baseline. At the end of T1, almost half (n = 60) remained on ASIA scale A, and another half had moved to lower scales. The improvement in scales at the end of T1, T2, and T3 is shown in Table 3. Gender was not a significant factor in the state of efficacy.

Table 3 Change in American Spinal Injury Association scales of patients (gender wise) from admission to discharge at the end of each treatment period

			No. of patients				
			At the end of treatment phase				
Gender	Treatment phase	Admission	A; no. (%)	B; no. (%)	C; no. (%)	D; no. (%)	E; no. (%)
Women	T1 (n = 59)	A (n = 32)	13 (41)	7 (22)	12 (38)	–	–
		B (n = 10)	–	7 (70)	2 (20)	1 (10)	–
		C (n = 15)	–	–	14 (93)	1 (7)	–
		D (n = 2)	–	–	–	1 (50)	1 (50)
	T2 (n = 23)	A (n = 4)	2 (50)	2 (50)	–	–	–
		B (n = 6)	–	4 (67)	2 (33)	–	–
		C (n = 12)	–	–	12 (100)	–	–
		D (n = 1)	–	–	–	–	1 (100)
	T3(n = 11)	A (n = 1)	1 (100)	–	–	–	–
		B (n = 4)	–	3 (75)	1 (25)	–	–
		C (n = 6)	–	–	5 (83)	1 (17)	–
Men	T1 (n = 167)	A (n = 121)	60 (50)	16 (13)	45 (37)	–	–
		B (n = 22)	–	11 (50)	11 (50)	–	–
		C (n = 21)	–	–	17 (81)	4 (19)	–
		D (n = 3)	–	–	–	1 (33)	2 (67)
	T2 (n = 83)	A (n = 28)	18 (64)	10 (36)	–	–	–
		B (n = 16)	–	16 (100)	–	–	–
		C (n = 39)	–	–	39 (100)	–	–
	T3 (n = 39)	A (n = 7)	4 (57)	3 (43)	–	–	–
		B (n = 12)	–	12 (100)	–	–	–
		C (n = 20)	–	–	20 (100)	–	–

Table 4 Adverse events observed during each treatment period (safety population)

AE parameter; no. total AEs; no. (%)	T1 (n = 226) 57 (25.2)	T2 (n = 106) 9 (8.5)	T3 (n = 50) 6 (12)
Fever	23	3	1
Headache	15	5	3
Loose motions	3	–	–
Abdominal pain	2	–	1
Constipation	2	–	–
Itching	2	–	–
Pain	2	–	–
Fever and headache	1	–	–
Fever, anorexia, and hematuria	1	–	–
Headache and vomiting	1	–	1
Headache and vertigo	1	–	–
Weight loss	1	–	–
Nausea	1	1	–
Redness and itching	1	–	–
Acidity	1	–	–

AE adverse event

Safety Evaluation

No death or serious adverse events occurred during the study period. No teratoma formation was observed during or after the study. Adverse events observed during each treatment period are tabulated (Table 4). Mild fever was the most frequent adverse event during the study and resolved without sequel.

The present study is the first of its kind to demonstrate the adequate efficacy of hESC in SCI patients with a good tolerability profile. The results of the present study have given a new ray of hope to SCI patients. Given the improvement shown by our patients, we propose that hESC transplantation into SCI patients presents a unique opportunity to address this greatly unmet medical need.

Anesthesia and hESC Injectable Procedures

A major concern for physicians is hESC implantation with reduced pain. Nutech Mediworld has been using specialized procedures to implant

hESCs in an SCI patient. Nutech Mediworld has thus developed a novel approach: the use of epidural and caudal routes.

An epidural route injects hESCs into the region outside the dura mater of the meninges, whereas hESCs implantation through the sacral membrane—approximately three centimeters above the tip of the coccyx and in continuum with the epidural space—is achieved by caudal route. The anesthesiologist plays a significant role in hESC transplantation by evaluating a patient's condition and protocol development along with suitable health-care management. This specialist encourages the optimal use of multimodal regimens as well as the implementation of novel techniques, ensuring improvement in pain control and the minimization of adverse events.

hESC implantation is not an easy task, as determination of the anatomic location of the implantation site is a major concern. High cell concentration within the region of interest is the physician's main target. However, we wish to prevent the dwelling of cells into other undesirable sites/organs. Intracoronary, transendocardial, transpericardial, intraventricular, intravenous, intramyocardial, and other routes of catheterization/administration have been previously reported. Further developments of catheterization systems for clinical studies involve other routes for administering hESCs such as intramuscular, intravenous, intrathecal, epidural, caudal, brachial plexus, popliteal, and/or deep spinal muscle injection. At our hospital, the epidural as well as caudal routes—procedures requiring anesthesiologists—have been used regularly for hESC implantation in SCI patients. The present study explains the caudal and epidural method of implantation.

Non-neuronal and neuronal cell lines—obtained from a single, spare, expendable, pre-implantation stage fertilized ovum taken during the IVF process, with due consent—are cultured, maintained, and stored in syringes for further use. The pre-filled, frozen syringes are thawed when required. The cells undergo quality analysis for determination of integrity, viability, and microbial contamination (Gupta and Barthakur 2014).

The paraplegic patients receiving caudal, epidural, and both procedures were the ages of 66, 20, and 43, respectively. Similarly, the quadriplegics receiving caudal, epidural, and both were 27, 12, and 23, respectively. The patients were under the care of skilled and experienced

anesthesiologists and physicians. SCI patients being readied for hESC therapy were provided with the facts, implications, and consequences of the therapy. Each patient gave video and signed consents before treatment. In addition to understanding the general protocol and time involved in the procedure, patients were advised to discuss with their clinician and physician whether medications could be taken on the day of the injection.

The physician took a record of each patient's allergic reactions and medications, and patients were asked to avoid drinking and eating after midnight. Medicines were taken with a sip of water. Alcohol and smoking were restricted before, during, and after the procedure. Patients were asked to change into a hospital gown, to allow physicians to clean the injection area and easily visualize the injection site. After moving the patients to the operation theatre, the hESCs were transplanted with a procedure involving trained anesthesiologists.

Epidural Procedure

An 'epidural' injection or catheter infusion involves the region outside the dura mater of the meninges. The patient was positioned in knee abdominal position in left lateral posture. The lumbosacral or lumbothoracic area (per requirement) was thoroughly cleaned with antiseptic agents followed by draping with a cut sheet. Because the epidural procedure might lead to discomfort, the area was locally anaesthetized with 2% lignocaine and a 26-gauge needle.

Following the method of loss of resistance (LOR), the epidural catheter was introduced and fixed depending on the level of injury. The patency of the epidural catheter was assessed by injecting normal saline. After ascertaining the strength, approximately 0.5–0.7 mL of hESCs were transplanted per vertebral space. This was followed by the multiple dosing of hESCs using the same catheter in situ through an infusion pump at the rate of 60 mL/hour. Because the hESCs possess a shelf life of half an hour, they were introduced in five to 10 minutes. In the case of higher level of injury, that is, high thoracic or cervical injury, the lumbar catheter was replaced by a single-shot cervical epidural injection. Patients were

able to receive fluids intravenously, and pulse, blood pressure, and oxygen levels were checked constantly. After the procedure, the catheter was removed from the back, and the patient was asked to lie on the bed until ready to move.

Because tenderness may be felt at the site of needle insertion for a few hours after the implantation, patients were able to receive an ice pack, which could be applied for 10–15 minutes once or twice a day. Patients were asked to rest for the remainder of the day, with normal activities typically being resumed the following day. A temporary increase in pain is possible for several days after due to the pressure of the fluid injected or the local inflammatory response.

The epidural procedure was contraindicated if the patient was on anticoagulant therapy (which might lead to coagulopathy) or suffering from fever, local sepsis, or local infection.

Caudal Procedure

Administration through the sacral membrane—approximately three centimeters above the tip of the coccyx and in continuum with the epidural space—can be achieved by a 'caudal' injection.

Using the LOR technique, hESCs were transplanted through one of the supplemental routes, the caudal epidural space. The patient was asked to lie in a fetal position, and the underlying sacral hiatus was located via the skin folds of the buttocks. With the tip of the coccyx in the natal cleft, the thumb of the same hand was used to palpate the sacral cornua. The sacral area was cleaned thoroughly with an antiseptic solution and was properly draped. The area was numbed with a local (lignocaine 2%), and a special needle of 26 G (two inches) was introduced via the sacrococcygeal membrane at an angle of 45 degrees. Positioning is accurate when a distinct 'pop' is heard. The needle was further penetrated parallel to the sacrum, and 5 mL of air was injected with hands positioned over the side of the needle tip. On feeling no air nor tissue resistance, hESCs were introduced with a 26 G needle. A number of complications can arise following a caudal procedure, and the physician must take great care at the insertion site. If the needle has been inserted correctly, it will

swing easily from side to side at the hub while the shaft is held like a fulcrum at the sacro-coccygeal membrane, and the tip moves freely in the sacral canal. An early resistance of insertion will show incorrect placement. A caudal injection is not advised if the patient refuses or if there is infection at the site, hypovolemic shock, coagulopathies, or preexisting neurologic disease.

During the treatments, no adverse effects or complications were observed. Clinical as well as radiological improvements were followed by regeneration of the spinal cord (Fig. 6).

A preper anesthesia process focuses on rapid recovery and ensures reduced pain. It also results in few complications and minimal systematic changes during the entire transplantation period. The use of anesthesia during stem cell transplantation in the human body has also been reported in many studies (Sharma et al. 2014; Negrin 2015). Lignocaine is a popularly used local anesthetic for spinal anesthesia and is mainly used for short surgical procedures due to its predictable onset and dense sensory and motor-blocking capacity for moderate duration. Many reports, however, suggest the neuro-toxic effects of lignocaine, thus doubting the use of lignocaine for spinal anesthesia (Schneider et al. 1993; Hampl et al. 1995; Freedman et al. 1998). However, some studies favor the use of

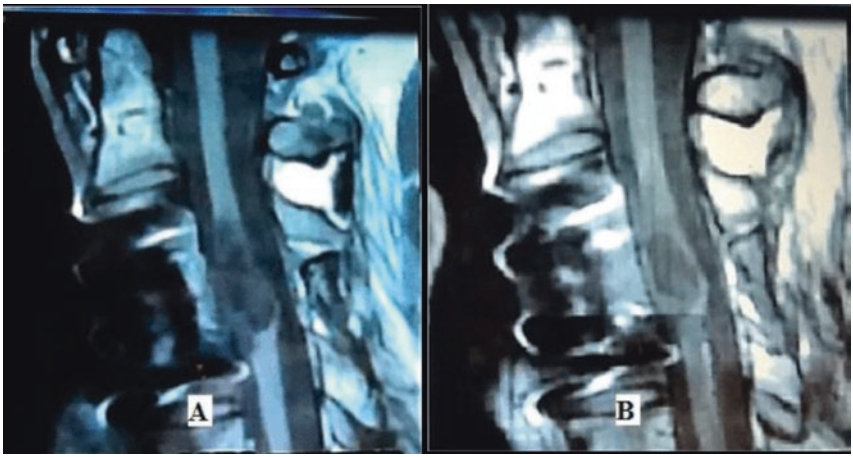

Fig. 6 Tractographic images of a SCI patient before and after hESC therapy using magnetic resonance imaging (MRI)

lignocaine as an excellent and safe modality for patients undergoing surgery (Srivastava et al. 2004). When lignocaine was used as an anesthetic agent prior to the procedure, no neuro-toxic effects were observed.

The anesthesiologist can provide specialized pre- and post-operative medical care (White et al. 2007), train others participating in the procedure, and evaluate patients before and after the procedure, adding depth to the physician-patient relationship. The complexity of the procedure's associated patho-physiology and risks require 'one-on-one' attention from the trained, experienced anesthesiologist. During transplantation, pain control is more effective due to the anesthesiologist-patient relationship developed during various consultations (Yosaitis, Manley, and Plotkin 2005) (Fig. 7).

Patient care and management should be a multidisciplinary strategy rather than a sub-specialty limited to one medical profession. The anes-

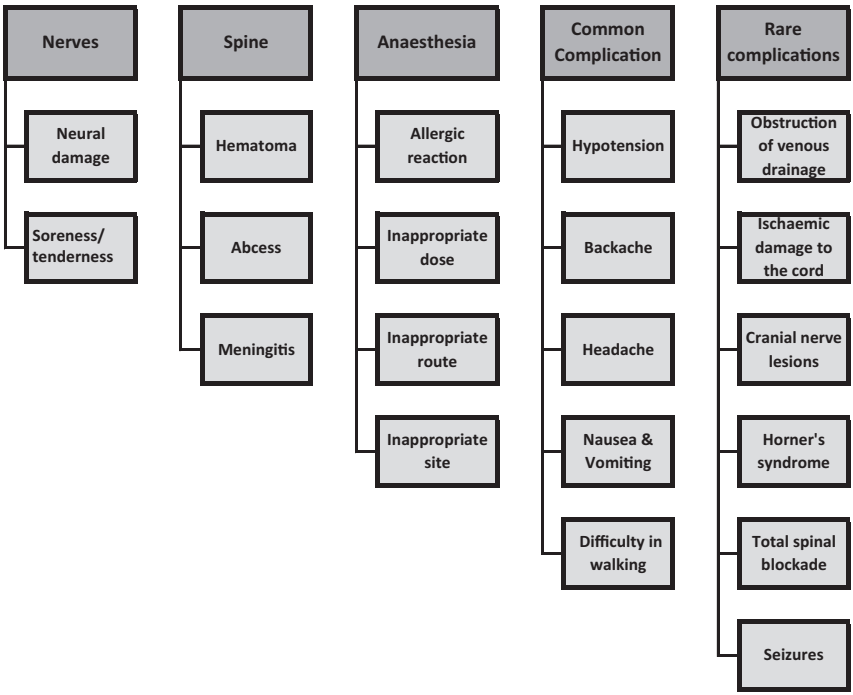

Fig. 7 Risks associated with epidural and caudal route of administration

thesiologist's role would ideally reach beyond the time of hospitalization, as effective pain control and appropriate hESC implantation is as important as achieving successful convalescence.

An interdisciplinary approach applied for the rehabilitation of patients with SCI also includes a team consisting of clinicians skilled in hESC therapy and physiotherapists. The patient data suggests positive role of physiotherapy in improving the mobilization in patients with SCI receiving hESC therapy (Shroff et al. 2016). It reveals an improvement in mobilization in patients with chronic SCI after receiving a combination of hESC and physical therapy. The physical therapy aided in training of cells and took care of atrophy of limbs, whereas hESC therapy resulted in an overall improvement of the patients. This has been observed due to the reduction in the orthotic devices and use of mobility aids. A previous study also showed remarkable improvement in the clinical, locomotive, as well as functional symptoms of the patients, where 81.72 % were able to walk with the support of calipers and mobility aids after receiving hESC therapy (Shorff et al 2015).

Conclusion

The case studies detailed in this chapter are the first of their kind to demonstrate the adequate efficacy of hESC in SCI patients with a good tolerability profile. Our patients gained voluntary movement of the areas below the level of injury as well as improvements in bladder and bowel sensation and control, gait, and hand grip. The MRI and tractography images taken before and after therapy confirmed the improvements observed. We did not observe any difference in the response to therapy between men and women.

Patient improvement was reflected in MRI scans and tractography reports that showed regeneration of the lost axonal connections. Fear of teratomas and immune rejection hinder the use of hESC therapy, but none of the patients in our study had a teratoma or an immune response; patients were not given steroids or immunosuppressants. Adverse events were mild and resolved without any sequel, with headache and fever

being the most common. It has been reported that inadvertent dural puncture can lead to the post-dural puncture headache (Crawford 1980). We began using hESCs for treatment in the year 2002, and our first patient received only four doses that year; no adverse events occurred until 2004, and the patient had experienced great benefits (Shroff and Barthakur 2015).

The results of the present study have given a new treatment option to SCI patients. We considered all patients for analyses regardless of the level and extent of injury.

This now-permanent disability affects the everyday lives of those with SCI. Even small clinical improvements can contribute to a better well-being and more productive life. hESC transplantation in SCI patients presents a unique opportunity to address this mostly unmet medical need.

Notes

1. The data in this chapter first appeared in open-access articles distributed under the terms of the Creative Commons Attribution License in the following peer reviewed journals:

    1. Geeta Shroff, Puneet Agarwal, Avinash Mishra, and Nayan Sonowal. 2015. Human Embryonic Stem Cells in Treatment of Spinal Cord Injury: A Prospective Study. *Journal of Neurological Research*, 5(3): 213–220.
    2. Geeta Shroff and Rakesh Gupta. 2015. Human Embryonic Stem Cells in the Treatment of Patients with Spinal Cord Injury. *Annals of Neurosciences*, 22(4): 208–216.
    3. Geeta Shroff, Nayan Sonowal, and Avinash Mishra. 2015. Role of Anesthetists in Human Embryonic Stem Cells Transplantation in Patients with Spinal Cord Injury. *Journal of Anesthesia & Clinical Research*, 6(5): 1–6.
    4. Geeta Shroff. 2016. Human Embryonic Stem Cell Therapy in Chronic Spinal Cord Injury: A Retrospective Study. *Clinical Translational Science*, 00: 1–8.

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Geeta Shroff is Founder and Director of Nutech Mediworld, New Delhi, India. Dr. Shroff has developed the technology to isolate human embryonic stem cells (hESC), culture them, prepare them for clinical application, and store them in ready-to-use form with a shelf life of six months. Further, this technology is being used clinically to treat patients. Since 2002, more than 1400 patients suffering from various conditions categorized as incurable—spinal-cord injury, diabetes, multiple sclerosis, Parkinson's disease, cardiac conditions, cerebral palsy—have been treated successfully by Dr. Shroff, and the number is steadily growing. A graduate in Medicine from the University of Delhi, Dr. Shroff did

her postgraduation work in Gynecology and Obstetrics. She specialized in treating infertility and is a trained embryologist and a qualified IVF practitioner. After eight years of valuable clinical experience at Safdarjung and Batra hospitals, large multi-specialty hospitals in Delhi, Dr. Shroff set up her own IVF practice in 1996. She began research on human embryonic stem cells in 1999 and pioneered hESC therapy. She has presented her work at various national and international forums. Dr. Shroff envisions making hESC therapy available globally so that it becomes the first line of treatment for many of humankind's worst afflictions.