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# Mesenchymal Stem Cells in Spinal Cord Injury

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N.K. Venkataramanaa and Rakhi Pal

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## 1. Introduction

Spinal cord injury is the most devastating neural injury associated with road traffic accidents or fall from height. Due to the compact arrangement of nerve fibers injury often leads to significant deficits. In addition the cellular components of the spinal cord are highly susceptible to injury. Together with the brain the ability of self-repair in comparison to other tissues of the body is poor.[1]. Recently it is noted that the tissue response of the spinal cord to injury is distinctly different from that of brain. The structure, cellular arrangement, vascularity, blood spinal cord barrier, and lack of exposure to inflammatory cells are some of the limiting factors for repair. Added to it receptor and membrane specializations that allow chemical and electrical neuro transmission is prone to major ionic shifts. Though regeneration of spinal cord in teleost fishes and urodele amphibians is established, no adult mammal is able to regenerate. Hence, any insult can result in permanent and significant loss of body function. The therapies currently practiced (surgery, drugs, rehabilitation), are grossly inadequate. The available surgical treatment could only achieve prevention of further injury, maintain and support blood flow, relieve the compression and secure stabilization of spine for early mobilization and rehabilitation. Thus any new treatment for spinal cord injury that enables recovery of function is the need of the hour and could be a significant advancement in clinical care. Biological therapies are now being developed to augment the endogenous repair capabilities. They are aimed at preservation of tissue, promotion of cell survival, activation of neuronal regrowth, reduction in growth inhibition, scarring and cavitation, promotion of myelin repair thus enhancing neuronal circuits.

We have studied and evaluated such applications to attempt spinal cord regeneration. Adult human mesenchymal stem cells were the obvious choice due to their self-renewal property, ease of availability, hypo-immunogenic property, non-teratogenicity, multi-potentiality with high genetic stability.

## 2. Incidence

More than half of all spinal cord injuries occur in the cervical area; and a third of them affect thoracic region. And the rest afflicts lumbar region. Most of the affected ones are young, in their teens or twenties.. The leading causes of acute spinal cord injury include vehicular accidents-41%, violence-22%, falls-21% and sports-8% [2]. Population studies shows the incidence that vary between 2-20%. The official figure is 12% majority being, due to trauma. The total number of people suffering a spinal cord injury in the US alone is 200,000; and 11,000 being added annually. The United Kingdom had over 700 new spinal cord injuries in 2004 (according to the International Campaign for Cures of Spinal Cord Injury).

Spinal cord injury (SCI) is the third most prevalent disease in our country after diabetes and myocardial infarction. More than 12% of the Indian population suffers from the complications associated with spinal cord injury and at least 10,000 are being affected annually [3]. Majority are in the age group 21-36 years, the most productive years of life and 10-12% of severe head injuries are associated with spinal injury. Awareness of this fact is important to protect spine during pre hospital care. The clinical dictum is to suspect spinal injury in all high speed injuries.. Penetrating injuries are relatively rare.

## 3. Pathophysiology

The biological response to spinal cord injury is customarily categorized into 3 phases that follows a distinct but somewhat overlapping temporal sequence: acute or primary (seconds to minutes after the injury), secondary or sub-acute (minutes to weeks after the injury), and chronic (months to years after the injury) [4] Table 1. Primary injury is due to direct impact, damaging the neurons, cell membranes, disrupting blood supply, and destabilizing the spinal column. Secondary damage soon follows causing oedema, inflammation and free radical production. A series of molecular changes then produce a cascading effect with liberation of toxins compounding the primary injury. This can continue for few days to even up to six months. [5,6,7] Diverse type of cells and molecules from nervous, immune and vascular system are known to be involved in each phase. Most of the involved cells reside within the spinal cord; also some other cells are recruited through the circulatory system [8]. Hypotension and hypoxia can induce secondary permanent damage.

The onset of acute phase begins within seconds after an insult to spinal cord injury and is marked by both local and systemic events. Cascade of sequential pathological changes can occur during this phase. Local events such as cord compression, release and accumulation of various neurotransmitters such as catecholamines and excitotoxic amino acids to a toxic level enough to kill neural cells have been postulated to occur within seconds of injury [8]. Soon after trauma, hypotension, shock, low cardiac output and respiratory failure and hypoxia occur due to autonomic system failure. Between 15 to 30 minutes of post trauma, edema in white matter and hemorrhage in gray matter have been reported. Electron microscopic studies

Primary events (0-2 hrs)	Secondary events (1-6 hrs)	Spinal shock (12 hrs –3 weeks)	Post spinal shock Reflexes reappear
Mechanical compression of neural elements by bone fragments, disc material, and ligaments, laceration, shear and distraction	<ol style="list-style-type: none"> <li>1. Toxic metabolites</li> <li>2. Electrolyte loss</li> <li>3. Hypoperfusion of gray matter</li> <li>4. Loss of autoregulation and microcirculation</li> <li>5. Vasospasm, thrombosis and hemorrhage.</li> <li>6. Accumulation of neurotransmitters : like Glutamate leading to excitotoxicity</li> <li>7. Elevated calcium levels</li> <li>8. Cell membrane disruption and loss of cell integrity</li> <li>9. Cytotoxicity/free radicals/apoptosis/ prostaglandin release and lipid peroxidation</li> <li>10. Demyelination</li> <li>11. Edema</li> <li>12. Invasion of glial cells and activation of resident microglial cell population.</li> </ol>	<ol style="list-style-type: none"> <li>1. Neurogenic shock.</li> <li>2. Respiratory distress.</li> <li>3. Impaired autonomic functions.</li> <li>4. Quadriplegia.</li> <li>5 Anesthesia below affected level.</li> <li>6. Delayed gastric emptying.</li> <li>7. Paralytic ileus</li> </ol>	<ol style="list-style-type: none"> <li>1. Superficial abdominal reflex</li> <li>2. Cremasteric reflex</li> <li>3. Bulbocavernous reflex</li> <li>4. Withdrawal reflex</li> <li>5. Beevar sign etc</li> </ol>

**Table 1.** Illustrates the different phases of spinal cord injury and the cascade of events associated with it.

revealed accumulation of intra- and extracellular fluids in the intercellular space. Ischemia or local anemia has been reported within first few hours after severe trauma using angiographic methods. A major reduction in spinal cord blood flow and lack of perfusion has been observed. This ischemic zone encompasses a large portion of gray matter and surrounding white matter. The main reasons postulated for ischemia are vasospasm (due to vasoconstrictors and vasoactive amines), thrombosis, and platelet aggregation and hemorrhage [9]. By 4th hour, axonal degeneration followed by vesicular disruption in myelin sheaths and ischemia becomes evident. In other patients who do survive the initial injury, hyperaemia and other vascular changes become prominent in 12 to 24 hours. These reactions are mediated through prostaglandins, catecholamines and other agents [10]. At the end of 24 hours, necrosis starts and remains active for another 24 hours which triggers the inflammatory response and disruption of cell membranes resulting in release of intracellular contents of neurons and endothelial cells lining them. This progressive, coagulative and patchy necrosis generally occupies the previous hemorrhagic region and develops infarcts. Increased intracellular calcium influences enzymes, such as phospholipases and phosphatases, to promote the breakdown of the cell membrane. This results in liberation of free fatty acids, which are converted to prostaglandins which

further increases the constriction of the blood vessels (vasospasm), which in turn contributes to final cell death [11].

The secondary mechanisms are still ill understood. In literature (shows) there are approximately 25 well established secondary injury mechanisms are described [12, 13]. Secondary phase sets in minutes and lasts from days to months. Some classical examples of secondary injury mechanisms are continuation of events from the acute phase as outlined by Charles. They are vasospasm, cell death from direct insult, ischemia, edema, derangements in ionic homeostasis and accumulation of neurotransmitters. In addition some novel features, marks the secondary phase such as free-radical production, lipid peroxidation, nitrous oxide excess, conduction block, excess noradrenaline, energy failure and decreased ATP, immune cells invasion and release of cytokines, inflammatory mediated cell death, neurite growth-inhibitory factors (Nogo-A, Rho-A, oligodendrocyte myelin glycoprotein (OMgp) myelin-associated, glycoprotein (MAG), and chondroitin sulfate proteoglycans, central chromatolysis,). vertebral compression / column instability, demyelination of surviving axons, initiation of central cavitation, astroglial scar launch, plasma membrane compromise /permeability, mitochondrial malfunctions and activation of death signals causing apoptosis [8] are the remaining..

The third phase (chronic phase), along with the events in secondary phase, such as demyelination, apoptosis, central cavitation, glial scar formation, is marked by the emergence of new types of pathologies both at micro and macro level [8]. At microlevel, death of oligodendrocytes, susceptible to Reactive Oxygen Species (ROS), loss of electrical impulse conduction by axons due to demyelination and altered neurocircuits and alteration of ion channels and receptors occur [9]. At macrolevel, formation of the glial scar represents an attempt by Glial cells to contain the injury site and promote healing. In addition to reactive astrocytes, scar formation also involves oligodendrocyte precursor cells, microglia, and macrophages. The pathobiology of glial scar is due to reactive gliosis and extra cellular matrix (ECM) remodeling [8]. These changes during reactive astrogliosis have the potential to alter astrocyte activities both through gain and loss of functions that could be beneficial as well as detrimental to surrounding neural and non-neural cells [16, 17, and 18].

More than a quarter of spinal cord injured patients develop cavities which eventually lead to Syringomyelia [19]. Pathogenesis of post traumatic syrinx is not clear. Widely accepted theory recognized two steps in the pathogenesis, namely formation of cavity followed by its enlargement and extension. Microscopic examination demonstrated gliosis, which is an astrocytic response to adjacent tissue damage, appears as high MRI signals around the syrinx. [20]. The initial cystic lesion results from multiple factors like mechanical damage, local ischemia [19], arterial and venous obstruction, liquifaction of hematoma, by lysosomal and other intracellular enzymes [21].

Beside, chronic phase also initiates number of neuroprotective and regenerative responses. But they are insufficient for regeneration of the nerve root by Schwann cells or oligodendrocytes. Some compensation by spared neurons (sprouting) often with inappropriate connectivity.

Finally the reactive astrogliosis itself hinders the axonal regrowth and the functional recovery of the injured spinal cord [22].

### **3.1. Impediments for regeneration**

Cord tissue comprises of several components with variable sensitivity to injury.[1].Injury often causes cavitation of epicenter due to cell death, ischemia, mechanical injury, excitotoxicity and neuro inflammation. This cavity can enlarge extending the injury up and down. In addition it becomes a physical barrier for regeneration and cell transmission and cell migration. Body attempts to contain the injury and promote healing resulting in gliosis. This astrocytic gliosis becomes an impediment to the growth of axon described by Raymon Y Cajal in 1928 [1]. These gliotic cells also secrete inhibitory molecules for the axonal growth and connectivity. Inflammation slows down the initial angiogenesis response and oligodendrocytes (secrete Nogo molecules), glycoproteins Semaphoring 4D and Epherin B3,also have been shown to have inhibitory role.[21,22,23]

## **4. Tools for assessing spinal cord injury and repair**

### **4.1. Molecular, genetic, and in vitro tools**

Techniques now have been developed that allow researchers to isolate and grow populations of neurons to investigate the effects of specific proteins and molecules on neuronal injury and repair. Neurons can be grown in isolation or with glial cells such as oligodendrocytes or Schwann cells to study the processes of axonal outgrowth and myelination using DNA or protein analysis. Furthermore, the elucidation of the signaling pathways responsible for this switch in response may lead to the discovery of a strategy for enhancing axon regeneration.

Often, *in vitro* assays are tested along with animal models which allow better understanding of the effects detected *in vitro* and to be validated in a more complex system. The best studied example includes chondroitin sulfate proteoglycans, a potent inhibitor of neurite outgrowth in *in vitro* experiments. Analysis with animal models demonstrated that the levels of these proteoglycans are enhanced, or up-regulated, during central nervous system (CNS) injury and led to the development of a strategy to break down these substances and promote the regrowth of axons in the intact rat spinal cord after an injury [21].

### **4.2. Animal models for spinal cord injury**

No single animal model has dominated for research in this area. Two broad classes of models have accounted for the great majority of studies. Both involve surgical exposure of the cord. Most commonly used models are transection or partial injuries for detailed studies of regeneration and experimental contusion and compression. Allen's weight drop model, the oldest method in use and produced by dropping a known weight onto the dorsal side of the exposed spinal cord. This is mainly to address the early processes of

injury. Besides the above mentioned category, microlesion formation and transgenic models, [25] Photochemical SCI model, excitotoxic spinal cord injury have also been developed in recent years [26].

During the last two decades, various researchers have shown interest in developing variety of animal models based on the above two categories, that mimic different attributes associated with spinal cord injuries. Depending upon the purpose of the study and the specific aspect of the injury to be investigated, researchers determine which animal model most closely replicates the injury in humans. Commonly used animal models for the investigation includes [8] Primates – to test the safety and efficacy of the therapy, [9] Cat – to examine and define spinal cord circuitry, [10] Mouse and rat – mainly used for the investigations of molecular, genetic and anatomical response to injury and to modify genes to test the effect of restoration or loss of function.

The kind of inquiries currently in focus can be addressed with rodent models, for which the maximum number of biological reagents and tools are available. In time, there may be a need to examine the conclusions of rodent studies in other models, to deal with questions of species differences (biological responses, chromosomal arrangements, genetic variability and the spatial arrangement of the nerve tracts) and mechanical scale (animal size, limited number of animals for experimentation) and ethics [9].

## **5. Assessing SCI and repair mechanisms**

### **5.1. Conventional treatment strategies**

Treatment for Spinal cord injury starts at the site of accident or trauma. Manual spine immobilization or using cervical collar and spine board, followed by administration of analgesics to reduce pain is an established practice to achieve comfort to the patient. Careful monitoring of airway, respiration, and arterial pressure is essential. Hypotension, hypoxia are deleterious and should be avoided at all cost. From the scene of trauma, the patient is moved to the medical center and assessed further with neurological status and clinical level of injury. Base line clinical status is established and documented. In parallel other systemic injuries were also evaluated. ASIA impairment scale modified BENZEL scale and FRANKEL scales are commonly used to evaluate progress. MRI is the gold standard in imaging to delineate the anatomy of injury. In addition, size and extent of cord contusion, hemorrhage and edema have prognostic significance. Throughout its mandatory to avoid secondary insults to spinal cord. Several drugs have been tried with no demonstrable benefit. There is no role for steroids and Methylprednisolone. All attempts of direct surgical repair of spinal cord have failed.

### **5.2. Experimental strategies**

Almost every aspect of the management of SCI is controversial, due to lack of good-quality evidence. Currently all the modes of the experimental therapy falls into any of the following

categories: neuroprotection, repair/regeneration, enhancing the plasticity and replace/assist function. The details of all can be found at ICORD website (<http://icord.org/>).

### 5.3. Neuroprotection (randomized clinical trials)

Clinical trials should augment the neurological recovery data with outcome measures designed to assess the functional significance of the neurological recovery. To date more than 70 clinical trials have been done on functional recovery of Spinal Cord Injury with drugs and other therapeutic intervention (<http://clinicaltrials.gov/>). Of those, drugs which have direct application in treatment regimen for SCI and the reason for their pitfalls are discussed here.

#### 1. Pharmacological therapy

The first and extensive studied drug is Methyl prednisolone sodium succinate (MPSS), an anti-inflammatory corticosteroid exerting its function as antioxidant, enhancer of spinal cord blood flow, by reducing calcium influx, posttraumatic axonal die back and attenuating lipid peroxidation. The drawback of this drug is that it did not rescue neurons from cell death and [16] its high rates of adverse events such as the occurrence of pulmonary and gastrointestinal complications and others. [8, 9, 15].

A noncompetitive N-methyl-d-aspartate receptor antagonist, gacyclidine (GK-11), showed promise as a neuroprotective agent as evidenced by walking recovery, motor performance, attenuation of spinal cord damage, reducing apoptosis of oligodendrocytes via inhibition of proNGF production in microglia [18] etc, in rat model. However, this agent is no longer being pursued for SCI [11] and the use of minocycline following contusion of cord requires further investigation before clinical trials are implemented [17].

Minocycline, an antibiotic and anti-inflammatory substance facilitated overall motor recovery and attenuated mechanical hyperalgesia in a rat model [8], but did not increase the survival of the preganglionic parasympathetic neurons (PPNs) [20].

2. GM-1(Sygen), a ganglioside found in the neuronal cell membranes, was found to promote recovery in a number of animal models. In human trials it resulted in statistically significant improvement in ASIA motor score but failed to demonstrate a significant difference in its primary outcome measure, a 2-point improvement on the modified Benezel walking scale [8, 9].
3. Erythropoietin, a potent cytokine [25], and its analogues have been thoroughly investigated [26] and shown to protect neuronal cell in vitro from apoptosis and also suppress the up-regulated expression of TGF- $\beta$  [27, 25] reduces the inflammation, and restores the vascular integrity [21].

#### 4. Immunomodulatory treatment

Inflammatory processes that occur at the injury site of the spinal cord are both beneficial and harmful. Phagocytic macrophages have been indicated in secondary destruction of neural tissue post SCI [28; 29] but are not sufficient as compared with peripheral nerve injury. Rapalino *et al.*, [30], has demonstrated that implantation of activated macrophages in the site



of injury in adult injured rats results in partial recovery. On the contrary, Popovich *et al.*, [31] suggested that depletion of macrophages may result in preservation of myelinated axons and functional recovery following injury. A phase I clinical trial demonstrated the safety of autologous macrophage transplantation into the damaged spinal cord within 14 days of injury.

5. Neurotrophic factors: Neurotrophic factors have been documented to improve cell survival and axonal regeneration and various approaches have been developed to deliver these factors to the site of injury. Stem cells from different sources like bone marrow [32, 33], adipose tissue, dental pulp [34], Wharton's jelly, olfactory ensheathing cells [35], neural stem cells [36] and embryonic stem cells [37] when transplanted *in vivo* have shown significant recovery.
6. In a controlled double-blinded study, 20 patients receiving thyrotrophin releasing hormone treatment showed significantly higher motor, sensory, and Sunnybrook scores than placebo treatment. But because of patients lost to subsequent follow-up, data were not highly informative [18]. Another study in rats treated with thyrotrophin-releasing hormone showed significant improvement in Neural Scores 14 days post-injury, but there were no significant differences in morphometric parameters between saline-and TRH-treated rats [19]. TRH has disadvantages, including its analeptic, endocrine, and autonomic effects, but a new generation of TRH analogs has been developed that have the protective effects of TRH without its adverse effects [20].

## 6. Repair and regeneration

A variety of promising substances have been tested in animal models, but few have had potential application to human spinal cord injury (SCI) patients. This category of treatment includes both the pharmacological intervention using FDA approved drugs and cell transplantation. (The latter will be discussed in detail in the forthcoming titles). Several drugs were tested for their efficacy in restoring spinal cord function as evidenced by multiple preclinical studies. Some of the Drugs such as Cethrin [47-50], rolipram [41-45], ATI-355 [45-51], chondroitinase [51-56] and riluzole [57-64] were thoroughly reviewed which are not limited to neuroprotection, axonal regeneration, motor neuron recovery, reduction in muscle spasms, enhanced sprouting of corticospinal axons, improved behavioral outcome and corticospinal plasticity, recovery of forelimb function, inhibition of apoptosis and suppression of glial scar formation with varying degree of success. The major drawback of the pharmacological intervention is their side effects and direct application in human trials.

### 6.1. Plasticity enhancement and rehabilitation

An inability to perform self-care activities is considered a "burden of care" by the medical community. The individual with acute SCI faces many challenges with the resumption of self-care tasks. Hence considerable efforts have been taken by the therapist in order to guide the

patient move their upper limb and lower limb and support their body weight after a spinal trauma. Upon discharge from a hospital setting, family members, other caregivers, or both share the burden of care. Medical insurance programs have required reliable data on which to determine benefits, including coverage of durable medical equipment, treatment, and care giving assistance. Task specific training i.e., activities of daily living (ADL) which include self feeding, bathing, bowel and bladder maintenance, dressing, hygiene maintenance, computer usage etc., plays a central role for the patient to be independent. Other techniques such as body weight support and treadmill training using upper and lower limb orthosis and knee orthosis, have shown recovery in maintaining the body gait and postures. Tilt table standing, robot-aided gait training, electric stimulated wheel chairs are also used in recent days for posture maintenance. Recreation and leisure skill development such as reading, writing, painting, exercises, All-Terrain Vehicles (ATVs) (cycling, fishing, horseback riding, climbing, diving, etc) arm ergometry and Nautilus-type machines. Although these techniques are considered to be promising, less is known about their mechanism and efficacy on the functional recovery. Hence a deeper understanding of the underlying mechanism for adaptation and plasticity after spinal cord injury is needed to improve rehabilitation regimes [65-81].

## **6.2. Non pharmacological intervention for the treatment of SCI**

Functional electrical stimulation (FES) is the technique of applying safe levels of electric current to activate the damaged or disabled nervous system. Although no absolute contraindications exist for the use of externally applied FES, a patient with a cardiac demand pacemaker or an automatic implanted defibrillator should be approached with extreme caution. Some of the relative contraindications for FES include patients with cardiac arrhythmias, congestive heart failure, pregnancy, electrode sensitivity, and patients with healing wound(s) that could be stressed during stimulation (i.e., muscle stimulation would adversely move healing tissues). As with any implant in the body, individuals with implanted FES systems need to obtain antibiotic prophylaxis when undergoing invasive procedures such as oral surgery. Functional uses for FES after SCI include applications in standing, walking, hand grasp (and release), bladder, bowel, and sexual function, respiratory assist, and electro ejaculation for fertility. [82-88]. Functional magnetic stimulation (FMS) can be defined as a technology that applies a time varying magnetic field to produce useful bodily function. There were no significant side effects of magnetic stimulation that were reported. However safety consideration such as magnetic effect, electric effect and power dissipation should be kept in mind during stimulation. A few reports have shown that repetitive transcranial magnetic stimulation may result in increased seizure activities [89-94].

Hypothermia, CSF Drainage, durotomy and subarachnoid perfusion, Functional electrical stimulation, Electromagnetic stimulation, hyperbaric oxygen were tried with some success. But none of them reached to the level of functional therapeutic options.

## **6.3. Replace or assist function**

Over the past 2 decades, advances in understanding the pathophysiology of spinal cord injury (SCI) have stimulated the recent emergence of therapeutic strategies. Functional repair of the

injured central nervous system (CNS) is one of the greatest challenges addressed by neurobiologists. The rapidly growing field of stem cell biology offers a promising future for cell replacement and neural regeneration therapies. Stem cells have seen its good days with success in Parkinson disease and Huntington's disease and hold a long history of research on the possible use of progenitor cells in the treatment of SCI. The application of cell-based therapies to SCI is a natural expansion of research in other fields, such as cancer, diabetes, and heart diseases.

#### Spinal Cord Injury and Stem cells: Some Cellular transplantation strategies

Spinal cord injury though uncommon leads to profound lifelong disability and systemic effects. So far no single therapy have proved its efficacy, therefore combination therapies might hold the future design. In order to repair the injured spinal cord, it is essential to reduce secondary damage and promote regeneration. Several biological agents such as proteins, antibodies, enzymes and cells were used to achieve this goal.

The adult spinal cord has an endogenous progenitor cell pool said to have been located, in the ependymal region around the central canal. [95-97]. While others believe their presence throughout the spinal cord [98]. The response of these endogenous cells post injury is insufficient and does not bring about adequate recovery following SCI [95-97]; probably due to the insufficient cell numbers, microenvironment at the injury site, and presence of tissue debris. Neural inflammation, immune mediated destruction and loss of vascularity also becomes major hindrance. There could be an imbalance between the degree of repair vs damage. Cell transplant strategies have the potential of reducing such secondary damage and promoting regeneration by replacement of dead cells and production of Neurotrophic factors promoting regeneration [99-101].

Oligodendrocytes and astrocytes are the major supportive cells within the central nervous system and are responsible for myelination of axons, so it is believed that replacement of this cell population will support the frame work in regenerative processes.

Immature glial cells have been shown to reduce the inhibitory properties of the lesion epicentre and promote axonal growth [102]. Immature oligodendrocytes provide remyelination after injury [103], whereas immature astrocytes promote axonal growth and survival after injury [104]. A recent study supports the idea of ensuring both of these cell types, astroglial, are replaced, since oligodendrocytes precursors failed to remyelinate the spinal cord in the absence of astrocytes.

Cao *et al.*, [99] has demonstrated that after the transplantation of stem cells into lesioned adult rat spinal cord most of these transplanted cells have differentiated into astrocytes and no neurons or oligodendrocytes were observed. This indicates that it would be essential to transplant a progenitor cell population capable of trans-differentiating into a mixed lineage *in vivo* or should be able to secrete neurotrophic factors *in vivo*. Studies have elucidated that MSCs do have the capacity to Trans-differentiate into the astroglial lineage and also secrete cytokines which may be essential for regeneration in spinal cord injury.

#### 6.4. Proposed scope of using stem cells / regenerative medicine

Type of stem cells	Species	Injury type	Site of injection	Results	Salient features	Reference
BM MSC						
Transgenically labeled BMMSC (autologous) and lineage restricted neural precursor (LRNP) (2x10 <sup>6</sup> cells)	Inbred Fisher-344 rats	Partial cervical hemisection injury	Intravenously, Intraventricularly, Intrathecally and Lumbar puncture	Intravenous route – least efficient. Intrathecally and intraventricular administration shows enhanced cell migration and grafting	Axonal growth was observed around the transplanted cells. Grafted LRNP cells differentiation into mature neurons, their survival and integration within the host spinal Cord might lead to functional recovery.	Ajay bakshi et al
BMSCs and GCSF	Male Wistar rats	Compression lesion	Intravenously (BMSCs) and subcutaneously (GCSF)	Higher BBB scores and better recovery of hind limb sensitivity	Significant increases in the spared volume of white matter due to the synchronized action of various factors released by MSCs	Lucia Urdžíková et al
Bone-marrow-derived mesenchymal stem cell (autologous and allogenic)	Adult Beagle dogs		Intrathecal	Improved neurological signs in pelvic limbs Significant high Olby scores	Recovery may be due to the synchronized action of various factors as evidenced by high rate of mRNA expression for neurotrophic factors.	Dong-In Jung et al

Type of stem cells	Species	Injury type	Site of injection	Results	Salient features	Reference
LA MSC						
Adipose-derived stem cells (allogenic)	Adult mixed-breed dogs	Epidural balloon compression	Injured site	Nerve conduction velocity was significantly improved GFAP, Tuj-1 and NF160 were observed	Neuronal transdifferentiation Survived MSCs produces large amounts of bFGF and VEGFR 3 which aid the recovery	
Cotransplantation of Mouse Neural Stem Cells (mNSCs) With Adipose Tissue-Derived Mesenchymal Stem Cells	Adult Male Sprague-Dawley rats	Clip compression	Epicenter of the injured spinal cord	AT-MSCs inhibited the apoptosis of mNSCs mNSCs transplanted with AT-MSCs showed better survival rates	Biomolecular substances secreted by ATMSCs improve mNSC survival and inhibit mNSC apoptosis, mainly VEGF	Jin Soo Oh et al
WJ MSC						
Human Umbilical Cord-Derived Schwann-Like Cell Combined with Neurotrophin-3	Adult Female SD rats	Transection	Lesion site	NT-3 administration significantly promoted the survival of the grafted cells Improved motor function and promotes neurite outgrowth	GDNF, BDNF, NT-3 and bFGF provided neurotrophic support	Guo Yan-Wu et al
Human umbilical cord mesenchymal stem cells	Female Sprague-Dawley rats	Weight drop method (contusion)	At the dorsal spinal cord 2 mm rostrally and 2 mm caudally to the injury site	Significant Recovery of hindlimb locomotor function Increased length of neurofilament-positive fibers and increased numbers of growth cone-like structures	Transplanted cells survived, migrated over short distances, and produced large amounts of glial cell line-derived neurotrophic	Hu SL et al

Type of stem cells	Species	Injury type	Site of injection	Results	Salient features	Reference
				Fewer reactive astrocytes were observed	factor and neurotrophin-3.	
Human umbilical cord mesenchymal stem cells	Adult Female Sprague-Dawley rats	Transection	Lesion site	Promotion of regrowth of injured corticospinal fibers change in the distribution of astrocytes in spinal cords reduction in the activation of microglia	Production of large amounts of human neutrophil-activating protein-2, NT-3, bFGF, glucocorticoid induced TNF-receptor, and VEGFR 3 in host spinal cord, may have helped in spinal cord repair.	Chang-Ching Yang et al
<b>GLIAL CELLS</b>						
Multineurotrophin-Expressing Restricted Precursor Cells	Adult Female Fischer 344 rats	Contusion	Lesion site	Improved transcranial magnetic motor-evoked potential responses Improved Electrophysiological and locomotor functional recovery	Grafted GRPs formed normal-appearing myelin sheaths around the axons in the ventrolateral funiculus (VLF) of spinal cord and restores the conduction.	Qilin Cao et al
<b>NSC/ NSPC</b>						
poly(lactide-co-glycolide) (PLGA) polymer seeded with human neural stem cells	African green monkey	Hemisection	Lesion site	Enhanced hindlimb motor neuron performance	Major mechanism of action of implanted cells may be due to trophic support rather than	Pritchard et al

Type of stem cells	Species	Injury type	Site of injection	Results	Salient features	Reference
					neuronal replacement	
Spinal cord-derived NSPCs and BMSCs	Adult male rat	Clip compression	Lesion site	No functional improvement was seen in either transplant group. But significant inverse correlation between the functional scores and the number of transplanted astrocytes was observed	Differentiation of NSPCs into astrocytes and oligodendrocytes promoting remyelination, and potential axonal guidance	Parr et al
Neural stem cells	Marmoset	Contusion	Lesion site	Recovery of motor function was observed mainly in the hindlimbs. Significantly higher spontaneous movement	Grafted human NSCs survived and differentiated into neurons, astrocytes and oligodendrocytes and restores motor function	Iwanami et al
Epidermal Neural Crest Stem Cell (EPI-NCSC)	Wild type C57BL6/J and C57BL/6-TgN (ACTbEGFP) 10sb	Contusion	Intraspinal	Differentiated into gabaergic neurons and myelinating oligodendrocytes	combination of pertinent functions including cell replacement, neuroprotection, angiogenesis and modulation of scar formation	Sieber
Spinal cord-derived neural stem/progenitor cells (NSPCs) and Bone Marrow-derived	Rat	Compression	Lumbar puncture	Expression of oligodendrocyte markers	wide dissemination of cells in the subarachnoid	Mothe et al

Type of stem cells	Species	Injury type	Site of injection	Results	Salient features	Reference
mesenchymal stromal cells (BMSCS)					space of the spinal cord	
Immortalized human NSC line over expressing VEGF (F3.VEGF cells)	Adult Sprague–Dawley female rats	Contusion	2 mm rostral and 2 mm caudal from the lesion epicenter	Elevated the amount of VEGF in the injured spinal cord tissue and increased phosphorylation of VEGFR flk-1. Enhanced cellular proliferation and tissue sparing.	VEGF increased the number of early proliferating cells that differentiated into mature oligodendrocytes.	Kim et al
Poly(lactic-co-glycolic acid) (PLGA) seeded with neural stem cell (NSC)	Adult dog	Hemisection	Lesion site	Grafted NSC survived the implantation procedure and showed migratory behavior.	Ectopic expression of a therapeutic neurotrophin-3 gene was observed.	Kim et al
hESC derived PROGENITOR CELLS						
hESC-derived oligodendrocyte progenitors (OPC) and/or motoneuron progenitors (MP)	Adult rats	Complete transection	Site of injury	Locomotor function was significantly enhanced. OPC and MP survived, migrated, and differentiated into mature oligodendrocytes and neurons.	The recoveries can be attributed to the reconnection of the axons above and below the lesion site.	SLAVEN ERCEG et al (
hESC derived Oligodendrocyte Progenitor Cells	Female Sprague Dawley adult rats	Contusion	Lesion site	Transplanted cells survived, redistributed over short distances, and differentiated into oligodendrocytes.	Widespread oligodendrocyte remyelination throughout the white matter.	Keirstead et al

**Table 2.** Provides a list of preclinical animal studies conducted for spinal cord injury



#### 6.4.1. Current status of cell replacement therapy

During the last 2 decades, the search for new therapies has been revolutionized by the discovery of stem cells, which has inspired scientists and clinicians to search for stem cell-based reparative approaches to many diseases. The adult spinal cord harbors endogenous stem/progenitor cells, collectively referred to as neural progenitor cells (NPCs) that might be responsible for normal turnover of the cells. However, the proliferative activity of endogenous NPCs is too limited to support significant self-repair after SCI. Thus, various cellular transplantation strategies have been adopted in models of SCI.

Current goals of cell replacement approach are broadly classified into two broad types: 1) regeneration and 2) repair. Alternatively the cell transplanted may promote protection to the endogenous cells from further damage.

A summary of cell therapy approaches has been listed in Table 2 mentioned above.

#### 6.4.2. Different cell types proposed to have therapeutic potential

##### *Human Embryonic Stem Cell derived progenitor cells*

Cocultures of hESC derived oligodendrocytes with or without motor neuron progenitors have been used for the treatment of SCI by different researchers with different injury models [14, 17]. The functional recovery concluded by both study is in vivo differentiation of the transplanted cells into oligodendrocytes and neurons promoting remyelination and axonal re-growth.

##### *Adult derived stem cells*

##### *Bone marrow derived stem cells*

In a hemisection model of rats, Bakshi et al has shown that BMSC co-transplanted with that of neural progenitors shows better cell migration and grafting when injected intraventricularly or intrathecally. However, intravenous route shows the least cell migration to the site of injury. Alternatively, Urdzиковá and his team reported that when BMSCs were transplanted intravenously with GCSF in subcutaneous region, spared white matter increases in size and enhanced recovery of hind limb sensitivity was observed. A canine model of injury using both auto and allogeneic BMSCs transplanted intrathecally shows improvement in neurological signs. But the mechanism of recovery observed was the synchronized action of the growth factors released by the grafted cells [11].

Strangely no functional recovery was observed in rat model of SCI wherein a co culture of Spinal cord-derived NSPCs and BMSCs were transplanted at the lesion site. Alternatively a reverse correlation was observed between the functional scores and number of astrocytes transplanted [25]. But the same group of cells when injected via LP shows potent oligodendrocyte marker [21].

##### *Adipose tissue derived stem cells*

In 2009, Hak-Hyun Ryu and his colleagues reported the use of adipose derived stem cells on a canine model of SCI using compression method. ADMSCs show better recovery by signifi-

cant increase in nerve conduction, neuronal transdifferentiation and production of bFGF and VEGFR3 in large quantity. In yet another study [2] using rat as animal model of SCI, transplanted ADMSC and Mouse Neural Stem Cells (mNSCs) and observed that ADMSC protect mNSCs from apoptosis and increases the survival rates by secreting biomolecular substances, preferably VEGF in various conditions like hypoxia, oxidative stress and combined injury.

#### *Human umbilical cord mesenchymal stem cells*

In two trans-section and one contusion injury model of rats studied using Human umbilical cord mesenchymal stem cells had revealed that the grafted cells survived, migrated and produced large amount of GDNF, BDNF, NT-3, bFGF [9] glial cell line-derived neurotrophic factor, [13] neutrophil-activating protein-2, glucocorticoid induced TNF-receptor, and VEGFR 3 [16].

#### *Glial precursor cells*

Improved transcranial magnetic motor-evoked potential responses and improved electrophysiological and locomotor functional recovery was observed in rat contusion model of spinal trauma using Multi-neurotrophin-expressing glial-restricted precursor cells. The reason behind the functional recovery in restoring conduction was proposed to be formation of myelin sheath around the axons by the grafted cells [12].

#### *Neural stem cells and Neural progenitor cells*

Various animal injury models were studied for the transplantation of NSC/NPC. This include primates and rodents model. NSC in PLGA scaffold was tested in African Green Monkey using hemisection and Pritchard concluded the regulatory mechanism as the signaling by various factors released by NSCs [10]. In a contusion model of injury using marmoset, Iwanami et al [18] reported the differentiation of NSPCs into astrocytes and oligodendrocytes which promotes remyelination and promotes functional recovery. In another contusion injury model, the efficacy of EPI-NSC in restoring function was due to differentiation of grafted cells into Gaba-ergic neurons and myelinating oligodendrocytes resulting in neuroprotection, angiogenesis and scar modulation [18].

Expression of a therapeutic neurotrophin-3 gene, which leads to the recovery, was observed when PLGA coated with NSC was grafted in a canine hemisection model [20]. While others [25] observed no functional improvements in either groups transplanted with spinal cord-derived NSPCs and BMSCs on rats at the lesion site, Mothe et al observed recovery by injecting the cells via LP. Differentiation of SC derived NSPC into astrocytes and oligodendrocytes were observed by both the teams. Elevated amount of VEGF in the injured spinal cord tissue and increased phosphorylation of VEGFR flk-1 enhanced cellular proliferation and tissue sparing and increase in the density of blood vessels was the result reported by Kim et al using immortalized human NSC line over expressing VEGF (F3.VEGF cells) in a contusion model of injury in rats [15].

### 6.5. Clinical trials for SCI

Cell transplantation therapies have become a major focus in pre-clinical research as a promising strategy for the treatment of spinal cord injury. Various types of stem cells such as bone marrow stromal cells (BMSCs), adipose tissue Mesenchymal stem cells (ADMSCs), Schwann cells, olfactory ensheathing cells (OECs), neural stem cells or progenitor cells have been reported for their potential to form myelin, promote axonal regrowth and guidance, bridging the site of injury.

More than a dozen of clinical trials have been registered in the official website of clinical trials (<http://www.clinicaltrials.gov>). A brief listing of the selected trials is given below.

The results obtained are as follows:

S. No	NCT study number	Title/ Brief summary	Study type/ phase	Study status
1	NCT01325103	To evaluate autologous bone marrow stem cells transplantation as a safe and potentially beneficial treatment for patients with spinal cord injury	Interventional, Phase I	Active, not recruiting
2	NCT01490242	Phase I/II, multicenter, prospective, non-randomized, open label study to evaluate the safety/efficacy of autologous bone marrow-derived stem cell transplantation in spinal cord injury patients.	Interventional, Phase I / II	Recruiting
3	NCT01393977	To study the efficacy difference between Rehabilitation Therapy and Umbilical Cord Derived Mesenchymal Stem Cells transplantation	Interventional, Phase II	Recruiting
4	NCT01328860	1. To see if Bone Marrow Cell harvest and transplantation are safe in children and 2. To determine if late functional outcome is improved following Bone Marrow Cell transplantation.	Interventional, Phase I	Recruiting
5	NCT01446640	A phase I/II trial designed to establish the safety and efficacy of intravenous combined with intrathecal administration of autologous bone marrow derived mesenchymal stem cells	Interventional, Phase I / II	Recruiting
6	NCT01162915	A Phase I, single-center trial to assess the safety and tolerability of an intrathecal infusion (lumbar puncture) of autologous, ex vivo expanded bone marrow-derived mesenchymal stem cells	Interventional, Phase I	Active, not recruiting
7	NCT01321333	A Phase I/II Study of the Safety and Preliminary Efficacy of Intramedullary Spinal Cord Transplantation of Human Central Nervous System (CNS) Stem Cells	Interventional, Phase I / II	Recruiting

S. No	NCT study number	Title/ Brief summary	Study type/ phase	Study status
		(HuCNS-SC®) in Subjects With Thoracic (T2-T11) Spinal Cord Trauma		
8	NCT01186679	Surgical Transplantation of Autologous Bone Marrow Stem Cells With Glial Scar Resection for Patients of Chronic Spinal Cord Injury and Intra-thecal Injection for Acute and Subacute Injury	Interventional, Phase I / II	Completed
9	NCT01274975	To assess the safety of intravenous autologous adipose derived mesenchymal stem cells transplant in spinal cord injury patients.	Interventional, Phase I	Completed
10	NCT00816803	To assess the safety of autologous bone marrow derived cell transplant in chronic spinal cord injury patients.	Interventional, Phase I / II	Completed
11	NCT01217008	To evaluate the safety of GRNOPC1 administered at a single time-point between 7 and 14 days post spinal cord injury	Interventional, Phase I	Active, not recruiting
12	NCT01231893	Assessment of the safety and feasibility of transplantation of autologous olfactory ensheathing glia and olfactory fibroblasts obtained from the olfactory mucosa in patients with complete spinal cord injury.	Interventional, Phase I	Recruiting

In an article published, Wolfram Tetzlaff et al has reviewed in detail, all the types of cells being used in the treatment of spinal cord injury from the available pre-clinical literature. Their review shows that rodent stem cells have been most extensively studied for SCI. Limited studies have been done on human stem cells. Majority of trials are with bone marrow stromal cells. Also reported was, while chronic treatments were rare and often failed to yield functional benefits, all the preclinical studies conducted, was in acute and subacute stage [8].

Also Fehlings et al [9] in his recently reviewed article has shown the efficacies and limitations of every type of cells, either alone or in various combinations as registered for trial studies, in use and has demonstrated the potential use of other promising candidate stem cells evaluated in pre-clinical studies but are not yet in Clinical Trials. Also they have made recommendations for the conduct and evaluation of pre-clinical studies and clinical trials of cell therapies for SCI [9].

However no clinical intervention is risk free and we require understanding more on the pathophysiology of SCI and the clinical potential of stem cells to translate the use of the same as a therapeutic agent.

NPCs/OPCs:

Geron conducted a Phase 1 clinical trial in the United States in October 2010, to evaluate the safety of human embryonic stem cell-based product candidate, GRNOPC1, in patients with thoracic spinal cord injuries. Accordingly, GRNOPC1, an investigational product for treatment of Spinal Cord Injury, is a population of living cells containing oligodendrocyte progenitor cells (OPC).

HUMSCs:

WJCs can undergo repeated freeze–thaw cycles without a significant loss of viability, mesodermal differentiation potential, and without accumulating karyotypic abnormalities and thus represent a potential for the treatment of the neurodegenerative disorders including SCI. Two studies so far have examined the use of WJCs in SCI models, but were poorly conceived and designed.

### 6.6. Ongoing clinical trials for spinal cord injury using stem cells

Based on the encouraging preclinical animal results, Sarel *et al.*, has conducted a phase II clinical trial of a cell therapy for patients with acute spinal cord injury using monocytes isolated from peripheral blood of human donors. They were able to stimulate by co-incubation with skin tissue, producing a distinct cellular phenotype which is said to be associated with wound healing. These features of skin-co incubated macrophages suggest possible mechanisms by which they may support an immune response that promotes neuronal cell survival and repair.

Jones *et al.*, 2004 [105] observed the long-term outcomes after complete spinal cord injury followed by subsequent treatment with a therapy consisting of autologous incubated macrophages that have been pre-incubated with autologous skin and injected into the lesion site. The study so far has been conducted on 14 patients. Recovery of clinically significant neurological function has been observed in several subjects after treatment, whereas untreated patients with complete SCI rarely recover significant function.

Auerbach *et al.*, 2004 [106], has conducted open-label, non-randomized trials to assess the safety of autologous macrophages in 16 patients with acute complete spinal cord injury. The macrophages were prepared from monocytes isolated from patient blood and co-incubated with autologous skin tissue. The cells were then injected into the spinal cord parenchyma within 14 days of injury. The study shows that administration of autologous macrophages has a favorable benefit to risk ratio for the treatment of patients with acute, complete spinal cord injury.

Keirstead *et al.*, 2005 [107] have shown human embryonic stem cells differentiate into oligodendrocytes in high purity and showed regeneration of the spinal cord in rat. On the basis of this study Geron Inc is currently conducting a FDA approved phase-I clinical trial.

Moviglia *et al.*, 2006, [108] demonstrated a case report of two patients who were administered BM-MSCs co cultured with an autologous pure population of T cells, intravenously 48 hours prior to transplantation of trans-differentiated NCS. This was followed up with 6 months of

neuro-rehabilitation. The authors conclude that *in vitro* cultures of MSCs and anti CNS T cells can induce transdifferentiation of MSCs into neural stem cells.

Kang *et al.*, 2005 [109] transplanted human umbilical cord cells into a 37 year old female with T11/T12 complete injury and have observed recovery but have not ruled out the fact that the laminectomy itself may have released compressed areas of the spinal cord and brought about recovery.

Zhou *et al.*, 2004 differentiated BM-MSCs into neural stem cells and transplanted them into SCI patients. 3 patients reported adverse events of intracranial infection requirement treatment. This study has not mentioned the baseline status of the patients, neither the details of the follow-up study conducted nor the details of the intervention.

Deda *et al.*, 2009 [110], reported that autologous hematopoietic progenitor stem cells are an effective and safe method for treatment of chronic SCI. In this study autologous hematopoietic progenitor stem cells were injected at the site of injury and three weeks post transplantation the patients have demonstrated improved sensory and motor functions.

## 7. Our experience

Realizing the unmet medical need in producing reasonable clinical recovery in spinal cord injury we have designed a preclinical experimental animal study. We developed a rodent model of spinal cord contusion injury and transplanted bone marrow derived mesenchymal stem cells both at the site of injury and into the CSF using lumbar puncture technique. The results were very encouraging [111]. Motivated by this an initial pilot study was conducted on 10 patients with chronic spinal cord injury. The initial results showed only partial sensory improvement. Only 2 patients showed minimal motor improvement but not clinically useful. Surprisingly 4 of them showed reasonable improvement in bladder function. This fact has triggered further interest in us to pursue this and try different methods to improvise the clinical results. Though an attempt was made to quantify the recovery, none of the existing methods were satisfactory.

But this study has raised several questions like a)Timing of intervention) Route of cell administration c)Dosage of cells D)Type of cell e) Number and interval of doses f)Autologous vs allogenic MSC g) problems of chronic injury h)Method of monitoring, evaluation and quantifying the results.

In our further study we attempted to address some of these questions: 1) route-Intrathecal, direct at the site of injury, Direct delivery into the cord during surgery 2) excision of scar 3) scaffold to bridge the damaged ends of the cord 4) number of injections 5) number of cells 6) Cell type – mesenchymal autologous, allogenic & mononuclear 7) source-bone marrow, adipose and Wharton jelly 8) additional systemic injections. 100 volunteers with clinically complete cord injury were recruited. Clinical, MRI and tractography were done at baseline and at periodic intervals to monitor the course of events post stem cell infusion.

## 8. Study plan

The objective of this study was to demonstrate the safety and feasibility of various stem cells as a possible therapeutic strategy for Spinal cord injury. For this, 52 volunteers were recruited and grouped into 4, on the basis of stem cells they received for the treatment. Group 1 received autologous bone marrow derived mononuclear cells (BMMNCs) for transplantation, group 2 were infused with autologous bone marrow derived Mesenchymal stem cells (BMMSCs), while group 3 were transplanted with different allogeneic stem cells (subgroup 1: Bone Marrow derived Mesenchymal cells, subgroup 2: Wharton's Jelly derived Mesenchymal stem cells (WJMSCs) and subgroup 3: Adipose Tissue derived Mesenchymal cells (ADMSCs). Also, in this study, we demonstrated, delivery of stem cells via 3 different routes (laminectomy, lumbar puncture, site of injury guided by CT scan and intravenous delivery) were safe and feasible and do not cause any infections and adverse reactions post transplantation.

### a. Regulatory approval, Informed consent:

As per national guidelines, approval from institutional ethics committee (IEC) was taken and informed consent was obtained from every patient who participated in the study. Any deviations, drop-outs and adverse events were documented and the IEC informed.

### b. Patient selection:

Patients were enrolled for this study as per the inclusion and exclusion criteria designed by and adapted in a pilot clinical study [1]. The inclusion Criteria in the study was as follows

- i. the patients could be of either sex,
- ii. must be between the ages 18 and 55 years,
- iii. the level of spinal injury between C4 and T10 level (neurologic),
- iv. (SCI was clinically complete and categorized as per the American Spinal Injury Association (ASIA) impairment scale.

Exclusion criteria for the study was

- i. Difficulty in assessing the size and location of the injury multiple sites of injury,
- ii. gun shot or penetrating injuries,
- iii. serious pre-existing medical conditions, disease or impairment that precluded adequate neurologic examination
- iv. Respiratory insufficiency requiring support.
- v. if he/she is enrolled in any other clinical trial
- vi. Not able to understand and comply with follow up
- vii. Diagnosed with infections like HIV, HCV, CMV and VDRL.
- viii. Fixed deformities.

**c. Screening of the patients:**

Before enrollment each patient was screened for HIV: Human Immunodeficiency Virus; HBV: Hepatitis B Virus; HCV: Hepatitis C Virus; CMV: Cytomegalovirus; and VDRL: Venereal Disease Research Laboratory, by a nationally certified testing laboratory.

**d. Isolation and propagation of stem cells:**

**i. Autologous Bone Marrow derived Mesenchymal Stem Cells:**

Patients willing to undergo autologous cell transplantation were screened 7 days before the aspiration for infectious disease mentioned above. Thereafter, BM-derived MSC were isolated and expanded using a method reported previously [1].

Briefly, 60 ml BM was aspirated aseptically from the iliac crest of each patient under aseptic conditions. The BM was diluted (1:1) with Knockout Dulbecco's modified Eagle's medium (KO-DMEM) and centrifuged at 1800 r.p.m. for 10 min to remove anticoagulants. The supernatant was discarded and the BM washed once with culture medium. Mononuclear cells (MNC) were isolated by layering onto a lymphoprep (Axis Shield, Norway) density gradient. The MNC present in the buffy coat were washed again with culture medium. The mononuclear fractions containing MSC were plated at a density of 1000 cells/cm<sup>2</sup> onto T-75cm<sup>2</sup> flasks and cultured in KO-DMEM. The media were supplemented with 10% fetal bovine serum (FBS), 200 mM Glutamax and Pen-Strep. The cultures were maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 2 days. The non-adherent cells were removed after 48 h of culture and replenished with fresh medium. Subsequently, the medium was replenished every 5th day until the required number of cells obtained. Once confluent, the culture flasks were washed with Dulbecco's Phosphate Buffered Saline (DPBS) and harvested using 0.25% Trypsin-EDTA solution and re-plated in 5 cell stacks (Corning, USA) for further expansion till the required number of cells obtained. On the day of transplantation the cells were harvested and suspended in saline solution, packed in sterile container and given for the transplantation procedure.

**ii. Autologous Bone Marrow derived Mononuclear cells (MNCs):**

All the patients were examined by a designated medically qualified staff member to establish their eligibility for bone marrow aspiration. Briefly, 60 ml BM was aspirated aseptically from the iliac crest of each patient under aseptic conditions. The BM was diluted (1:1) with Knockout Dulbecco's modified Eagle's medium (KO-DMEM) and centrifuged at 1800 r.p.m. for 10 min to remove anticoagulants. The supernatant was discarded and the BM washed once with culture medium. Mononuclear cells (MNC) were isolated by layering the bone marrow samples onto a lymphoprep (Axis Shield, Norway) density gradient. The MNC present in the buffy coat were washed again with culture medium and then with saline for 2-3 times, resuspended in the same and given for infusion.



## 9. Scaffolds

Scaffolds are basically structures to support and connect the cut ends of spinal cord. They are used after scar excision or otherwise in chronic injuries to bridge the healthy ends. The stem cells are deposited over the membrane. It helps to hold the cells in place, and grows along using this as support. We have used Gelfoam as well as a special biological membrane, which is inert and biocompatible made out of Chitosin. It is a thin and transparent glucosamine polymer. Stem cells have grown in sheets over this membrane in our invitro studies.

In acute phase, the chemical changes resulted out of injury presumably attracts stem cells even after remote injection whereas in chronic injuries there are additional problems.

1. Scar intervenes ends of normal cords 2. In severe injuries there is thinning and atrophy causing anatomical discontinuity. 3. Due to ongoing degeneration there is a functional void between the two ends, with or without an abnormal cord intervening. The main purpose of scaffolds is to bridge this gap and create continuity for the cells to reach both ends.

## 10. Screening of potential donors for bone marrow aspiration

Potential voluntary donors were interviewed, counseled and examined by the investigator or a designated medically qualified staff member to establish their eligibility for bone marrow aspiration. Donors were informed with full description about the nature and purpose of the aspiration and written consent were obtained from them before proceeding with study. Some of the inclusion criteria include (i) the donor must be healthy (ii) may be of either sex (iii) must be between 18-30 years of age (iv) able to understand the voluntary donation program, and ready to provide voluntary written informed consent. The donors were excluded if (i) diagnosed with a past history of illness such as autoimmune disorders, tuberculosis, malaria and any other infection, any illness which precludes the use of general anesthesia, history of malignancy, diabetes, hypertension, significant heart disease, genetic or chromosomal disorders, history of any inherited disorders, hemoglobin less than 10, and pregnant women. Also, at the time of obtaining informed consent they were screened for infection with human immunodeficiency virus (HIV), hepatitis B (HBV), hepatitis C (HCV), cytomegalovirus (CMV), and syphilis (VDRL) and excluded, if found positive.

## 11. Allogeneic BM-MSCs

As per the donor selection criteria, donors were recruited and bone marrow samples were aspirated from the iliac crest of the donors and further processed for the isolation of mononuclear fraction using Lymphoprep (Axis Shield, Norway) density gradient. Thus obtained fraction was seeded in T-75cm<sup>2</sup> and cultured at 37°C in 5% CO<sub>2</sub> atmosphere. The non-adherent cells were removed after 48 hours by replacing the medium and the adherent cells were grown

for additional 4 or 5 days till it reached 80-85% confluency. On confluency, confluence, adherent cells were detached by treatment with a Trypsin-EDTA solution and re-plated at a density of 1000 cells/cm<sup>2</sup> in 5 cell stacks and cultured in the same condition for 14-16. The cell stacks were checked regularly and replenished with medium on every 5th day. The cells were then harvested at 80-90% confluency and cryopreserved in 10% Dimethyl Sulfoxide (DMSO, Sigma-Aldrich) and 85% Plasmalyte (Baxter, USA) and 5% Human Serum Albumin (HSA, Baxter) in liquid nitrogen till further use.

## 12. Adipose tissue derived mesenchymal stem cells

The use of lipoaspirate as a source for stem cells with multipotent differentiation potential offers a far less invasive procedure for cell sampling than the aspiration of bone marrow (BM), and numbers of stem cells obtained are reportedly higher in lipoaspirate than its BM counterpart. Lipoaspirate, an otherwise disposable byproduct of cosmetic surgery, has been shown to contain a putative population of stem cells, termed adipose-derived stem cells (ADSCs) that share many similarities to marrow stromal cells (MSCs) from BM, including multilineage differentiation capacity. Furthermore, these cells also show high colony-forming unit frequencies as well as an apparent pluripotent ability to differentiate to cells of a neuronal phenotype [9, 10].

This protocol describes the preparation of MSCs from human lipoaspirate obtained from cosmetic surgery. Briefly, the liposuctioned fat first washed thoroughly in phosphate-buffered saline (PBS) with antibiotic solution (Penstrep, 2X), until the bottom layer containing blood cells contaminant was clear, before being subjected to enzymatic digestion using collagenase type I (0.2%, diluted in KO-DMEM) for 45-60 minutes at 37°C in shaking condition, in order to obtain a soupy single-cell suspension. After digestion, the action of collagenase was neutralized by the addition of FBS. The suspension was then mixed well and passed through 40 µm cell strainer before being subjected to centrifugation at 1400 rpm for 10 minutes. After centrifugation, cell pellet, termed as stromal vascular fraction (SVF) is resuspended in KO-DMEM and seeded in a T-75cm<sup>2</sup> flask at a density of 1,000 cells/cm<sup>2</sup>. The non-adherent cells were removed after 48 h of culture and replenished with fresh medium. Subsequently, the medium was replenished every 4th day and the cells were harvested at 80% confluency and replated in 5 cell stacks to obtain the sufficient number of cells required for the infusion. The plates were checked for confluence every day and the cells are fed with fresh medium. After the cell stacks were confluent enough, the cells were harvested using Trypsin-EDTA solution and cryopreserved in liquid nitrogen till further use.

## 13. Wharton's Jelly derived mesenchymal stem cells

Studies have demonstrated the multipotent properties of mesenchymal stromal cells isolated from the inner matrix of the Wharton's Jelly derived from the umbilical cord. These cells have

also been demonstrated to differentiate into neuronal lineage and supporting glia [11, 12]. Based on these studies, in the current trial we have attempted to understand the therapeutic potential of WJ-MSCs in spinal cord injury.

After appropriate informed consent, a clean, healthy, straight clamped umbilical cord approximately 10 cms in length was collected in sterile normal saline bottle and transported to the laboratory. Briefly, the umbilical cord was washed with normal saline followed by DPBS (with 0.2% of Penstrep solution) wash for 3-4 times. This was followed by quick dip in 100% ethanol and was cleared off in DPBS. The tissue was washed free of contaminating blood with normal saline throughout the process and cut into 2-5 mm<sup>3</sup> pieces. Using sterile scalpel and forceps the cord was dissected, unfolded and the exposed arteries and vein were removed and discarded. The cord was then scrapped gently with scalpel to obtain the viscous, jelly like substance. The obtained suspension was passed through a 100 mm cell strainer to obtain single-cell suspension. The resultant suspension was then diluted with saline to reduce the viscosity of the suspension. Cells were centrifuged at 1400 rpm for 10 minutes at 37°C and the pellet was resuspended in KO-DMEM supplemented with FBS (10%), Glutamax (1%), Penstrep (0.5%), FGF-2 (1ng/ml) and cultured in T-75cm<sup>2</sup> flasks at 37°C in 5% CO<sub>2</sub> until confluent (80-85%). Upon confluency, the cells were harvested from the flasks and transferred to 5 cell stacks at a seeding density of 1000 cells/cm<sup>2</sup> for 10-12 days in order to obtain the required number of cells for the transplantation. The harvested cells were processed and frozen in cryobags in liquid nitrogen till use.

## 14. Characterization

### 1. Immunophenotype:

This is a technique used to study the expression of cell surface antigens on the MSCs using flow cytometry. Briefly, the cells were dissociated with 0.25% Trypsin-EDTA and resuspended in wash buffer at a concentration of  $1 \times 10^6$  cells/ml. 200 µL cell suspensions were incubated in the dark for 15 min at 4°C with saturating concentrations of phycoerythrin (PE) conjugated antibodies. The following markers were analyzed: CD34-PE, CD45-PE, CD73-PE, CD105-PE, CD166-PE, and CD90-PE (BD Pharmingen, San Diego, CA, USA). Flow cytometry was performed on a 5HT Guava instrument. Appropriate isotype-matched controls were used to set the instrument parameters. Cell viability was measured using 7-amino actinomycin D (7-AAD). Cells were identified by light scatter for 10,000 gated events and analyzed.

### 2. Multipotent differentiation assay

The mesenchymal properties of human stem cells isolated from various sources as described above, were investigated using specific differentiation kits for the three different lineages i.e., osteogenic, adipogenic and chondrogenic (as per ISCT criteria).

Briefly, *Osteoblast differentiation* was induced by culturing human MSCs in KO-DMEM supplemented with 10% FBS (Hyclone), 200 26mM Glutamax (Invitrogen), 10<sup>-8</sup> M dexamethasone (Sigma-Aldrich), 30 µg/ml ascorbic acid (Sigma-Aldrich) and 10 mM β-glycero-

phosphate (Sigma-Aldrich Chemical Private Limited, Bangalore, Karnataka, India) for 3 weeks. Fresh medium was replenished every 3 days. Calcium accumulation was assessed by von Kossa staining. The differentiated cells were washed with PBS and fixed with 10% formalin for 30 min. The fixed cells were incubated with 5% AgNO<sub>3</sub> for 60 min under ultraviolet (UV) light and then treated with 2.5% sodium thiosulphate for 5 min. Images were captured using an Nikon Eclipse 90i microscope (Nikon Corporation, Towa Optics, New Delhi, India; [www.nikon.com](http://www.nikon.com)) and Image-Pro Express soft ware (Media Cybernetics Inc., Silver Spring, MD, USA; [www.mediacy.com](http://www.mediacy.com)).

To induce *adipogenic differentiation*, human MSCs were cultured for 21 days in KO-DMEM supplemented with 10% FBS, 200 mM Glutamax, 1 µm dexamethasone, 0.5 mM isobutylmethylxanthine, 1 µg/ml insulin and 100 µm indomethacin (from Sigma-Aldrich). Inducing factors were fixed in 10% formalin for 20 min and 200 µl Oil Red O staining solution added and incubated for 10 min at room temperature. The cells were rinsed five times with distilled water. The images were captured using Nikon Eclipse 90i microscope (Nikon) and Image-Pro Express software (Media Cybernetics).

For *chondrogenic differentiation*, human MSCs were cultured for 21 days using Chondrogenesis differentiation kit (Life Technologies, USA) as per the manufacturer's recommendations and stained with Safranin O as specified. The images were captured using Nikon Eclipse 90i microscope (Nikon Corporation, Towa Optics, New Delhi, India).

#### **14.1. Karyotyping**

A standard G-banding protocol was performed by analyzing more than 200 cells per sample and reported according to the International System for Human Cytogenetic Nomenclature (ISCN). If the cells did not fall under the set standard of the above mentioned tests, they would not be released to the patient for transplantation was discarded appropriately.

#### **14.2. Quality control testing**

Based on the ISCT guidelines, certain quality control tests were performed on the end product before transplantation. These include Mycoplasma (using RT-PCR based method), Endotoxin testing by Limulus Amebocyte Lysate (LAL) method and cell surface markers like CD73, CD90, CD105, CD166, CD34, and CD45 via flow cytometry. The positive markers (CD73, CD90, CD105 and CD166) should be greater than 95% positive, while the negative markers (CD34 and CD45) must be less than 2% positive. 7-AAD (7-amino actinomycin D) was also analyzed via flow cytometry to determine the cell viability.

#### **14.3. Processing of cells for transplantation**

As described above, the cells were harvested and processed for transplantation. Briefly, the total cell count was calculated using a standard hemocytometer. The cells were washed several times with normal saline solution and finally resuspended in saline containing 0.2% human serum albumin. All the syringes and bottles were appropriately labeled. These were packaged

in a sterile container and dispatched in a transportation container maintained at 22°C to the hospital for transplantation via the shortest route.

#### 14.4. Route of administration

1. Intrathecal administration through Lumbar Puncture (LP) method: The patient was positioned in lateral decubitus position and the part prepared. Under aseptic conditions lumbar puncture was performed at lowest possible level usually L4-5 or L5-S1 levels. Once clear CSF was obtained the cells were delivered into the intra thecal space gently. The procedure was repeated as per the protocol.
2. Intra Venous – Regular intravenous infusion of cells in 50 ml saline administered into the peripheral veins of the hand.
3. At the site of injury-either by laminectomy or image guidance

Laminectomy-was performed where ever decompression was indicated with or without stabilization. Surgical technique includes prone position and exposure of lamina at appropriate level under general anesthesia. Dura was opened and injured cord was inspected.

Scar excision-In chronic injuries with glial scar or neuroma the intervening tissue was removed gently till healthy appearing tissue was seen under high magnification. Cavity was decompressed. The cells were injected into the ends of the cord tissue through an insulin syringe. If the edges are apart a scaffold or gelfoam was used to bridge the gap. Dura was closed water tight. If the cord was oedematous (acute injury) doroplasty was performed. Additional cells were delivered into intra thecal space and Laminectomy was closed using standard technique.

Image guidance method-In chronic complete injuries CT guided technique was used to deliver the cells directly at the site of injury.

Clinical assessment was performed on all patients based on the parameters of the ASIA impairment scale (American Spinal Injury Association). This was considered as the primary measurable outcome of the clinical study.

A	Complete: No motor or sensory function is preserved in the sacral segments S4-S5.
B	Incomplete: Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-S5.
C	Incomplete: Motor function is preserved below the neurological level, and more than half of key muscles below the neurological level have a muscle grade less than 3.
D	Incomplete: Motor function is preserved below the neurological level, and at least half of key muscles below the neurological level have a muscle grade of 3 or more.
E	Normal: Motor and sensory functions are normal.

**Table 3.** ASIA impairment scale

### **14.5. Follow up schedule**

At every follow-up, the patients were assessed clinically using the ASIA scale rating system and with the Barthel's index (BI) for degree of independence and patient rating. MRI was performed to observe structural changes, if any.

## **15. Isolation and identification of mononuclear cells and mesenchymal stem cells**

### **15.1. Autologous BM derived MNCs**

BM samples were aspirated from the patients (n=9) after getting proper consent and the samples were processed in cGMP compliant clean room facility for the isolation of the MNCs following the standardized protocol as described above. CD34 expression was analyzed using PE conjugated CD34 antibody in flow cytometer and cell count was performed prior to transplantation.

### **15.2. Autologous bone marrow derived MSCs**

BM samples obtained from the patients (n=11) after getting proper consent were processed in cGMP compliant clean room facility for the isolation propagation and expansion following the standardized protocol as described above. Flow cytometry analysis revealed that cell samples with positive markers are >95% and <2% of negative markers with >90% viability with 7AAD staining indicating the cells were mesenchymal in nature. Multipotent characteristics, as determined by Oil Red O stain, Von Kossa stain and Safranin O stain, respectively indicates the cell samples undergo adipogenic, osteogenic and chondrogenic differentiation. Karyotypes of all the cell samples were normal and no abnormalities/aberrations were found after ex vivo propagation figure. Endotoxin test using LAL method and Mycoplasma test using RT-PCR were found to be negative indicating the cells were safe for transplantation.

### **15.3. Allogenic BM derived MSCs**

BM samples obtained from the donors after appropriate informed consent and the samples were processed in cGMP compliant clean room facility for the isolation, propagation and expansion. Stem cells thus extracted are cryopreserved as master cell bank (MCB) in liquid nitrogen. From MCB, working cell banks (WCB) were raised in tissue culture plates until required number of cells obtained for the infusion. The cells were then harvested and frozen as investigational product (IP) until use. Prior to transplantation the cells were thawed and processed further. The cell samples were found to express positive markers >95% and <2% for negative markers, with >95% viability, when stained with 7AAD as determined by flow cytometry indicating the Mesenchymal nature of the processed cells. Multipotent characteristics, as determined by Oil Red O stain, Von Kossa stain and Safranin O stain, respectively, indicating the cell samples undergo adipogenic, osteogenic and chondrogenic differentiation. Karyotype of all the cell samples was normal and no abnormalities/aberrations were found

after ex vivo propagation figure. End product testing such as endotoxin test using LAL method and Mycoplasma test using RT-PCR were found to be negative indicating the cells were safe.

#### **15.4. Adipose tissue derived MSCs**

Mesenchymal stem cells isolated from fat samples received in a sterile container after liposuction were expanded in above mentioned conditions until the cells were confluent. Post confluent, the cells were harvested and stored frozen in liquid nitrogen as MCB, from which WCB were raised. IP were cultured on appropriate tissue culture plates on request. Prior to transplantation, in process test and end process test were done. Flow cytometric analysis showed that the cells express the surface markers with >95% for positive markers (fig) and <2% for negative markers (fig) with >90% viability. The cells were found to undergo adipogenic, osteogenic and chondrogenic differentiation as determined by Oil Red O stain, Von Kossa stain and Safranin O stain, respectively. All the samples showed normal karyotypes and no abnormalities/aberrations were noted after ex vivo propagation. A representative ideogram is illustrated in Figure. The cell samples tested for endotoxin using LAL method and Mycoplasma test using RT-PCR were found to be negative indicating the cells were safe to be infused.

#### **15.5. Wharton's Jelly derived MSCs**

Umbilical cords obtained postpartum in a sterile container were processed according to the standard protocol described earlier. The cells were further up-scaled and expanded in order to provide the required number of cells for the patient. The cultured cells were found to show normal spindle shaped phenotype when observed (fig). Flow cytometric analysis showed that the cells were positive with >95% for positive markers and <2% for negative markers (fig) with >90% viability. The cells were found to undergo adipogenic, osteogenic and chondrogenic differentiation as determined by Oil Red O stain, Von Kossa stain and Safranin O stain, respectively. All the samples showed normal karyotypes and no abnormalities/aberrations were noted after ex vivo propagation by standard G banding method. A representative ideogram is illustrated in Figure. The cell samples tested for endotoxin using LAL method and Mycoplasma test using RT-PCR were found to be negative indicating the cells were safe for the transplantation.

## **16. Clinical assessment**

### **16.1. Clinical examination and ASIA scale scoring**

Clinical assessment was performed on all patients based on the parameters of the ASIA impairment scale. This was considered as the primary measurable outcome of the clinical study.

## 16.2. Results

As per the inclusion and exclusion criteria mentioned above, 52 volunteers were recruited for this study. This includes 8 females and 44 males between the age group 17 and 66 years. Duration of injury varied between 15 days after injury to 20 years. All the patients were divided into 4 groups based on the type of cells received. The details of the patients recruited for this study are given in Tables below.

Case No.	Age	Sex	Level of injury	Duration of injury	No. of injection	Route of infusion
1	23	M	D11-D12	0 month	1	Laminectomy + IV
2	31	M	D4, C6-7	7 months	1	Laminectomy + IV
3	23	M	D11	4 months	1	Laminectomy + IV
4	26	F	C5-C6	1 year	1	Laminectomy + IV
5	21	M	C4-C5	1 year	1	Laminectomy + IV
6	26	F	C5-C6	1 year	1	Laminectomy + IV
7	53	M	C6-C7	3 years	1	Laminectomy + IV
8	23	M	C5-C6	3 years	1	Laminectomy + IV
9	31	M	C6-C7	4 years	1	Laminectomy + IV

**Table 4.** Group 1-Autologous Bone Marrow derived mononuclear cells (BMMNCs; n=9).

Case No.	Age	Sex	Level of injury	Duration of injury	No. of injection	Route of infusion
1	59	F	D3-D5	7 years	1	CT Guided
2	34	M	C7	6 years	1	CT Guided
3	56	M	D4	14 years	1	CT Guided
4	54	M	D5-D6	2 years	1	CT Guided
5	49	M	D6	4 years	1	CT Guided
6	26	M	D12	2 years	1	CT Guided
7	23	M	C4-C6	1 years	1	CT Guided
8	31	M	L1	4 years	1	CT Guided
9	42	M	D12	3 years	1	CT Guided
10	28	M	C5-C6	3 years	1	CT Guided
11	28	M	D5-D6	5 years	1	CT Guided

**Table 5.** Group 2-Autologous Bone Marrow derived Mesenchymal stem cells (BMMSCs; n=11)



Table 6: Group 3-Allogeneic BMMSCs or Adipose tissue derived MSCs (ADMSCs) or Wharton's jelly derived MSCs (WJMSCs) (n=26)

Case No.	Age	Sex	Level of injury	Duration of injury	No. of injection	Route of infusion
1	19	F	D4-D6	1 year	1	CT Guided
2	36	F	D10	1 month	3	Intrathecal
3	27	M	T12-L1	1 year	1	CT Guided
4	36	M	C6-C7	2 months	1	Laminectomy + IV
5	26	M	D3	3 months	1	Laminectomy + IV
6	46	M	C3-C4	0 month	1	Laminectomy + IV
7	29	M	Partial	1 year	3	Intrathecal
8	45	M	C1-L1	1 year	1	Laminectomy + IV
9	50	F	C2	1 year	1	Laminectomy + IV
10	46	F	Dorsal SCI	1 year	3	Intrathecal
11	25	M	D9-D10	1 year	3	Intrathecal
12	29	F	D4	1 year	3	Intrathecal
13	27	M	SCI	10 months	1	Laminectomy + IV
14	26	M	C7-T1	3 years	3	Intrathecal
15	27	M	Cervical	6 months	3	Intrathecal
16	51	M	D4-D6	20 years	3	Intrathecal
<b>(a)</b>						
Case No.	Age	Sex	Level of injury	Duration of injury	No. of injection	Route Of infusion
1	23	M	Cervical	2 years	3	Intrathecal
2	54	M	Thoracic	1 year	3	Intrathecal
3	54	M	C4-C5	6 months	3	Intrathecal
4	31	M	C2-D4	1 year	3	Intrathecal
<b>(b)</b>						
Case No.	Age	Sex	Level of injury	Duration of injury	No. of injection	Route Of infusion
1	47	M	Cervical	8 months	3	Intrathecal
2	37	M	D12	4 years	3	Intrathecal
3	42	M	Thoracic		3	Intrathecal
4	35	M	C3-C4	0 month	3	Intrathecal
5	27	M	C5-C6	6 years	3	Intrathecal
6	37	M	Thoracic	13 years	3	Intrathecal
<b>(c)</b>						

Table 6. (a): Subgroup 1: BMMSCs, (b): Subgroup 2: WJMSCs, (c): Sub group 3: ADMSCs

On an average, 2 million cells /kg bodyweight were transplanted via 3 different routes i.e., laminectomy, lumbar puncture, and intravenous injections. All the patients stood the procedure well, there were no postoperative complications and were discharged within a week's

time from the hospital, indicating that there were no immediate cytotoxic effects due to implantation of various cell types (as mentioned above) and the procedures were safe.

The ASIA rating scale did not reveal any significant changes or further worsening or deterioration in neurological or functional level pre and post stem cells therapy.

Of the total patient recruited for the study, 9 patients have shown notable clinical and functional recovery. While follow-up, one patient (G3C2; Table 4a) whose baseline report was as follows: Motor – Upper limb-5/5, Lower limb-2/5, Sensory – Loss of sensation at D 10 and below for all modalities, reported to be able to stand and walk with support and does swimming.

Another patient (G4C3; Table 5], at baseline with power at shoulder-grade 3/5, Power at elbow joint-3/5 in flexion and extension and hand grip-2/5, lower limbs-grade 0/5 with generalized wasting in all limbs and spasticity in both the lower limbs, anesthesia below C6 dermatomes and exaggerated deep tendon reflexes in lower limbs, has shown minimal recovery.

Slight improvement in Upper Limb sensation after the first dose, was reported by one patient (G1C5; Table 2). However at times, the patient had painful sensations.

Post therapy one patient (G4C4; Table 5) was able to feel the bladder fullness from 3rd month of transplantation. In subsequent follow-up, the imbalance while sitting on the wheel chair has partially improved. Also, improvement in touch and pain sensation up-to the right knee on the right side and up to the upper thigh in the left side were noted. Bladder sensations have improved to some extent.

Additionally two patients (G4C5; Table 5 and G3C4; Table 4c) has shown improvement in sensation and able to sit with support.

Two patients (G3C1 and G3C3; Table 4c &4b) were able to walk with the aid of walker post therapy. But however, the latter patient had a fall and is now back to baseline.

One patient (G3C5; Table 4c) has regained some sensation in abdomen and lower back area and below feet. The patient can now feel stretching sensation in toes when performing exercises and becoming more aware of bowel movements.

Out of the 52 patients treated, only 3 patients reported pain after infusion. And two patients were lost to follow-up.

#### Barthel's Index Score

Barthel's index (BI) was performed on all patients, pre-and post-transplantation of the cells. No significant improvement or appreciable changes were observed in the patients with long history of injury. However, patients with less than 6 months of injury have shown improvement in the scores.

Magnetic resonance imaging (MRI) of the spinal cord before and after stem cell infusion:

No change was observed in MRI findings at baseline and post-stem cell transplantation. Also, no adverse effects of transplantation were detected on the MRI post transplantation. Further, no changes in cystic regions or syringomyelia, and no further external compression of the cord

or formation of tumor-like masses in and around the injection site or along the cord, were visualized.

## 17. Discussion

Spinal cord essentially is a conduit integrating relay and transmission of signals and the functions of the body (motor, sensory and autonomic) with the higher centers (brain brainstem & cerebellum). SCI can be devastating with lifelong disability due to its complex architecture and compounding consequences that follows an injury. Disruption of such local integrative networks interrupts ascending and descending input and outputs resulting in dysfunction of motor, sensory, autonomic and dysregulation of various reflexes in the body. Majority are in the age range of 16-35. Damage to the spinal cord progresses rapidly in stages. In the last two decades, researchers have made their efforts to understand this complex pathobiology from several animal studies [6]. Ischemia, Scarring, cavitation, wallarian degeneration, axonal die back, excitotoxins, inflammation and several complex cellular and molecular changes are known to influence recovery of such injury. Several medical (pharmacological and others) and surgical attempts did not influence any substantial positive outcomes. Hence the attention was turned towards neurotrophic factors and cell based treatments. As a result spontaneous neurological recovery has been reported only in 6-13% of patients with only 2% gaining any functional recovery. [113-116].

Cell death is often rapid after SCI. The adult spinal cord harbors endogenous stem/progenitor cells, collectively referred to as Neural Progenitor Cells (NPCs) that might be responsible for normal turnover of the cells and repair process. Several studies have confirmed that new cells are born around the central canal from the ependymal precursors [1]. However, the proliferative activity of endogenous NPCs is too limited and grossly inadequate to support spontaneous repair after SCI. Hence various cell transplantation strategies have been adopted in models of SCI such as embryonic stem cells, Wharton's jelly, adult neural stem cells, bone marrow and adipose tissue derived Mesenchymal stem cells [13]. They are currently being studied as potential sources of neurons, glial cells or neurotrophic factors. Transplantation of these cells to create or regenerate spinal cord as an alternative therapy has generated lot of interest. This study clearly documents the feasibility of such cell replacement strategies [14].

Several studies have reported several protocols different timings and type of cells [117-120]. We have studied autologous BMMNCs, BMMSCs (autologous and allogenic), WJMSCs and ADMSCs (allogenic) have been used to study their therapeutic potential in spinal cord injury. Those who received autologous BMMNCs showed only minimal improvement. The reason may be due to variations in age; extent, duration of injury; and variance in cell quantity and quality.

Nevertheless, autologous BMMSCs had shown good improvement, the concern with the transplantation is the availability of cells in time and other problems as mentioned above.

Recently, allogenic MSCs from sources like Bone marrow, Adipose tissue and Wharton's jelly shown to have attracted many, to use it as source for treatment and conducting trials on them.

The advantages of allogenic cells over autologous cells for transplantation may be that, they are readily available with defined cell quality and quantity. This makes allogenic stem cells, a good choice to make extensive research on the feasibility in other therapeutic interventions. In addition it offers an opportunity to use the cells as early as possible. It has been the observation that early intervention within few days has yielded marginally better results suggesting an optimal temporal window for cell mediated therapy. [121]. several studies have indicated better results with early intervention and acute injury. [122, 123, 121]. The preclinical literature also has suggested that there is an earlier window for the optimization of cell therapies [125, 124]. Now its well known that these cells do HOME at the required site. Homing could be mediated by the ongoing cell reactions, products of cell death or inflammation or some chemo attractants. We believe timing of delivery of cells is crucial for these cells to impregnate in large numbers. In delayed or chronic injuries cell reach may be poor and once gliosis sets in cell penetration may be difficult. In addition spinalcord –csf barrier doesn't allow cell migration into the parenchyma.

The strategy to cord injury is twofold-Initial control of damage and minimizing secondary deleterious effects, and later promotion of recovery. Cell therapy can play a role in both provided they were given at the right time.. However there is no sufficient data to indicate the exact time of maximizing the benefit. In general those who are likely to be benefited must be treated before the molecular mechanisms cause the irreparable damage. [5] The drawback of our study is timing could not be controlled since they were inducted as and when they came to our clinical service. In addition its difficult to have clinical controls.

A canine study from South Korea 2009 used autologous and allogenic cells. Though autologous BM-MSCs had better results than allogenic both showed better results compared to controls [126]

Literature shows several routes of administration like intra arterial, intravenous, intra thecal and direct injections to the injured cord. Intra thecal injection was most frequently used method. [121]. Our study also demonstrates that administration of MSCs via multiple routes such as laminectomy, lumbar puncture and intravenous delivery, are feasible and safe. Though direct injection into the cord appears logical, the apprehension of enhancing the injury always exists. In our opinion it is invasive and should be reserved for those where decompression or stabilization is indicated and most suitable in chronic complete injuries.. Saberi et al [119] reported no serious adverse effect after intraparenchymal injections. They also reported transient low grade fever with nausea, vomiting and headache. But we did not encounter such complications in our series. The use of scaffold is complimentary and may have positive influence. [110]. In chronic injuries widening of anatomical gap between the functional tissues of the cord is a challenge and a possible reason for poor outcomes. Degeneration makes this anatomical and functional gap wider with time. Often this gap gets replaced by glial scar which becomes a physical barrier preventing cellular penetration, regeneration and migration. Scaffolds can act as an anatomical substrate on which these cells can grow and connect the physiological ends.

It appears that the cytokines and bio active molecules secreted by these cells play a significant role in acute as well as sub acute phases. Therefore it is essential to retain the cells at the required

area in sufficient numbers. We have included only complete injuries so as to remove the bias of spontaneous recovery. Based on our results and the positive role of anatomical continuity we feel partial injuries shall definitely benefit more. Cell therapy can augment the spontaneous recovery either by promotion or reducing the derogatory inhibitory influences.

In our findings delivery through lumbar puncture is simple and equally effective [111-112]. But cell survival in CSF and their functionality need to be enhanced. Retaining large number of cells at the site of injury is also a challenge. We did not encounter any adverse reaction or infection. After lumbar puncture majority had low pressure headaches which were treated with fluid therapy and analgesics effectively. Kishk et al reported neuropathic pain in 56% of their patients following intrathecal injection which was not noticed in our series.

Though the results of animal experiments are enticing the overall translation into clinical benefit is minimal and quite disappointing. Irrespective of site of injury, route of administration and type of cell the clinical recovery is very minimal and only less than 1/3rd showed signs of recovery. Useful functional recovery was seen only in 7-9%. This is rather disappointing. Therefore it appears that even cell therapy has its limitations. But there has been definite evidence of clinical recovery in few and are useful to understand the role of cell therapy. It appears we are somewhere closer to some success yet needs understanding to augment these benefits. Young age, focal segmental injury and early intervention seem to benefit or complement recovery. Though all our patients expressed subjective well being, ability to sit for longer periods and actively participation in physical exercises following cell therapy could be mediated by cytokines and growth factors. In addition trunk muscles just above the site of injury showed definite clinical improvement. This could explain the need of anatomical integrity for recovery and also their enhanced ability to sit longer. In the distal segment sensory, long tracts and bladder have shown signs of recovery in many, but few had clinically useful benefit. Motor recovery is the most difficult to achieve. Possibly due to loss of trophic influence from higher centers, vascularity which leads to loss of anterior horn cells. Presently available imaging and electro physiological methods are not sensitive enough to detect or monitor regeneration in spinal cord. Those who recovered could be potential partial injuries (anatomical continuity) although behaved as complete injuries clinically.. This could be the possible reason of useful clinical recovery observed in our study. Presently we feel role of cell therapy is only complimentary. MSCs are known for immunomodulation and once administered in the right time may help in minimizing neural inflammation and immune mediated damage. Early intervention might reduce gliosis and promote recovery through secretion of cytokines, bioactive molecules and growth factors. These cells also known for angiogenesis hence benefit by revascularization of spinal cord. Lastly the role of effective activation native progenitor cells to come the rescue of adequate repair needs further exploration. Preservation and promotion of recovery of ant horn cells and reestablishing neuronal functional circuits should be the focus.

Going forward, SCI appears to be the most difficult clinical challenge today. Our understanding of its pathobiology is not complete. The challenges are local (site of injury), peripheral (body below the site of injury) and central (higher centers). The future strategy need to target all the three. Augmenting central influences; sustaining muscles with proper neurotization, rehabilitation, promoting recovery and regeneration at the site seems to be the goal. Several

methods and mechanisms alone or in combination need to put in place. Rehabilitation does play a significant role in those with clinical recovery. We speculate that combination of rehab and regeneration may be better. The local neuronal circuits within the segments of the cord must be sustained to retain the integrity of the reflex arc. This appears complex and needs further experimental and clinical data to understand the underlying mechanisms.

## 18. Conclusion

In conclusion, the surge of research activities in the cell therapies for SCI has yielded only mixed results. While the pre clinical studies are quite promising it is difficult to reproduce similar results in the clinical scenario.. Knowing the mechanisms involved stem cells seems to have specific role and prospects for future studies. Future direction should focus on enhancing the benefits of cell therapy by combination of methods systematically addressing the challenges involved. Our study documents safety and influence on recovery to some extent paves the way for further preclinical and clinical studies with proper design. Such larger clinical studies only can overcome the present diversity in methods and outcomes.

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## Author details

N.K. Venkataramanaa<sup>1</sup> and Rakhi Pal<sup>2</sup>

1 Department of Neurosurgery. BGS Global Hospital, Kengeri, Bangalore, India

2 Advanced Neuroscience Allies Research Foundation, India

The authors state that there is no conflict of interest.

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