Role of Bone marrow Mesenchymal Stem Cells in motor recovery of spinal cord injury in rats

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CERTIFICATE

This is to certify that the thesis entitled **"Role of Bone marrow mesenchymal stem cells in motor recovery of spinal cord injury in rats"** was done in the Department of Physical Medicine and Rehabilitation, Christian Medical College and Hospital, Vellore.

Also certified that this thesis is the bonafide work of Dr.George Tharion, from this Department, under my guidance and supervision.

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Introduction

Spinal cord injury is a major catastrophy with no solutions for cure, and realistic expectations and possibilities for spinal cord repair are being acknowledged recently. The incidence of spinal cord injuries is approximately 30 per million. The incidence of spinal cord injuries is highest in the age group of 16 to 30 years. More than 80% of spinal cord injury occurs in males[1]. Unfortunately most of the victims are young, in the prime of life, left with permanent paralysis of limbs, bladder, bowel and sexual functions. This results in far reaching social, vocational and psychological consequences. Prevention of complications, stabilisation of spine and active rehabilitation and long term follow up constitute the mainstay of the management of persons with spinal cord injury. Early rehabilitation in an organised multidisciplinary SCI team has been shown to be beneficial with lower mortality, decreased pressure sores, and slightly greater chance of neurological recovery and shorter lengths of stay in the hospital[2]. Surgical stabilisation helps in early mobilisation, but there are no significant differences in neurological level changes between early surgery, late surgery and no surgery[3]. There was no evidence that routine early surgical intervention or decompression improved neurological outcomes[4]. The national spinal cord injury study NASCIS trials I, II, and III on early steroids to limit cellular damage from secondary injury processes suggested this to be part of the early management of SCI. The conclusions of this study have been challenged due to a variety of reasons[5].

Rehabilitation focuses on functional restoration, capitalising on the residual abilities assisted with devices and equipment. Devices like specialised light weight wheel chairs and orthotic support are commonly used to aid in ambulation. Functional electrical stimulation is used in a variety of neuroprosthesis to substitute for different functions lke gait training. Sacral anterior root stimulator is used to improve functions of bladder, bowel and sexual

function. Body weight support treadmill training could be useful for incomplete injuries. Robotic devices, and development in neuroprosthesis facilitate movement and compensates for the loss of function. Drugs like monosialo tetra hexosyl ganglioside (GM Ganglioside), anti Nogo antibody, omega poly unsaturated fatty acids have shown good results in the experimental spinal cord injury settings. 4 aminopyridine, a potassium channel blocker enhances the axonal conduction of demyelinated fibres but with equivocal results on clinical study[6].

The organisation of spinal cord is unique in that unlike brain the grey matter is located in the centre, and the white matter arranged in the periphery. Spinal cord functions are organised into functional segments grossly. The lesion to grey matter at a spinal cord segment damages the function of that particular segment of the spinal cord, where as the damage to the segment of white matter damages the long tracts which ascend to or descend from the brain, affecting the functions of the body below the level of the segment of injury. The common strategies for the regeneration of damaged spinal cord include methods to prevent progression of secondary injury, provide agents to promote remyelination, remove natural inhibitors of regeneration, provide guidance to direct axons to their target, creation of bridges to promote scaffolds which facilitates axonal growth, or replace lost cells . Stem cell transplantation is used to replace lost cells, but could also play additional role including delivery of molecules that promote regeneration, immunomodulation, enzymatic break down of inhibitory scars, reprogramming of endogenous stem cells, elimination of cellular debris and inhibitory substances, creation of novel bridges and matrix, and improvement in vasculature to facilitate recovery. Stem cells derived from the recipients themselves have the advantage of being naturally compatible without any immunological conflicts, thus avoiding need for immuno-suppression[7].

Stem cells have several characteristics that make them unique in comparison with other cells. They exist as unspecialized cells and maintain undifferentiated phenotype until they receive appropriate signals. They have capacity for extensive self-renewal and this property can be utilized by culturing large number of stem cells in vitro for therapeutic use. Under the influence of specific biological signals they differentiate into specialized cells with a phenotype which is distinct from its precursor[8]. Thus the definition of a stem cell must include two essential characteristics, self-renewal and multipotency. Undifferentiated cells that are found in a differentiated adult tissue are considered as stem cells, which contribute to tissue maintenance and repair. These cells are capable of self-renewal but do not replicate indefinitely in culture. Adult stem cells may differentiate to produce progenitor, precursor and mature cells, but these activities are limited to cells contained in a tissue of origin. Adult stem cells usually comprise a small minority of the total tissue mass and as such are quite difficult to identify and isolate. The concept of stem cell plasticity refers to the phenomenon of trans differentiation which is the ability of an adult stem cell from one tissue to differentiate as specialized cell type of another tissue[9]. Bone marrow is a source for hematopoietic cells as well as non-hematopoietic mesenchymal stromal/stem cells (MSCs). Mesenchymal stem cells are multipotent, self-renewing, that can differentiate into osteoblasts, chondrocyte and adipocytes. Bone marrow derived mesenchymal stromal cells, is autologous, easily obtainable, can be expanded quickly and can also be differentiated into neural cell types *in vitro* and *in vivo*. It was observed that transplanted MSC into a spinal cord lesion site enhance axonal regeneration and promote functional recovery in animal models. In addition, it is believed that MSCs lack B7 co stimulatory molecules, CD80 , CD86 and are non immunogenic upon allogenic transplantation. The overall effect of MSC is to rescue neuronal cells by neuro protection, immune modulation, and possibly re myelination and neuro regeneration. MSC have greatly increased the hope to treat the untreatable central

nervous system injuries by meeting these requirements. Human mesenchymal stem cells are isolated from the bone marrow aspirate. They represent a very minor fraction (0.0001%) of the total nucleated cell population in marrow, but can be plated and enriched using standard cell culture techniques.

In this study the role of bone marrow stromal cells for restoration of motor control was observed in rat models of spinal cord injury. Different doses of cells were delivered to the spinal cord injured rats and were followed up to determine the effect of these cells in motor recovery. The responses were observed by assessing the behavioural motor scale as well as electrophysiologically by recording the electromyogram from the lower limb muscles of the rats following trans cranial stimulation of the motor cortex.

Aim :

To study the effect of Bone marrow Mesenchymal Stem Cell transplantation for motor recovery in rat models of spinal cord injury.

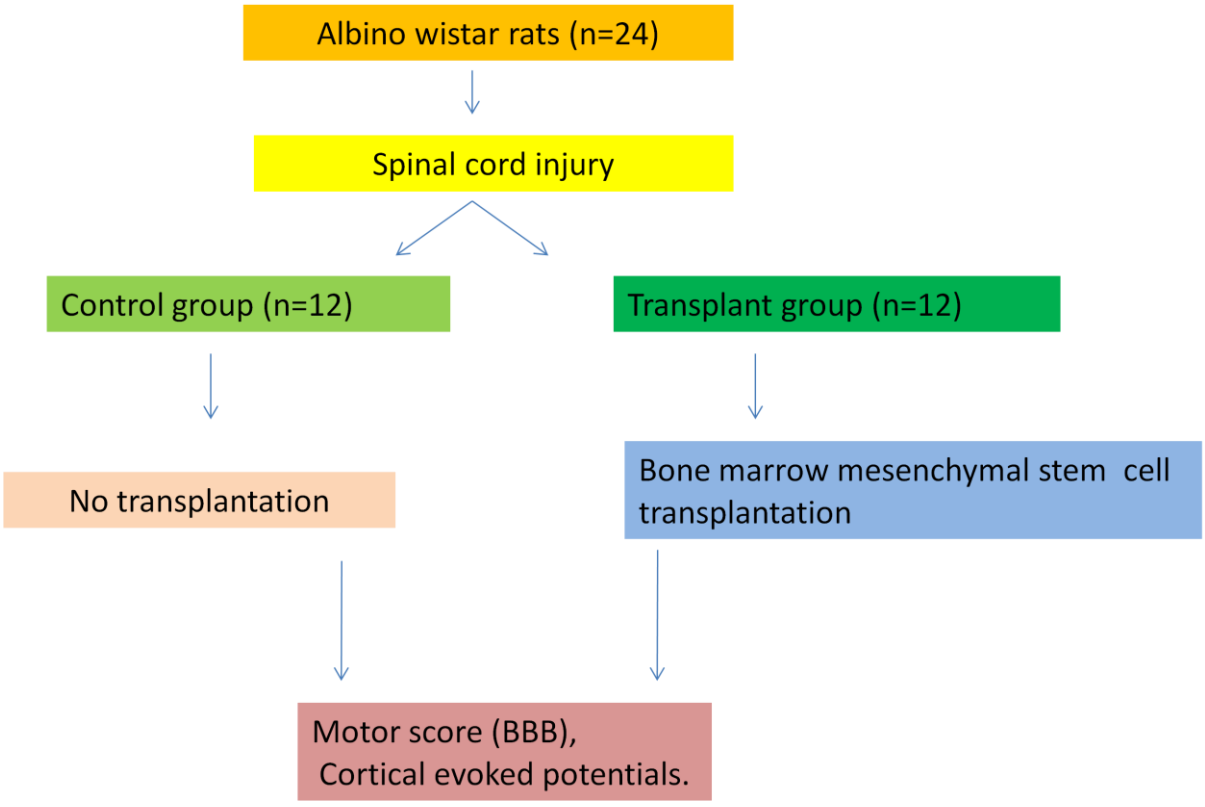
Objectives:

1.Bone marrow mesenchymal stem cell (MSC)culture and characterization .

2. Transdifferentiation of MSC to neuronal cells.

3.MSC transplantation to injured spinal cord segment of rat models.

4. Outcome assessment of transplantation by BBB scale, and EMG.



Review of Literature

Since time immemorial spinal cord injury was synonymous with morbidity and despair. Physicians were unable to successfully manage the cascade of interrelated problems and complications following spinal cord injury. Since last few decades spinal cord injury has been considered a manageable condition, compatible with reasonably good health with near normal life expectancy. Despite landmark achievements in medical field during the nineteenth century, such as Pasteur's discoveries in bacteriology, Lister's aseptic techniques, Roentgen's discovery of X rays and introduction of anaesthesia, management of spinal cord injury was poorly organised with grave outcome.. Sir Ludwig Guttman introduced the comprehensive care and interdisciplinary rehabilitation of persons with spinal cord injury, a model which has been widely replicated around the world. The success of specific units highlighted the fact that inadequate training, experience, number of staff, facilities, and equipment, not only placed the spinal cord injured person's life at risk, but often resulted in development of complications and poor functional outcomes.

Life expectancy of persons with spinal cord injuries has increased steadily over decades, but still remains below the able bodied individuals. Predictors of mortality include age, male sex, being complete injury, high neurological level, emotional distress, functional dependency, and poor adjustment to disability[10].

The motor system performs many different tasks, reflex and voluntary, with speed and accuracy because of two special features of the organisation. First, the processing of the sensory inputs and the commands to the motor neurons and muscles is hierarchically interconnected in areas of spinal cord, brainstem and forebrain. Each level has circuits that can organise or regulate complex motor responses through their output and input connections. Second, sensory information relating to the movement is processed in different systems that

operate in parallel. The spinal cord is the lowest level of this hierarchical organisation. It contains the neuronal circuits that moderate a variety of reflexes and rhythmic activities such as locomotion. The simplest normal circuit is monosynaptic. It includes only the primary sensory neuron and the motor neuron. Most reflexes are mediated by polysynaptic circuits where one or more inter neurons are interposed between primary sensory neuron and the motor neuron. Inter neurons and motor neurons also receive input from axons descending from higher centres. These supra spinal signals can modify reflex responses to peripheral stimuli by facilitating or inhibiting different populations of inter neurons. They also coordinate motor actions through these inter neurons. For example, when a joint is flexed, the descending commands that drive the flexor muscles also inhibit the opposing extensor muscle. All motor commands eventually converge on motor neurons whose axons exit the spinal cord to enervate the skeletal muscles. Thus motor neuron forms the final common pathway for all motor actions. By facilitating some circuits and inhibiting others, higher levels can let sensory input govern the temporal details of the evolving movement. The timing of activities of agonist and antagonist muscles is intrinsic to the spinal circuit and thus descending signals themselves need not be timed precisely. The patterns of coordination in spinal cord are relatively stereotyped. A cat with its cervical cord transacted can if provided with body support, walk in a moving treadmill and bring its paw around an obstacle after hitting it. But the spinal cat cannot lift its fore limb before impact with an obstacle an intact animal does, because this movement requires controlling of limbs using visual information[11]. At the spinal cord level, afferent impulses produce simple reflex responses. At higher levels in the nervous system, neural connections of increasing complexity mediate increasingly complex motor responses. In the intact animal, individual motor responses are integrated into the total pattern of motor activity. When the neural axis is transected the activities integrated below the level of section are released from the control of higher brain centres. This could be

due to removal of inhibitory control from higher centres or due to denervation hypersensitivity of centres below the level of transaction.

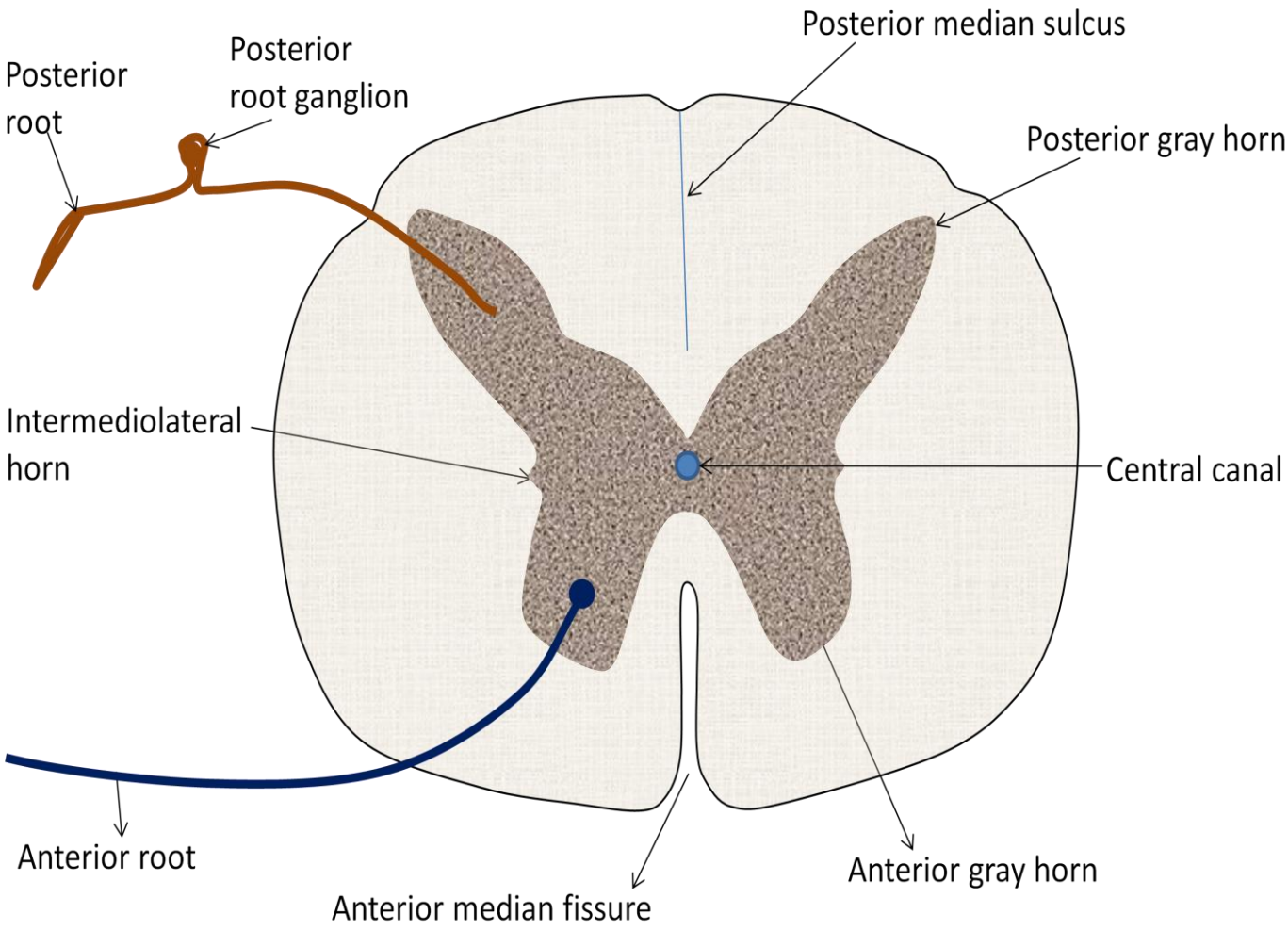
External anatomy: In adults, spinal cord extends from the medulla oblongata at the base of the brain to the lower border of the first lumbar vertebra. Spinal cord is enclosed in the bony vertebral canal, covered by three layers of connective tissue called meninges the pia, arachnoid and dura mater, and protected by cerebrospinal fluid to buffer the cord against any traumatic damage. The accepted definition of spinal instability by White and Punjabi is clinical instability where there is a loss of ability of the spine under physiological loads to maintain relationship between vertebrae in such a way that there is no damage to spinal cord or nerve roots[12].

The arrangement of the spinal cord appears to be segmental. 31 pairs of spinal nerves emerge at regular intervals thorough intervertebral foramen. Each pair of spinal nerves arise from the corresponding spinal segment, Spinal nerves are paths of communication between spinal cord and the specific end organ in the body. Two bundles of axons called the roots of the nerve connect each spinal nerve to the corresponding segment of the cord. The posterior root contains only sensory axons which conduct nerve impulses from the sensory receptors in the skin, muscles and internal organs to the central nervous system. The posterior root has a swelling at the intervertebral foramen known as the dorsal root ganglion which contain the cell bodies of the sensory neurons. The anterior or ventral root contain axons of motor neurons which conduct nerve impulses from spinal cord to muscles and effector organs.

Internal Anatomy; The anterior median fissure and the posterior median sulcus divide the cord into two sides. The grey matter is arranged like the letter H surrounded by the white matter. The grey matter contains primarily of cell bodies of neurons, neuroglia and

inter neurons. The white matter consists of bundles of myelinated and unmyelinated axons of sensory and motor neurons.

Internal anatomy of spinal cord:



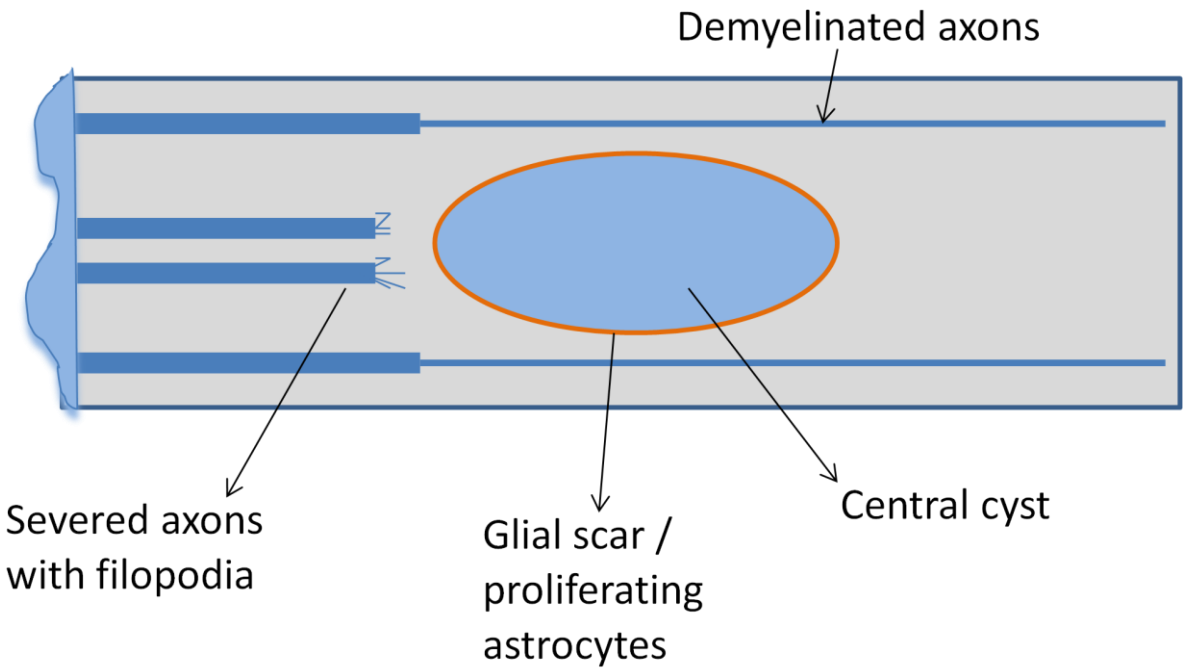
The gray matter on each side of the spinal cord is divided into regions called horns. The anterior or ventral horn contains cell bodies of somatic motor neurons and which enervate skeletal muscles. The posterior or dorsal gray horn contains somatic sensory nerve cell bodies. Between the anterior and the posterior gray horns are the lateral gray horns which contain the cell bodies of the autonomic sympathetic neurons which regulate the activities of heart, smooth muscles and glands. The white matter is also arranged into three broad areas called the columns: anterior/ventral white column, posterior/dorsal column, and the lateral column. Each column contains distinct bundles of axons known as tracts. Axons that conduct the impulses towards the brain are the ascending tracts or sensory tracts, and those that conduct impulse down the spinal cord from the higher centres are known as the descending tracts. Nerve impulses from sensory receptors propagate up the spinal cord to brain along two main routes on each side. The lateral and anterior spinothalamic tracts convey impulses for sensing pain and temperature. The posterior columns of either side convey touch and proprioception. Cerebral cortex plays a crucial role in precise voluntary muscular movement control. Motor output travel down the spinal cord along two descending pathways, direct and indirect. The direct pathways include lateral and anterior cortico spinal and cortico bulbar tracts. They cause precise voluntary movements of the skeletal muscle. The indirect pathways include rubrospinal, tectospinal and vestibulospinal tracts. They control automatic and coordinating movements of the body and maintain muscle tone and equilibrium.

The corticospinal neuron is clinically known by the term upper motor neuron. This synapses with the lower motor neurons which in turn synapse with the muscles. Damage to spinal cord causing lesion in the axons of the upper motor neuron results in a constellation of signs and symptoms called the upper motor neuron syndrome. This includes loss of motor control, spasticity, hyper reflexia, clonus and Babinskis sign. The damage to lower motor neuron in the central gray matter of the spinal cord leads to lower motor neuron signs like loss of

muscle tone or hypotonia, hyporeflexia, ie loss of tendon reflexes, and gross muscle wasting. Following an acute spinal cord injury, spinal cord shows a period of spinal shock, a transient suppression and gradual return of reflexes.

Spinal cord is supplied by a single anterior spinal artery and two posterior spinal arteries. This is replenished by several radicular segmental arteries. Posterior spinal artery is a branch of vertebral artery and supplies posterior one third of the spinal cord. The anterior spinal artery also a branch of the vertebral artery supplies the anterior two third of the spinal cord. The areas at T1 to T4 and T12 to L2 form the water shed regions and particularly prone to ischemic damage. Due to the dual posterior and single anterior blood supply, ischemia often affects the anterior part of the cord known as the anterior cord syndrome, with motor loss, loss of pain and temperature, but preserved touch and position sensation.

Pathology: The secondary injury cascade causes further neuronal damage due to the biochemical changes. Ischaemia of gray matter due to vasoconstriction mediated by vasoactive substances like serotonin, thromboxanes, platelet activating factor, is followed by oedema at the site of injury. There is rise of intracellular calcium, rise in exitotoxic aminoacids such as glutamate and aspartate. Intra cellular calcium facilitates activation of phospholipases, production of free radicals and free fatty acid metabolites which cause damage to cell membrane. This releases potassium and its level rises in the extra cellular space leading to conduction block. Micro haemorrhages appear in this area of damage. Iron from this haemorrhage catalyses the per oxidation of lipids, leading to further tissue damage.



Schematic diagram of spinal cord pathology following trauma

This leads to the formation of central cavity surrounded by astrocytic scar with a small rim of white matter with demyelinated axons in the periphery of the spinal cord

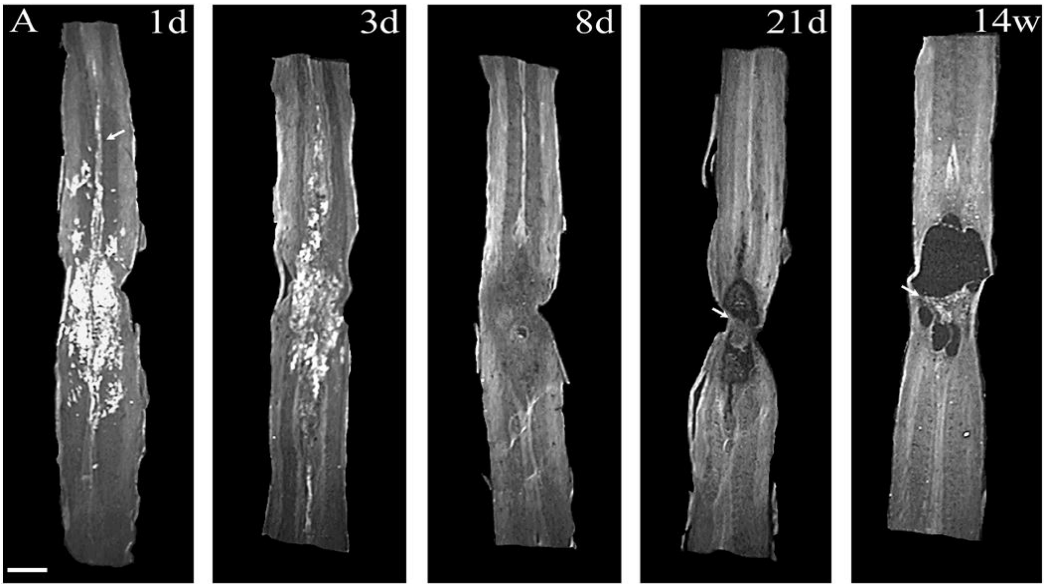
Neutrophils migrate to the site of injury which releases lysosomal enzymes and free oxygen radicals. This is followed by the invasion of macrophages that phagocytose cell debris. Demyelination of white matter begins within 24 hours. Small CSF filled cysts develop that are surrounded by demyelinated nerve fibres [13, 14, 15].

Classification of Spinal Cord injury: The international standards for neurological classification of spinal cord injury provide a simplified universally accepted procedure for classifying spinal cord injury. The procedure involves systematic evaluation of dermatomes and myotomes. This also provides definition for complete and incomplete injuries. A complete injury is defined as an injury where there is lack of any sensory or motor function in the lowest sacral segment. An incomplete injury is where there is at least partial sensory or

motor function in the lowest sacral segment. The examination observes the evaluation of sensation at key sensory points and motor grading of key muscles representing myotomes. The neurological level of injury is the most caudal segment of the spinal cord with normal sensation and motor function bilaterally. This also includes a scale of impairment called American Spinal Injury Association Impairment scale (ASIA) which classifies SCI into five categories of severity, labelled A to E based on the degree of sensory and motor loss[16]. Persons with motor complete lesions have a poor prognosis for recovering ability to walk. Only 2-3 % persons classified initially as having an initial A convert to D. Persons with preserved pin prick sensation in the lowest sacral dermatome has 70% chance of regaining ambulatory capacity by 1 year, while persons with only light touch sensation in the same region are unlikely to regain ambulatory capacity[17]. The first step in the management of the person suspected to have sustained spinal cord injury is ensuring adequate air way, and immobilising the spine with a collar and spinal board before transporting to the medical facility. Once medically stabilised, Xrays , CT/MRI are done to assess the damage to bone and soft tissues. Closed reduction is performed by proper positioning and applying a series of increasing distracting forces. Surgical treatment of acute spinal cord injury is performed either to stabilise an unstable spine or to decompress any compressed natural elements. Role of surgery in complete lesions is to provide early stability and rapid involvement in of the injured person in rehabilitation. Pharmacological treatment of acute spinal cord injury includes high dose methyl prednisolone in the first eight hours of injury. This appears to interrupt the secondary injury cascade by inhibiting lipid per oxidation and reducing level of free radicals. This method as a standard of care is controversial because of concerns regarding methods of data analysis, choice of statistical methodology, randomisation imbalance, and inclusion of persons with minor motor deficits[18].

Rehabilitation goals after spinal cord injury include maximization of physical independence, and preventing complications. The rehabilitation team includes family members of the person along with doctors, physiotherapists, occupational therapists, nurses, psychologists, social workers, recreation therapists, and vocational therapists. Physical and Occupational therapists train persons with spinal cord injury in mobility, self care skills, and other activities of daily living skills including wheel chair skills or ambulatory skills using orthotic support. Electric stimulation of nerves and muscles are commonly used for therapy, but can also be used to restore functions when it is referred to as functional electrical stimulation. This can increase the muscle bulk, prevent pressure sores, prevent contractures, reduce osteoporosis, control spasticity, generate hand grip, improve bladder, bowel and sexual functions and enhance ambulation. A computer controlled functional electrical system is known as the neuroprosthesis[19].

Spinal cord injury causes a surge of events that eventually leads to loss of the central part of the cord, leaving a rim of demyelinated axons in the periphery of the cord. The traditional approach had been to limit the damage, since it is easier to limit the progression of injury than to repair the damaged cord. Initial studies towards this direction used peripheral nerve grafts and transplanted cells to bridge the gap of injury (Intercostal nerves, Schwann cells). The axonal growth was not satisfactory in the hostile environment of the distal cord. Following the injury the condition triggers a train of secondary events that progressively destroy the tissue adjacent to the lesion over period hours to weeks. Major components of secondary mechanism include vascular effects, haemorrhage, ischemia, metabolic derangement, ionic changes, glutamate induced exicotoxicity, free radical excess, cytokines and chemokines and inflammation leading to apoptosis of neurons and oligodendrocytes. This leads to the formation of central cavity surrounded by astrocytic scar with a small rim of white matter with demyelinated non-conducting axons in the periphery of the injured spinal cord[20].



Secondary injury cascade; dynamic changes following trauma showing initial hemorrhage progressing to cavity formation over time.

Cell based methods for spinal cord regeneration:

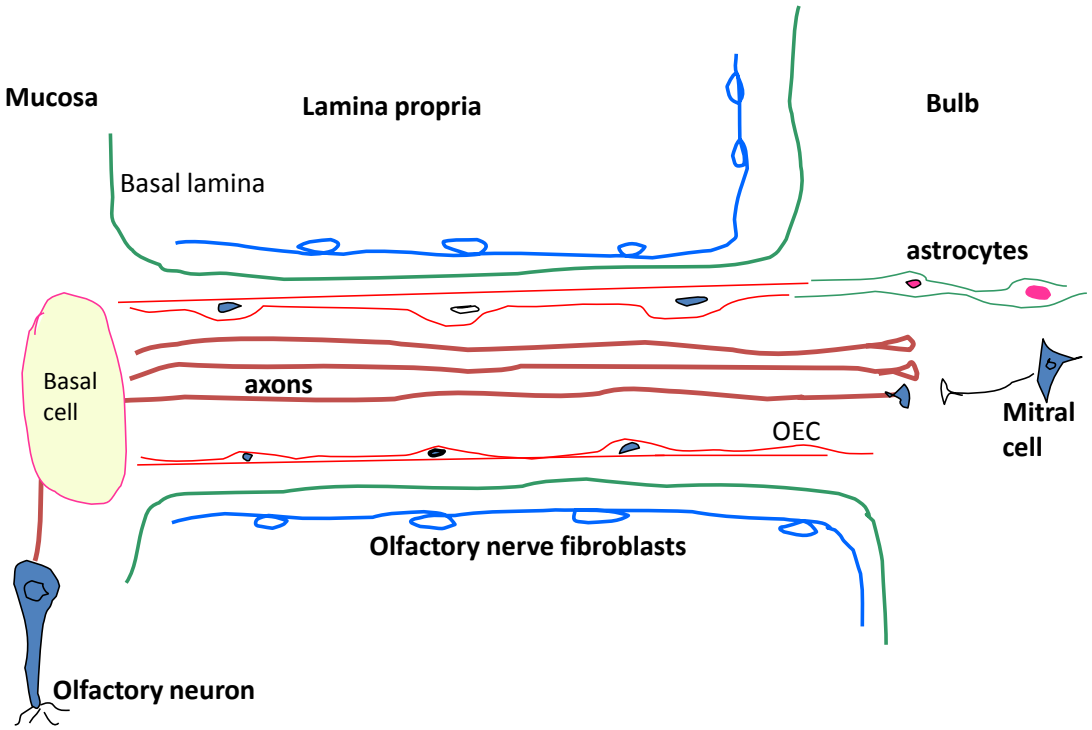
Use of cell therapy to restore motor function following spinal cord injury is being persued extensively across the globe as a probable solution for this conundrum. The reports that have captured scientific as well as media attention include transplantation of autologous olfactory ensheathing cells, foetal olfactory ensheathing cells, autologous activated macrophage transplantation, human embryonic cell derived pre-oligodendrocyte transplantation, and autologous bone marrow stromal cells transplantation which have entered initial clinical trials.

1. Human oligodendrocyte progenitor from human embryonic stem cells:

This method aims for remyelination of the preserved axons. Demyelination of spared axons is a prominent feature of spinal cord injury due to loss of oligodendrocytes. The initial insult is followed by a period of secondary degeneration characterized by oligodendrocyte death and subsequent demyelination of the axons. In order to address the demyelination in the clinical setting, human oligodendrocytes differentiated from embryonic stem cells are transplanted into rat models of spinal cord injury. These cells have demonstrated promising results in experimental models. High purity oligodendrocyte precursors were differentiated from human embryonic stem cells for transplantation. Oligodendrocytes provide trophic support for neurons and axons. Neuronal survival has been known to depend on factors that inhibit apoptosis. Factors produced by oligodendrocytes have been shown to induce sodium channel clustering along the axon and promote axon maturation and stability. These cells integrated into white matter, differentiated into oligodendrocytes, formed compact myelin and promoted recovery of motion. This study has now entered into Phase I clinical trial. Remyelination restores saltatory conduction in axon and locomotor deficits[21].

2. Autologous olfactory ensheathing cells:

During development, pathway taken by the growing nerve fibres depends on a large number of positive and negative signals recognised by the growth cones. These signals are located on other elements like neuronal and glial cells whose orientation determines the route taken. Following injury, in addition to local effects of damage, oligodendrocyes die, astrocytes proliferate, microglia invade, and large cavities filled with fluid are formed. Even though the cut ends of the axon continue to sprout locally for a long time it is unable to grow out towards its target.



Axons of olfactory neurons ensheathed by OEC which integrates with astrocytes in CNS

The olfactory neurons are formed continuously throughout adult life. These nerves contain unique type of ensheathing cells, known as the olfactory ensheathing cells. This cell resembles a non myelinating Schwann cell but encloses a large number of axons. Cultured and transplanted OEC in spinal cord demonstrated that it allowed the axons to align and grow across the lesion into the distal part of the tract. The axons having crossed the bridge may synapse locally. The olfactory ensheathing cells form a continuous channel or conduit for the axons to grow unhindered by the local inhibitory influences. These channels are important for effective axonal regeneration[22, 23].

The Olfactory ensheathing cell has properties of both Schwann cells and astrocytes, with a phenotype closer to Schwann cell. It exists both outside central nervous system (like Schwann cell) and inside (like astrocyte) and it is able to assist axonal growth throughout adult life[24]. The most pronounced difference between OEC and Schwann cells is in their ability to interact with astrocytes. It has been demonstrated that when Schwann cells make contact with astrocytes they form a distinct cellular boundary without mixing. OEC do not form a boundary and can migrate freely among the astrocytes,. OEC do not induce reactive or hypertrophic responses in astrocytes that occur with Schwann cells as measured by astrocyte size, expression of GFAP, and growth inhibitory molecules like chondroitin sulphate proteoglycans. Thus axons are able to traverse the graft axon boundaries following OEC transplantation[25]. Following focal lesion of corticospinal tract in rats, transplantation of OEC led to a directed elongation of regenerated corticospinal axons across the lesion and into the distal white matter[26]. Following successful reports in animal models of spinal cord injury autologous transplantation was performed in a phase I clinical study which demonstrated clinical safety, but with no motor recovery[27].

3. Foetal olfactory ensheathing cells:

Olfactory cells derived from foetal olfactory bulb has been cultured and transplanted in patients spinal cord injury and favourable outcome has been reported in large number of patients in China[28].

4. Schwann cells:

It has been observed that intraspinal Schwann cell graft limit injury induced tissue loss and promotes axon regeneration and myelination and that this response can be improved by adding neutrotrophic factors or anti-inflammatory agents. Implantation of Schwann cells in rat models of spinal cord injury promotes regeneration of sensory axons. It has been observed that Schwann cell transplantation did not cause the axons to cross the bridge of implanted tissue. Schwann cell transplantation needs to be combined with other interventions to modify the permissiveness of the graft cord interfaces to allow axonal growth. Neurotrophic factors, Brain Derived Neurotrophic Factors (BDNF), Neurotrophin- 3 , (NT-3) or high dose methyl prednisolone facilitated the axonal growth. In general, Schwann cell implantation alone into the site is not sufficient to promote axonal response that could lead to biologically significant functional recovery. It has been observed that transplanted Schwann cells did not migrate into surrounding spinal nervous tissue and therefore cannot myelinate axons that have regenerated through and beyond the graft. It has been clear that the grafting Schwann cell alone will not result in substantial functional recovery and additional intervention need to be combined with Schwann cell based repair strategies. Approaches to obtain axonal growth include decreasing inhibitory nature of the scar, preventing axons from the recognising the inhibitory molecules, and facilitating intrinsic ability of the neurons. Regeneration of supra spinal axons beyond the intra spinal graft is essential to achieve the cortical control of motor function following spinal cord injury[29,30,31,32].

5. Activated Macrophage transplantation:

The concept of repairing the damaged spinal cord and the role of immune system has been investigated. In experimental animals with spinal cord injury, local injection of homologous macrophages induced partial recovery after their activation by incubation with autologous peripheral nerves. Macrophages incubated with autologous skin as been found to be equally effective. The exposure of the macrophages to the injured regenerative tissue like the excised skin is thought to sensitise the macrophages towards a wound healing property. The skin co incubated macrophages have been reported to demonstrate a distinctive profile of cytokine secretion and cell surface markers. These cells have the potential to influence nerve cells immune cells and glial cells that are present in the injured spinal cord. In rat models of spinal cord injury, the skin activated macrophages promoted neurological recovery and reduced cavity formation. Unlike single molecule therapy, autologous macrophage therapy is intended to exploit the numerous activities that are characteristic of these cells including the clearance of tissue debris from the lesion site, secretion of protective and healing molecules and modulation of immune system. All these contribute to improved cell survival and regrowth[33]. A phase I clinical trial among 8 patients with spinal cord injury has been conducted. A single dose of 4 $x10^6$ cells in 60µl were administered into spinal cord parenchyma at the lower border of the traumatic lesion. Three patients demonstrated partial recovery and the cell therapy were tolerated in these patients, and further clinical evaluation has been recommended. Transplanted patients underwent preoperative and follow up neurological assessment according to American Spinal Injury Association scale, electrophysiological monitoring of motor evoked and somato sensory potential, magnetic resonance imaging and safety monitoring. The two main problems in arriving a conclusion from the results was a lack of control group and the small number of patient cohort[34].

6. Neural stem cells:

Recent observations have raised the possibility that the brain has inherent capacity for self – repair in response to injury or disease through the use endogenous NSC or neural progenitor cells. NSC in the adult brain is able give rise to neurons in few restricted areas in vivo like subventricular zone in the wall of the lateral ventricle and subgranular zone of the hippocampal dentate gyrus. Poor regenerative capabilities of adult CNS is due to inhibition by the microenvironment factors present in most areas of the central nervous system. The number of endogenous NSC will be too small for effective cell repair. Strategies to overcome these obstacles could facilitate recovery from damage. In addition to micro environmental factors including cytokines and cell to cell interactions, fate of NSC or neural progenitor cells have been shown to be regulated by epigenetic modification of the cell specific genes through DNA methylation or chromatin structure. Neural stem cells have great potential as a therapeutic tool in a number of central nervous system disorders. However today little evidence exist regarding the efficacy of neural stem cells and restoring function. NSC can be isolated from embryonic or adult brain tissue. There are two major methods of applications for treatment of CNS disorders. First it can be transplanted as undifferentiated cells, whose subsequent differentiation could be controlled by signals derived from host CNS. Alternatively, NSC pre differentiated in vitro to desired neuronal cells which could be transplanted to host CNS. This is preferable as it has the ablility to direct the differentiation from stem cell to desired neuronal phenotype[35]. It appears that in vitro manipulation of NSC lineage fate prior to transplantation may be necessary to control terminal lineage of the transplanted cells. Although NSC exhibit remarkable degrees of plasticity with regard to lineage potential it may not be sufficient to overcome local barriers. Both embryonic and adult NSC differentiates into primary glia when transplanted into CNS. When pluripotent NSC were transplanted into injured spinal cord, the engrafted cells differentiated only to

astrocytes. Hence successful neuronal replacement may require transplanting cells committed to neuronal lineage rather than pluripotent lineage. Pluripotent NSC differentiates into oligodendrocytes and form myelin after engraftment. When NSC are transplanted into contused rat spinal cord majority of cells differentiate into astrocyte with no oligodendrocytes. Hence to obtain large number of oligodendrocytes from transplanted NSC it will be necessary to initiate oligodendrocyte lineage commitment invitro prior to transplantation. Transplantation of this progenitor cells expressing immature oligodendrocyte markers, into spinal cord of myelin deficient rat resulted in large areas of myelination[36,37].

7. Autologous bone marrow and Granulocyte macrophage colony stimulating factor:

Transplanted bone marrow cells into the spinal cord models were found to improve neurological deficits in the central nervous system injury models by generating neural cells or myelin producing cells. Bone marrow cells can also produce neuroprotective cytokines, which rescue neurons from impending cell death after the injury. Granulocyte macrophage colony stimulating factor has been observed to improve neurological outcome. Recombinant GM CSF has been used in patients with bone marrow suppression. It causes haemopoiesis by inducing the growth of several different haemopoeitic lineages. It also enhances the functional activities of mature effector cells involved in the antigen presentation and cell mediated immunity, including neutrophils, monocytes, macrophages, and dendritic cells. It has been reported that GM-CSF has prevented apoptotic cell death not only in haemopoeitic cells but also in neuronal cells. GM-CSF stimulates microglia cells to increase brain derived neurotrophic factor and decreased the neuronal apoptosis and thus improving functional out come in animal models of SCI[38].

8. Autologous Bone Marrow Mesenchymal Stem Cells:

Bone marrow provides a source of circulating erythrocytes, platelets, monocytes, granulocytes, and lymphocytes which are derived from haematopoietic stem cell. The marrow stroma is complex tissue that contains cells that are required for lineage commitment for haematopoietic cells. Although initially considered to be primarily haematopoietic support cells, the marrow stromal cells also contain non haematopoietic cells that can differentiate into a variety of mesenchymal cells including bone, fat and cartilage. MSC is thus a unique cell in the bone marrow that differentiate into non haematopoietic and non lymphocytic tissues. The stromal cells have a propensity to adhere to tissue culture plastic, a property which has been used as a means to isolate them from bone marrow. MSC are thus pluripotent cells capable of differentiating into various mesenchymal tissues, like chondrocytes, osteoblasts, myoblasts and adipocytes. Several criteria are required for transplantation of MSC in humans like safety, reproducibility and quality. For clinical use it is necessary to find a method to amplify MSC in a short time retaining all the properties[39].

MSC are plastic adherent cells with multi potent differentiation capacity which express CD73, CD90, CD105, CD106, CD146and HLA ABC (MHC Class I), while being negative for CD34, CD45 and surface HLA DR(MHC Class II). Human serum does not support the growth of MSC in vitro. Platelet derived growth factor PGDF is observed to be a critical factor in growth supplementation. DMEM supplemented with fresh frozen plasma and platelets provide optimal growth conditions[40].

Because MSC are easy to isolate, and have broad differentiation potential and proliferate in vitro, they are attractive candidates for cell therapy. Bone marrow is an accessible source for these cells. Ideally the cell for transplantation should be autologous easily obtainable, and effective in supporting host axonal growth when placed in vivo. The ideal time for

transplantation is the time after the initial inflammatory cascade has subsided and before the astrocytic scar is established. MSC transplanted into rat models have been observed to migrate towards the injury site. When MSC were transplanted 3 months after the injury, functional benefits were seen four weeks after transplantation which continued to one year. Grafted MSC formed cellular bridges across the cavity in the spinal cord and expressed astrocytic and neuronal markers[41].

It has been observed that these cells trans differentiate into neuronal lineage in vitro under specific conditions of inducing media. These findings have been controversial. It has also been suggested that they can form neural cells when exposed to CNS microenvironment. MSC form guiding strands for host axonal growth when transplanted to sites of spinal cord injury in rat models. In addition MSC establish a cellular matrix that support host axonal growth probably due to endogenous growth factor production by MSC[42]. MSC transplantation in spinal cord in rats can remyelinate the demyelinated spinal cord axons. Thus MSC has a potential to provide an efficient and renewable source of cells for auto transplantation at sites of demyelination or white matter diseases[43].

The use of autologous bone marrow has distinct advantages as it avoids problems associated with graft versus host interaction. Autologous bone marrow therapy is considered safe and not associated with malignancy potential. Extensive scientific data on previous experience in BMSC transplantation for haematological diseases are available which demonstrate safety. These advantageous make cell therapy using BMSC applicable and are being investigated for treatment of various neurological diseases. Bone marrow has a mixed cell population including hematopoietic stem cells, mesenchymal stem cells, endothelial progenitor cells, macrophages and lymphocytes. It has been observed that haemopoietic and mesenchymal stem cell has neuroprotective effect and permit neural growth[44, 45, 46].

9. Adipose stem cell:

Adult stem cells can differentiate appropriate neuronal phenotypes in damaged neuronal phenotype. Adipose tissue compartments are a particularly useful source of mesenchymal stem cells due to easy availability, clonogenic potential and robust proliferative capability. Adipose derived stem cell can differentiate into adipocyte, chondrocyte, myocyte, oesteoblast and also neural lineages. Adipose derived stem cells have therapeutic potential and functional recovery in rodent models of spinal cord injury in vivo have been reported after transplantation. Subcutaneous fat contains unpurified population of stem cells including adipose derived stem cells. The other cells include endothelial cells, smooth muscle cells, pericytes, fibroblast, leucocytes, haemopoietic stem cells or endothelial progenitor cells. Adipose derived stem cells are plastic adherent and thus the cells self select out of the mixed cell group during subsequent cell culture passages. When adipose derived stem cells were transplanted to animals with neural injury they migrated to the injured area and some cells stained positive for MAP-2, and glial fibrillary acidic protein[47,48,49].

Non cell based methods for spinal cord regeneration:

These include a variety of agents which have been found to support axonal growth following spinal cord injury in experimental conditions. Minocycline, a tetracycline is observed to prevent secondary inflammatory cascade, resulting in limited tissue damage. Rolipram blocks the phospho diesterase enzyme in the axonal terminal end, increase the cyclic AMP and promote axonal linear growth. Chondroitinase is a bacterial enzyme which lyses the proteoglycan scar formed by the astrocytes and facilitates the axonal regrowth.

4-Aminopyridine:

4-aminopyridine a potassium channel blocker, facilitate axonal conduction of the demyelinated axons. Toxins like charybdotoxin, BTK2 which are voltage gated potassium channel blockers can promote axonal conduction in demyelinated axons. Physical trauma to spinal cord results in demyelination of axons. Without insulating sheaths of myelin the surviving axons become less efficient to transmit electrical impulses. When the axons are demyelinated after injury large numbers of potassium channels which are juxtanodular at nodes of Ranvier are exposed and potassium ions leak out. 4-aminopyridine blocks this exposed potassium channels and restores the ability of the axons to transmit electrical impulses. Clinical studies using 4- aminopyridine demonstrated statistically significant improvement in reduction of spasticity and a positive trend towards improvement[50].

Sodium and potassium channel blockers:

HP184 (N-proxyl, 3 Fluoro,4 pyridinyl,1H-3-Methyl indole-amine hydrochloride) pharmacological blocker capable of antagonising both sodium and potassium channels have been developed and is undergoing studies to evaluate the effect on spinal cord injury both on walking function and on spasticity.

GM-1 ganglioside:

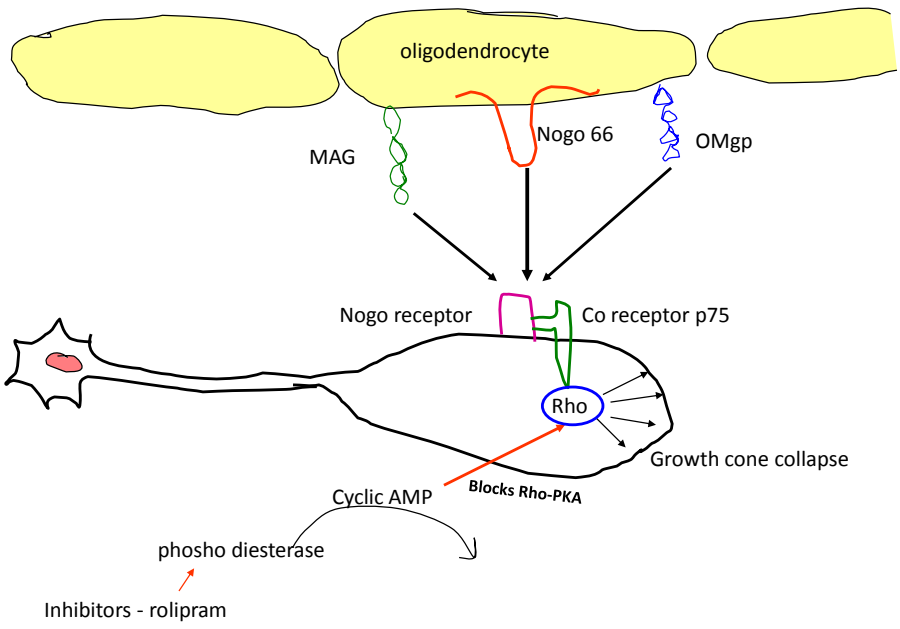
Mono sialo tetra hexosylganglioside GM-1 sodium salt is a naturally occurring compound which is located in the cell membrane of the neural cells. Preclinical studies demonstrating neuro protective and neuroregenerative actions by GM-1 in experimental models have been reported. Clinical trial showed variable outcome with no definitive conclusions[51].

Minocycline:

Minocycline is a broad spectrum of antibiotic, a lipophilic derivative of tetracycline that has demonstrated ability to provide neuroprotection. Mechanisms attributed for protective actions elicited by minocycline include ability to overcome glutamate mediated exicitotoxicity, anti-inflammatory effects by blocking the activation of microglial cells inhibiting Cytochrome C release, inhibiting caspase dependent apoptotic neuronal death and antagonising matrix metallo-proteases. Minocycline also reduces oligodendrocytes apoptosis and corticospinal tract die back in animal models of spinal cord injury[52].

Nogo:

The non-permissive environment for axonal growth is associated with oligodendrocytes and myelin. Monoclonal antibody directed against this substrate allowed the axonal out growth. Nogo is essential during development of the nervous system to permit and guide the axonal growth in a linear manner towards target.



Nogo receptors, molecular inhibitors and potential targets for treatment

Nogo A is a high molecular weight transmembrane protein and potent inhibitor of neural growth, produced by oligodendrocytes. Efforts to develop antagonists to target Nogo receptor is being studied[53, 54]. It has been demonstrated that oligodendrocyte myelin membrane act as an inhibitor for axonal growth. The major inhibitory elements which have been suggested to block axonal growth include chondroitin sulphate proteoglycan, myelin associated glycoprotein, and oligodendrocyte myelin glycoprotein. Nogo receptor is part of a receptor complex on the axonal membrane, which act through Rho at growth cone. Growth cone cytoskeleton is composed of filamentous actin fibres with distinct features of distal finger like filapodia and central fan like lamella podia . Blockade of Rho by elevated levels of cyclic AMP prevents the growth cone collapse and facilitate axonal growth. Agents like Roliparam which blocks the phoshodiesterase enzyme cause elevation of cAMP allowing axons to grow.

Methyl prednisolone:

Methyl prednisolone sodium succinate was studied in NASCIS I, II and III . Methylprednisolone elicits therapeutic effects due to improved protection from ischemic insults and calcium dependent degradation of neurofilament cytoskeleton proteins. In the NASCIS trial, methylprednisolone was administered as 30mg/kg of bolus over the first hour followed by an infusion of 5.4 mg/kg per hour for the next 23 hours. An analysis of all patients failed to demonstrate significant differences. Analysis of data in the study showed the patient who received methylprednisolone within the 8 hours of injury significantly improved motor and sensory function compared to placebo. Despite beneficial therapeutic effects demonstrated with methylprednisolone treatment, results from this trial have not been universally accepted. Concerns due to small sample size of the population for the groups showing beneficial effects, non-standardised medical and surgical protocols by different participating centers and lack of correlation with functional outcome reduced the enthusiasm for administration methylprednisolone in acute spinal cord injury[55, 56].

Materials & Methods

All experiments were conducted with the approval from Institutional Review Board and the Animal Ethics Committee.

Materials required:

Cell culture facilities

Phosphate buffered saline (PBS), Gibco,Catalog no: 10010.

RosetteSep antibody cocktail, Stem Cell Technologies Inc, catalog. No: 15168.

Ficoll-Paque, GE Healthcare Bio-Sciences, catalog .no: 17-1440-03.

T75 culture flask, Greiner.

Alpha - Minimum essential medium, Gibco,

Fetal bovine serum, Gibco, catalog.no: 10082.

L-Glutamine, Gibco, Catalog.no: 25030.

0.25%Trypsin-EDTA, Gibco, Catalog.No: 25200.

Dulbaco modified eagle medium, Gibco,catalog.No: 11995.

DiMethylSulphoxide, DMSO Sigma, Catalog no: C6295

Butylated hydroxyanisole (BHA), ACROS.

Potassium chloride (Kcl) , Qualigens.

Valproic acid, sigma, catalog. No:P4543.

Forskolin, Sigma.

Hydrocortisone, Sigma, catalog.No: H6909.

Insulin, Sigma, catalog.No: I9278.

bFGF, Invitrogen, catalog.no: 13256-029.

Hanks Balanced salt solution (HBSS), Gibco, catalog no: 14175.

100U/mL penicillin, 100µg/ml streptomycin and 25ng/ml of amphotericin-B.

Centrifuge, REMI.

Laminar flow hood, Kartos International.

CO2 incubator, Thermo Electron Corporation.

Inverted phase-contrast microscope.

Animal experiments:

Albio Wistar rats

Animal house with air-conditioned facilities,

Operation theatre for anaesthesia and surgery.

Ketamine

Xylazine
Operating microscope.
Surgical instruments:
Bone rongeur.

Surgical scissors.

Equipment for drop weight device and transducers to record forces.

Transcranial cortical stimulator

EMG recorder.

Gait analysis set up for rats and video recorder for BBB score.

3 D Injection device for cell transplantation.

Methods:

i) Bone marrow collection:

Rat Bone marrow:

Male Albino wistar rat was weighed and anaesthetized with appropriate dose of Ketamine and Xylazine by intraperitoneal injection using 31-gauge insulin syringe. The hind limb was disarticulated at the hip joint and the muscles were cleared to expose the femur. The tibia and femur of rat were dissected. The femur was then isolated from the leg bones by incisions at the knee joint. The ends of femur bone were opened using bone rongeurs to expose the marrow cavity. A syringe containing a 2 ml of phosphate buffered saline (PBS) was inserted into the narrow cavity and the bone marrow was collected in a test tube by flushing the marrow cavity with phosphate buffer saline. The bone marrow suspension was then taken to the culture lab for processing. Procedures followed as described below.

Isolation of Bone marrow stromal cells (BMSCs):

The mesenchymal stromal cells were separated from hematopoietic cells by using the Rosette Sep antibody cocktail. This cocktail cross links unwanted cells in bone marrow to multiple red blood cells (RBCs), forming immune rosettes. This increases the density of the unwanted (rosetted) cells, such that they pellet along with the free RBCs when centrifuged over a buoyant density medium such as Ficoll-Paque. Desired cells are not labelled with antibody and are easily collected as a highly enriched population at the interface between the plasma and the buoyant density medium.

2ml of bone marrow taken in a 15ml tube was incubated with Rosette Sep cocktail 50µl/ml of bone marrow at room temperature for 20 minutes. Bone marrow was diluted twice its volume with PBS and layered gently on top of Ficoll-Paque taking care to minimize

mixing of both the sample and Ficoll-Paque. The sample was then centrifuged at 1200 rpm for 25 minutes at room temperature and the enriched cells removed carefully from the (Buffy layer) interface between the plasma and the Ficoll fluid. The enriched cells were washed in 10ml PBS and centrifuged at 1000 rpm for 10 minutes at room temperature. The supernatant was discarded and cell pellet was dissolved in culture medium.

Cell Counting:

10µl of cell sample was stained with 30µl of tryptan blue (ratio of 1:4) and charged into the counting chamber using micropipette. The cell viability and numbers were calculated.

Culture of MSC

The cell culture medium consisting of Minimum Essential Medium (MEM Alpha) (Gibco), supplemented with 20% fetal bovine serum (Gibco), 2mM L-glutamine (Gibco-Invitrogen), 100U/mL penicillin, 100µg/ml streptomycin and 25ng/ml of Amphotericin-B. 30µl of the cell suspension was taken and mixed with 30µl of 0.4% trypan blue stain (Gibco). Viable cells were counted using haemocytometer under phase-contrast microscope and the number of cells/ ml was calculated. Approximately 8000 cells/cm^2 were plated on to polystyrene culture dishes (Greiner bio-one). The culture was maintained at 37°C, with 5% CO2 in air, at 95% humidity. The cells were fed every second day by replacing half of the culture medium with fresh complete medium.

Characterization of cultured cells

Monolayered cells grown on Poly-L-lysine coated cover slips were washed twice with PBS and fixed in 4% paraformaldehyde for 15 min. Fixative was removed by washing thrice in PBS. The cells were incubated in power block (proteinaceous blocking agent containing casein, PBS with 0.09% sodium azide) for 10 minutes at room temperature. This is to

minimize the non-specific binding of antibody to highly charged sites in the cover slips. The cells were then incubated with primary antibodies (i) for integrin β1 (rabbit polyclonal serum, 1:500 chemicon) and (ii) for CD54 (mouse monoclonal antibody, 1:100, Chemicon) (iii) for collagen type I (rabbit polyclonal antibody, 1:500, Chemicon) (iv) for CD14 (mouse monoclonal antibody, 1:1000, Chemicon) (v) for CD45 (mouse monoclonal antibody, 1:100, Chemicon), Neurofilament (1:100 Millipore), Neuron specific enolase (1:100 Millipore), Tau protein (1:100 Millipore) at 37°C for 1hour in moist chamber. Primary antibody was washed off with PBS and cells were treated with secondary antibody (Super Sensitive Polymer-HRP IHC Detection system (BioGenex, USA) for 30minutes at 37ºC. To detect the HRP, the cells were incubated with stable DAB substrate buffer containing peroxide and liquid DAB chromogen (3, 3'-diaminobenzidine), The insoluble, permanent brown precipitate of oxidized DAB in the cells demonstrated the presence of HRP and hence the binding of primary antibody to the antigen. After washing off the chromogen with water, the cells were counterstained with Delafield haematoxylin (Qualigens) to demonstrate the nuclei. The cover slips containing the stained cells were cleaned with xylene and mounted with DPX (Qualigens).

Neuronal Induction:

Cells were washed with PBS thrice and treated with pre-induction medium for 24 hours before induction. After adding induction medium morphological changes were analysed and stained for Tau protein, Neurofilament, Neuron-specific enolase for the confirmation of neuronal transdifferentiation.

Pre-induction medium:

DMEM, 1µM bFGF and 20% FBS.

Long-term neuronal induction medium:

DMEM, 2%DMSO, 200µM BHA,10mM Kcl, 2mM valproic acid, 10µM Forskolin, 1µM Hydrocortisone, 5µM Insulin.

Fabrication of impactor for Spinal Cord Injury in Rats

A device was developed which would deliver a force which can be used to create contusion model of spinal cord injury of the rat. Measures of evaluating this injury would also be used.

Rat models have been well accepted for studying spinal cord injuries since the pathological changes after injury is similar to that occurs in human injured cord. A device which will make injuries on the rats reproducibly was fabricated. This is important so as to make uniform injuries so that the effects of the interventions can be observed easily.

The weight-drop method is most widely used in spinal cord injury impactors. The basic concept is to drop a known weight from a known height onto the spinal cord. We have used this method for our impactor. The forces that usually correspond to a drop of a weight of 10 gms from a height of 12.5 - 50 mm, producing a mild to severe injury. The forces that are delivered to the spinal cord is approximately 1-2 N.

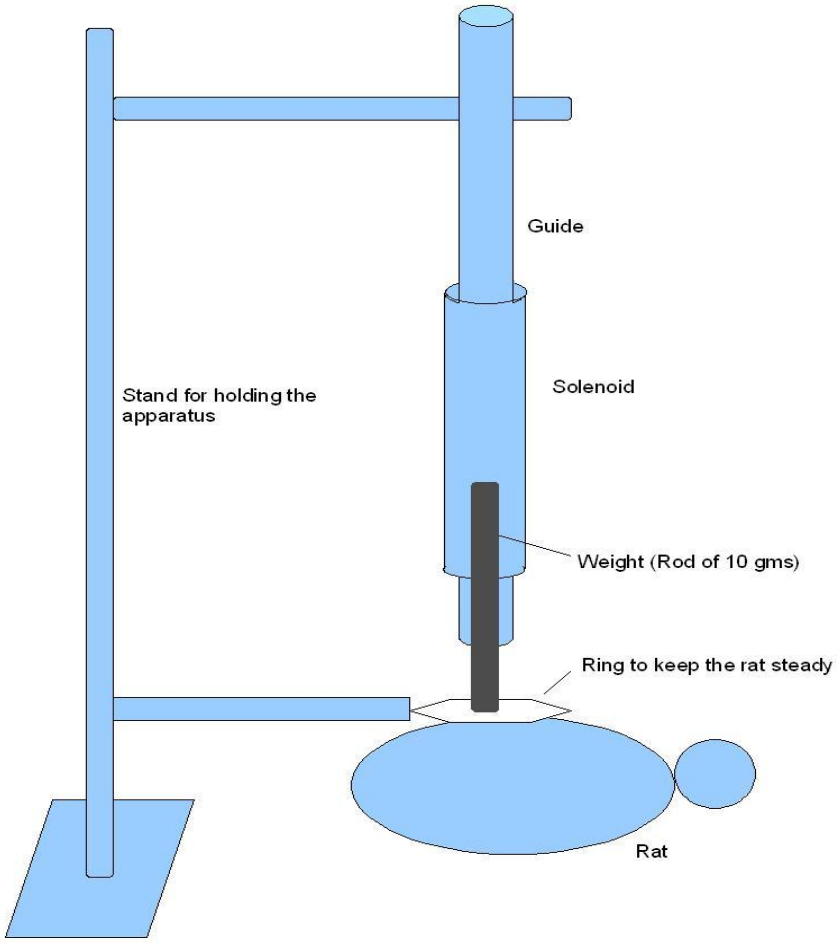
Description of the Impactor

The salient features of the impactor that was developed are as follows:

- a weight of 10 gms which can drop from up to a height of anywhere in between 2.5 mm to 20cm the heights corresponding to moderate and severe injury, respectively.
- the rod hits the spinal cord and retracts back after a fixed time (the retraction is effected by an electromagnet that is controlled by a micro-controller).

- The micro-controller uses the position sensed using a sensor (an inductance sensor) to control the retraction of the weight.
- The position is recorded in an oscilloscope and the force is then calculated after transferring the data into the PC.

A frame is provided which can be sutured to the back of the rat so that it does not move when the experiment is in progress. This keeps the distance of the falling height constant.



Schematic diagram of the apparatus for Spinal Cord Injury showing the impactor.

The guide is made of a long glass tube. The solenoid and the inductor for the position sensing is wound on it, both of which are connected to the control circuit in the adjacent box. The frame to which the rat is sutured is also shown.

Position Sensing: The position sensing is done using a coil (the length of the coil, L = 10 cms, the total number of turns, n ~ 400 turns) which makes an inductor. As the rod moves through this inductor the inductance changes. The inductor is connected in parallel to a capacitor making a tank circuit. The output is fed into a rectifier and the rectified signal is then fed into the micro-controller which reads the position and decides when the the solenoid should be pulled back.

Retraction: The retraction is affected by a solenoid which is powered by a motor driver chip controlled by the micro-controller. The solenoid is wound on a core of 4 mm and is made of a coated wire of thickness 0.24 mm. The length of the solenoid is 10 cms and it has ~ 1600 turns (there are four layers of winding, each layer having 400 turns).

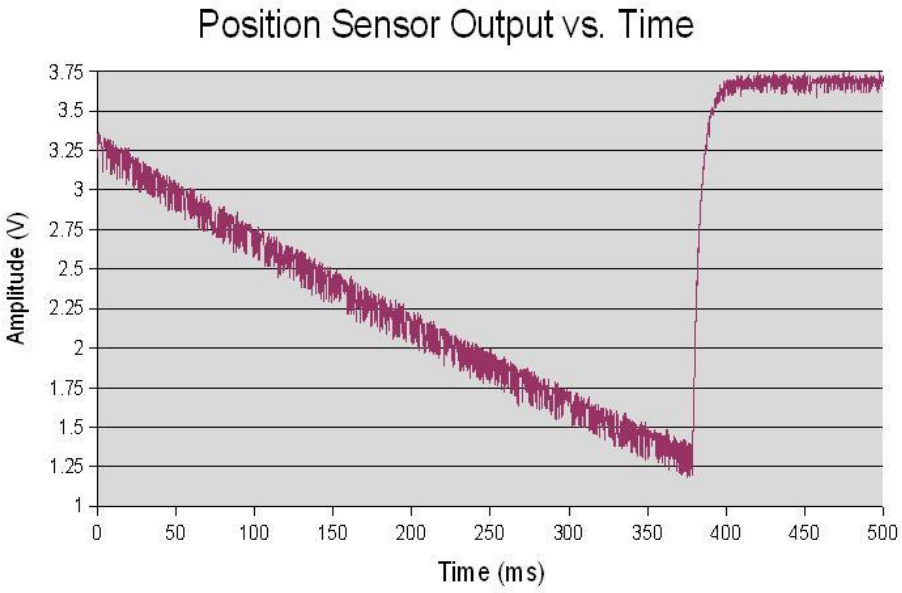
Though various designs were thought of for delivering the force, the weight-drop method was finally chosen owing to its simplicity and acceptability in the research community. A fixed weight dropping from a fixed distance would also give a very reproducible velocity-before-contact and therefore, a reproducible force delivered to the spinal cord resulting in a reproducible injury. The major factors that would effect the extent of the injury would be,

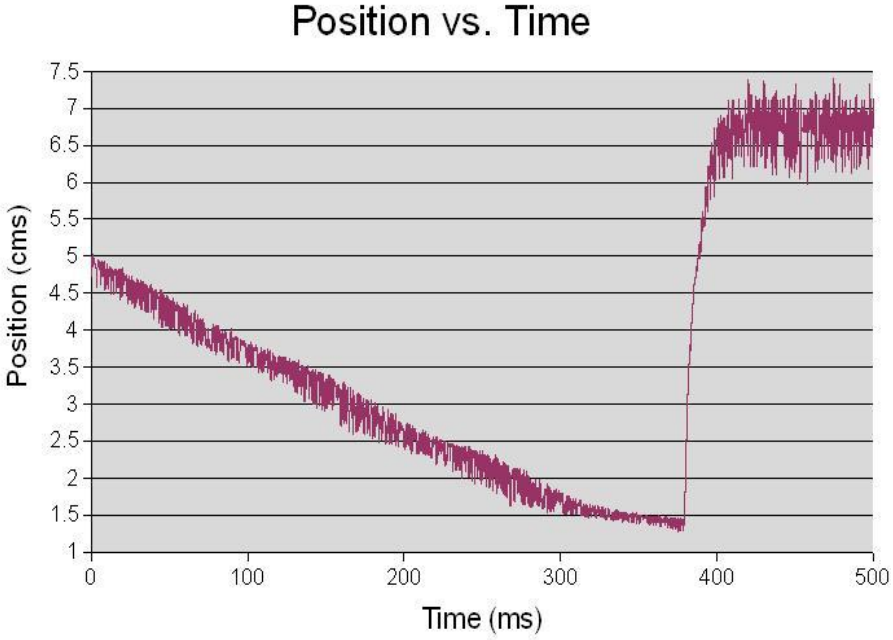
- the velocity of the rod before it hits the cord
- the time of compression of the rod
- the amount of compression of the rod

Assuming that the mechanical properties of the spinal cord of various rats will be the same, controlling the first two parameters should afford reproducible injuries to the rats. The first

parameter, i.e. the velocity of the rod before it hits the cord is fixed as the distance the rod falls due to gravity will be the same and is given by,

$$v = \sqrt{2 * g * h}$$





The second parameter is controlled by the solenoid that pulls the rod after a fixed amount of time after impact. The solenoid is energized and the resulting magnetic field pulls the rod away from the spinal cord. The instant of time when the solenoid is energized is decided by the position sensing circuit. The principle of the position sensing is to measure the change in impedance of a tank circuit formed by a capacitor in parallel to an inductor coil, inside which the rod that would strike the spinal cord would move. As the rod moves the inductance alters changing the impedance of the tank circuit. This was done mainly to reduce the noise in the calculation of the acceleration. Timing of signals are not disturbed using this formula. Notice that the acceleration peaks just before the retraction, corresponding to the rod hitting the spinal cord. At the same time the velocity also starts to increase in the positive direction (positive is defined as away from the ground). This impactor developed for creating spinal cord injury drops a weight of 10 gms from a height of anywhere between 2 cms to 20 cms. The position is sensed using a sensor that used change in inductance to calculate the change in position and recorded on an oscilloscope.

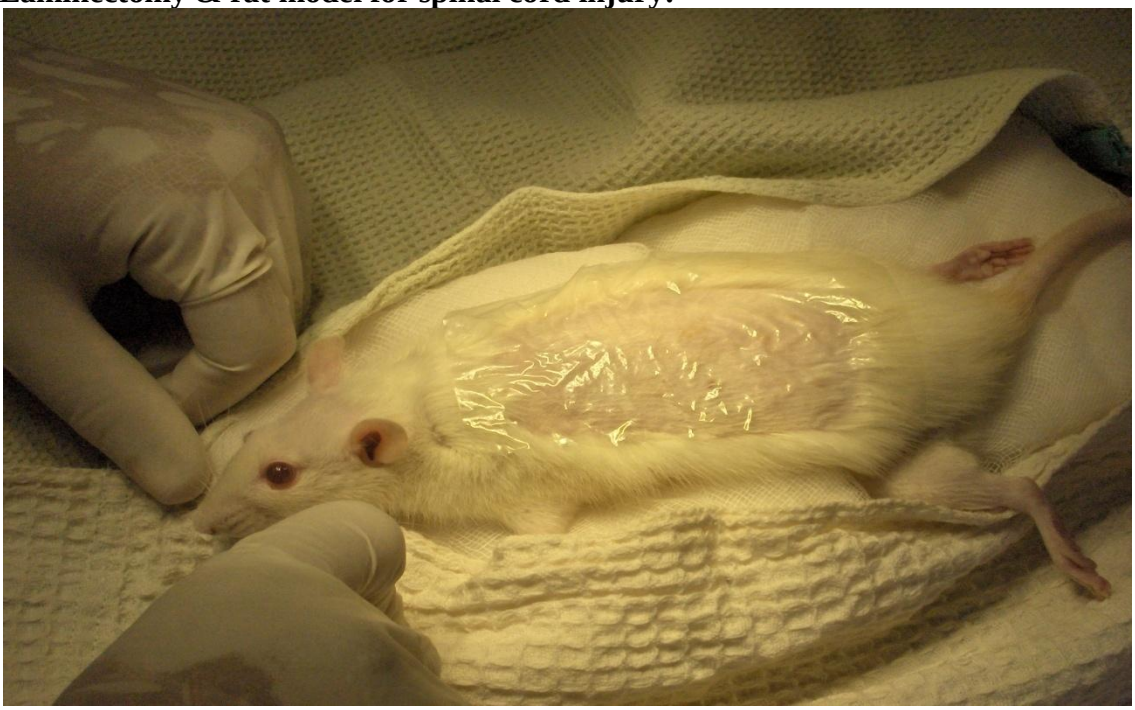
Animal Experiments

Female Albino Wistar rat, 100-300g in body weight were anaesthetised with ketamine and xylazine (90:10 mg/kg) administered intra peritoneally. The fur was shaved on the mid dorsal region and cleaned with Povidone – Iodine solution (7.5% w/v), finally with surgical spirit. Tega-derm applied over it, to prevent fur contamination during surgery. 2cm incision was made over the lower thoracic area, muscle and connective tissue were bluntly dissected to expose the T9-T11 vertebrae. A T10 laminectomy was completed using a microsurgery bone rongeur, taking care not to damage the spinal cord. Drop-weight injury was performed, with 10g weight rod falling from 20cm height on the exposed spinal cord. Absorbable suture were used to ligate the incised muscle and skin. Meloxicam 1mg/kg as analgesic, Enrofloxacin 2.5mg/kg as antibiotic, and Ringer lactate 5ml/100g were administered sub-cutaneously as post-operative care. Animals had free access to food and water throughout the study. Bladder and bowel expressed as post-operative care.

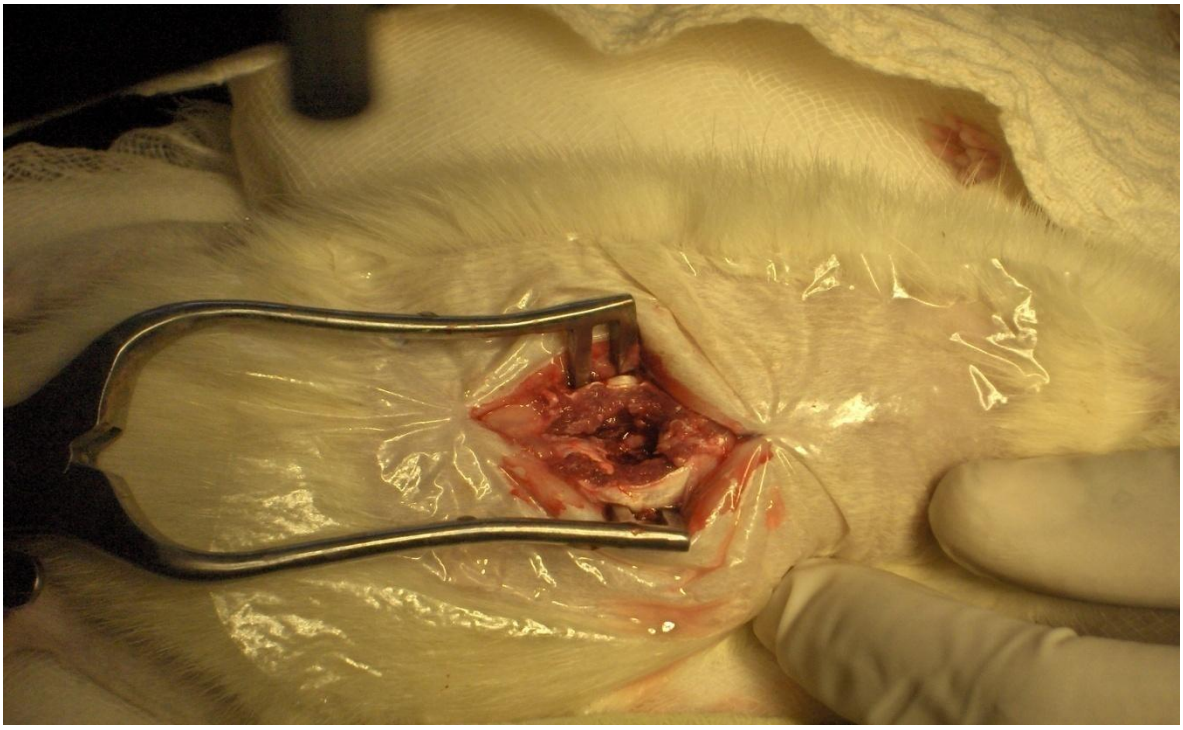
Operation theatre set-up:

The animals were anaesthetised by intraperitoneal injection of ketamine and xylazine. Sterile surgical instruments and operation set-up ready for laminectomy procedure.

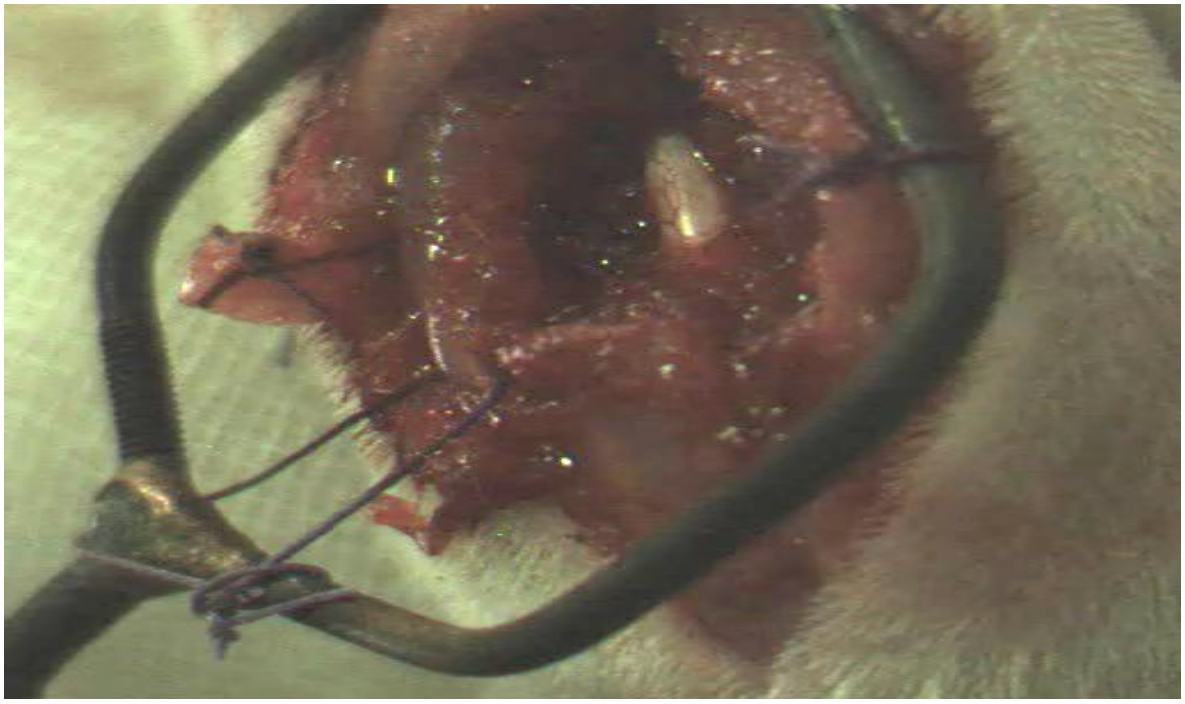
Laminectomy & rat model for spinal cord injury:



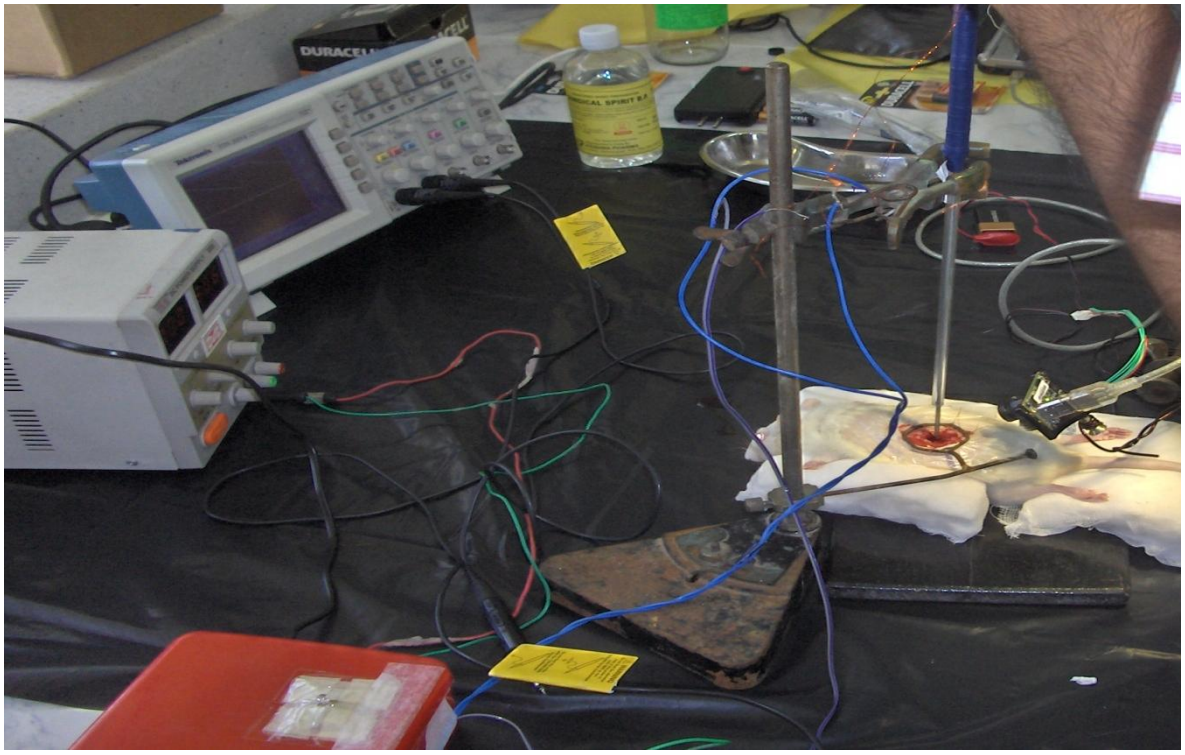
Dorsal side of anaesthetized rat was shaved and cleaned with povidione and surgical spirit. Tegaderm applied over it, to prevent fur contamination during laminectomy.



Incision of skin, subcutaneous tissue and location of spinous process, Laminectomy done at T10 level, without damaging the spinal cord.



Spinal cord was exposed in order to create spinal cord injury by drop-weight device.



Setup with the exposed rat spinal cord as the target. The guide glass-rod can be seen, with a portion of it wrapped in blue containing the solenoid coil. The red box in the foreground contains the electronics.

Transplantation

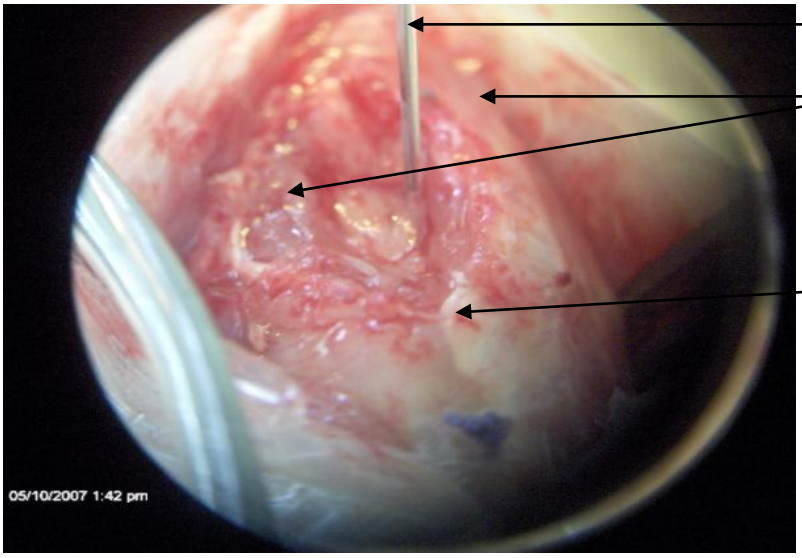
Transplantation of BMSC:

Two to six weeks following the drop-weight injuries, rats were re-anesthetized (intraperitoneal ketamine/Xylazine: 90:10 mg/kg), and the original incision was re-opened and the dorsal laminectomy was extended to the T9-T11 vertebrae. Under a surgical microscope, scar tissue from the previous injured site was removed until spinal cord tissue could be visualized. After removal of the scar tissue, the gap between the caudal and the rostral stumps was approximately 3mm in longitudinal extent.

All injections were made with the aid of a sterile Hamilton syringe attached to a micromanipulator. 5-50µl of cells was injected at different sites. Routine post-operative care was given.

Hamilton syringe is attached to micromanipulator, which can move up and down. Cells were transplanted intra-lesionally into the injured cord, as well as caudal and rostral to the injured site.

Magnified view of transplantation:



Injured spinal cord is exposed in between lateral sides, on which the injecting needle is positioned for transplantation.

Cells transplantation:

S.No	Test group (n=12)						Control group (n=12)		
	Duration between injury and gait (days)	Duration between transplant and gait (days)	Cell numbers (Lakhs)	Pre-transplant BBB	Post transplant BBB	Hindlimb EMG Amplitude	Duration between injury and gait (days)	BBB	Hindlimb EMG Amplitude
1	32	420	10.0	0	8	0.4687	323	1	-
2	45	113	10.0	0	1	-	191	0	0.2734
3	19	120	16.7	0	6	0.8593	144	1	0.2734
4	14	111	11.5	0	7	0.6835	118	0	0.0976
5	13	104	13.5	0	4	0.8203	106	2	0.0000
6	26	79	13.3	1	5	0.9375	104	0	0.0585
7	27	78	21.5	0	3	1.3378	101	0	0.0000
8	30	75	16.2	0	2	0.2734	97	0	0.0000
9	21	51	18.9	0	4	1.1328	87	1	0.1171
10	17	53	24.0	0	6	1.7187	85	0	0.0000
11	16	14	16.8	0	1	-	84	2	0.1171
12	13	49	20.5	0	4	1.0351	72	0	0.0000

Postoperative care:

Following the surgery, rats were nursed in cage and monitored until they recovered from anaesthesia. Rats were monitored twice a day throughout the post-injury survival period for general health and mobility. Bladders were manually expressed twice daily. Ringer lactate 10ml was administered subcutaneously twice daily on the first 5-10 postoperative days. To prevent urinary tract infection, prophylactic antibiotics were administered (Enrofloxacin 2.5mg/kg) once a day for first 10 postoperative days. Animals were monitored for urinary tract infections (UTI) for the entire period of the experiment. If an animal developed general debilitation, vaginal inflammation, or discharge indicative of a UTI, they were treated with the antibiotics. Inspection for skin irritation or decubitus ulcers or evidence of autophagia, was carried out daily.

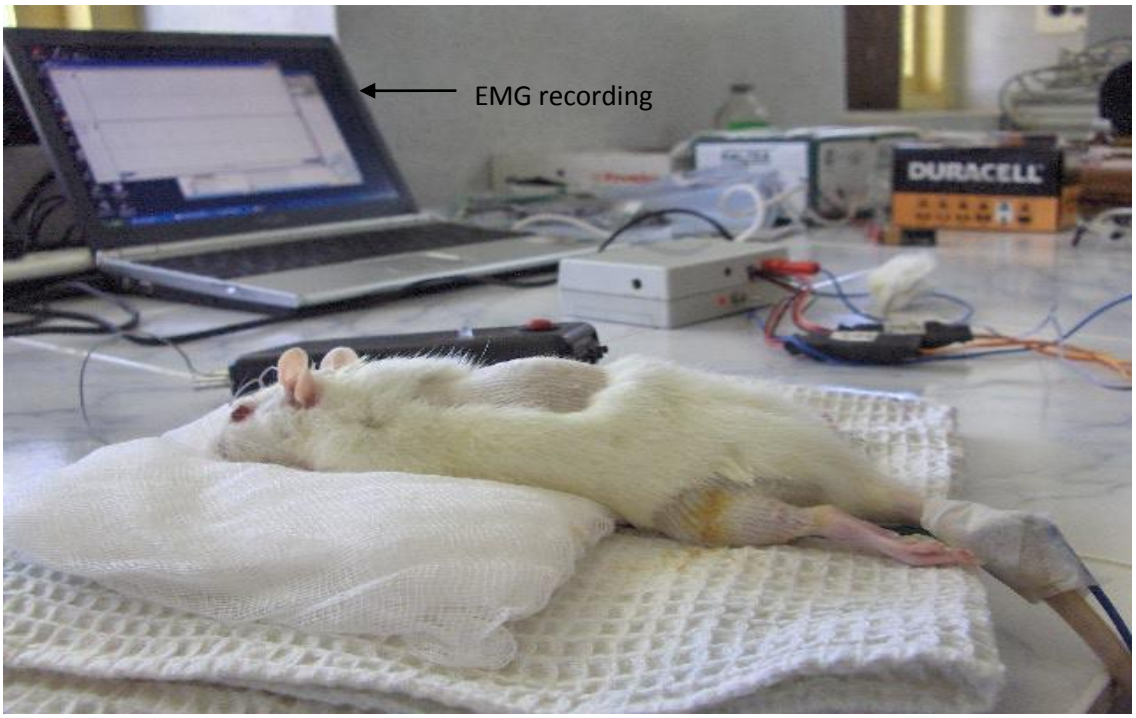
Behavioural assessment:

The BBB scale is an operationally defined 21-point scale, designed to assess hind limb locomotor recovery after impact injury to the thoracic cord in rats. This scale categorises combinations of rat hind limb joint movements, trunk position and stability, stepping, coordination, paw placement, toe clearance, and tail position, representing sequential recovery stages that rats attain after spinal cord injury. Open-field observations were made on rats placed in a floor area of 95×95cm. All rats received bladder expression before open field testing to eliminate behaviours due to bladder fullness.(Appendix: BBB Scale)

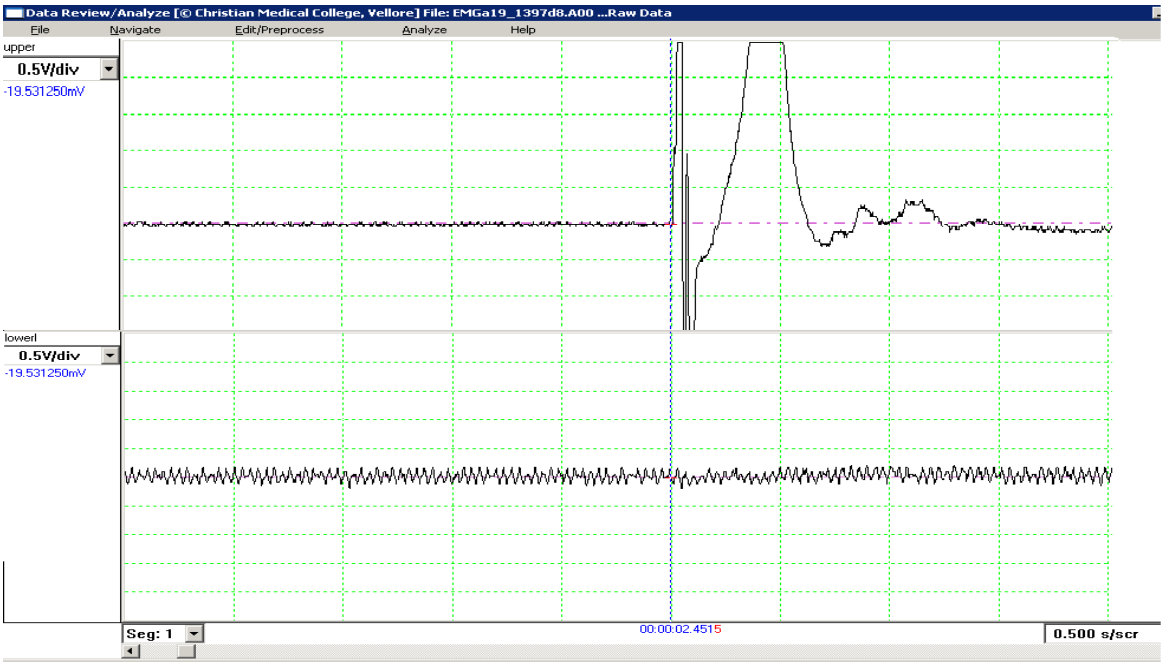
Transcranial stimulation & Cortical evoked potential study for cord integrity

Transcranial stimulation of motor cortex was done in the anaesthetized rats and the EMG signals were recorded from the upper limb and the lower limb muscles.

Among spinal cord injured rats which received MSC transplantation, EMG signals were observed in the lower limb muscles on stimulation of motor cortex.



EMG study before cell transplantation (after injury)



The upper wave of each set was collected from the upper limb (triceps muscle) and the lower wave from the lower limb (gastrocnemius) of the paraplegic rats.

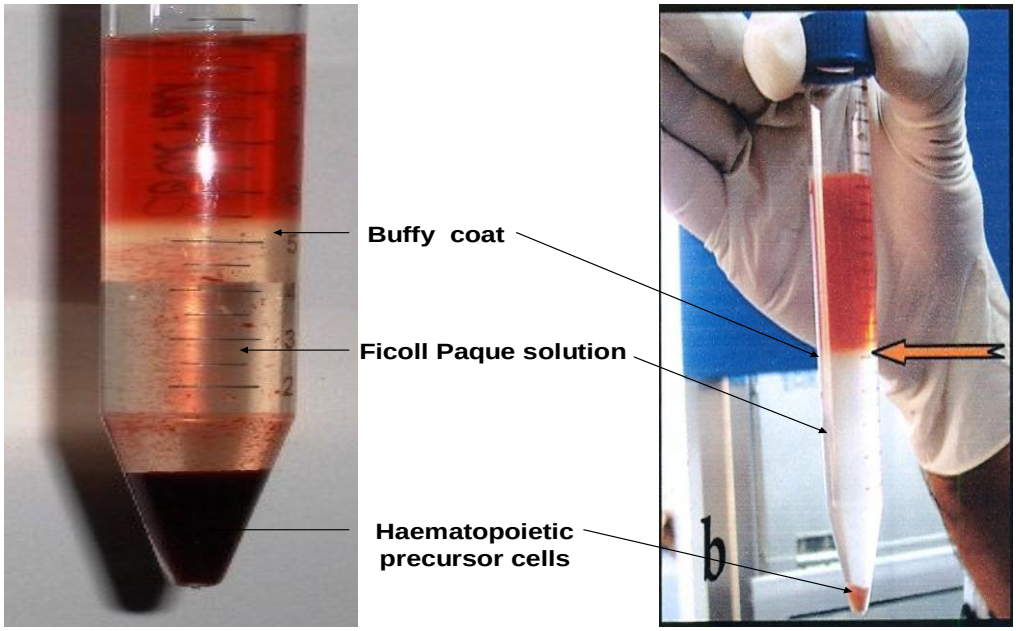
Statistical Analysis

The data collected for BBB assessment and EMG were analyzed using Wilcoxon test and Mann – Whitney test. P value < 0.05 was considered significant in this study.

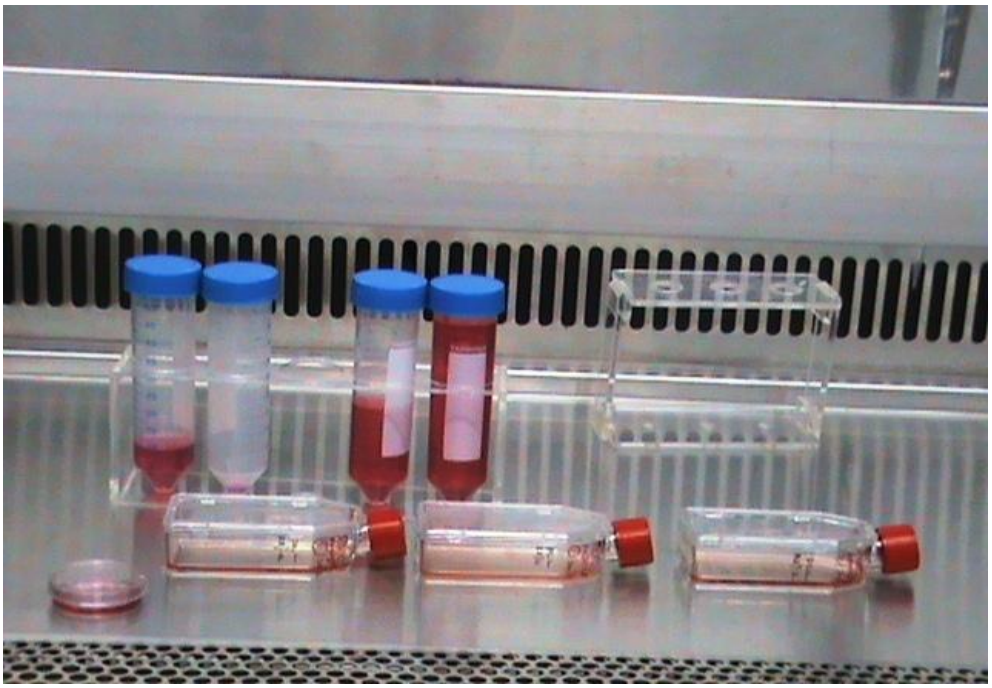
Results

Isolation of bone marrow mesenchymal cells:

Bone marrow mesenchymal stem cells were separated from rat bone marrow by ficoll-paque density gradient method. This yielded buffy coat as shown in the figure which was rich in mononuclear cells including BMSC.

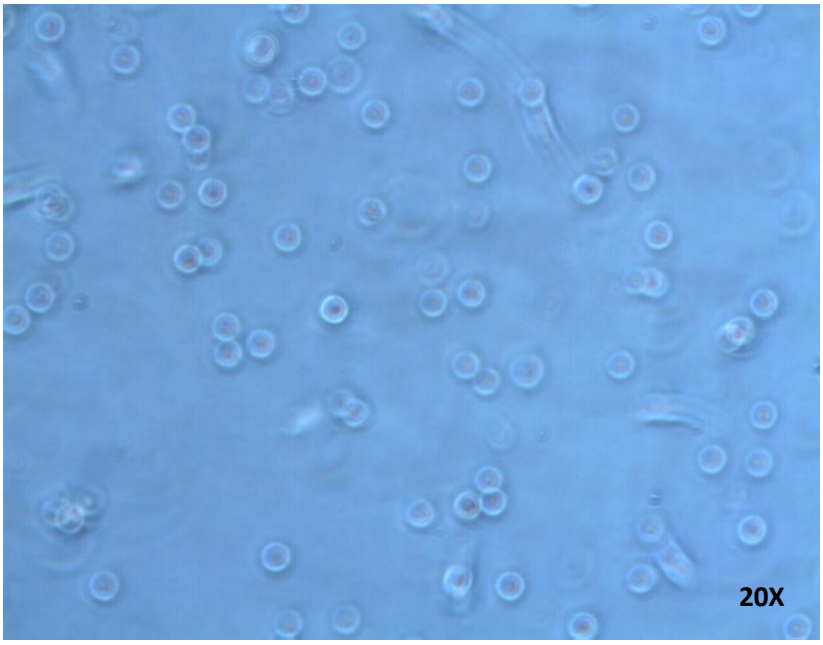


Buffy coat cells on plating on to plastic culture dish in the culture media allowed BMSC to adhere to the plastic and grow and thus selectively separated these cells as seen in the figure.



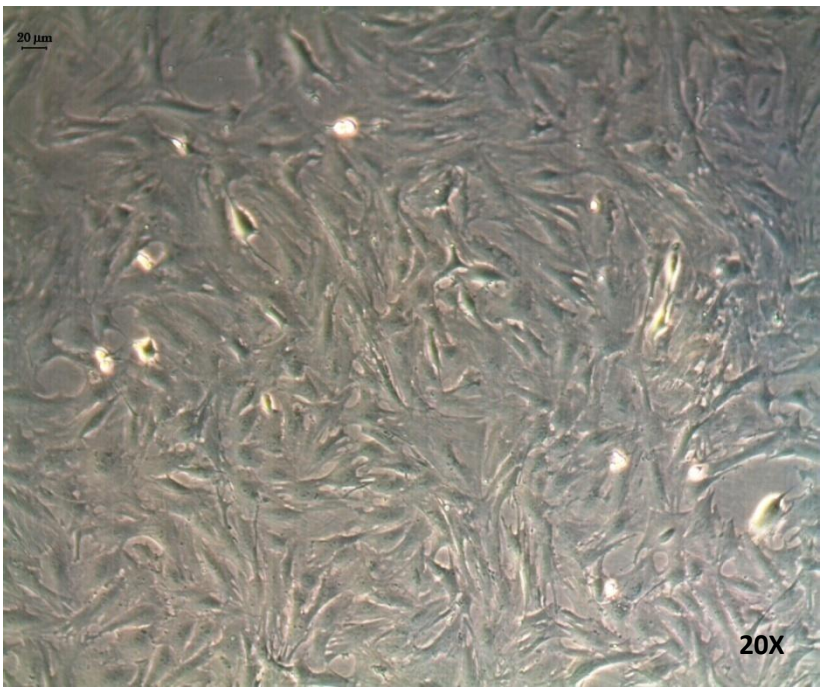
First day culture of Bone marrow stromal cells:

Culture on the first day showed rounded cells adherent to the plastic culture dish, a characteristic feature of the BMSC as seen in figure.



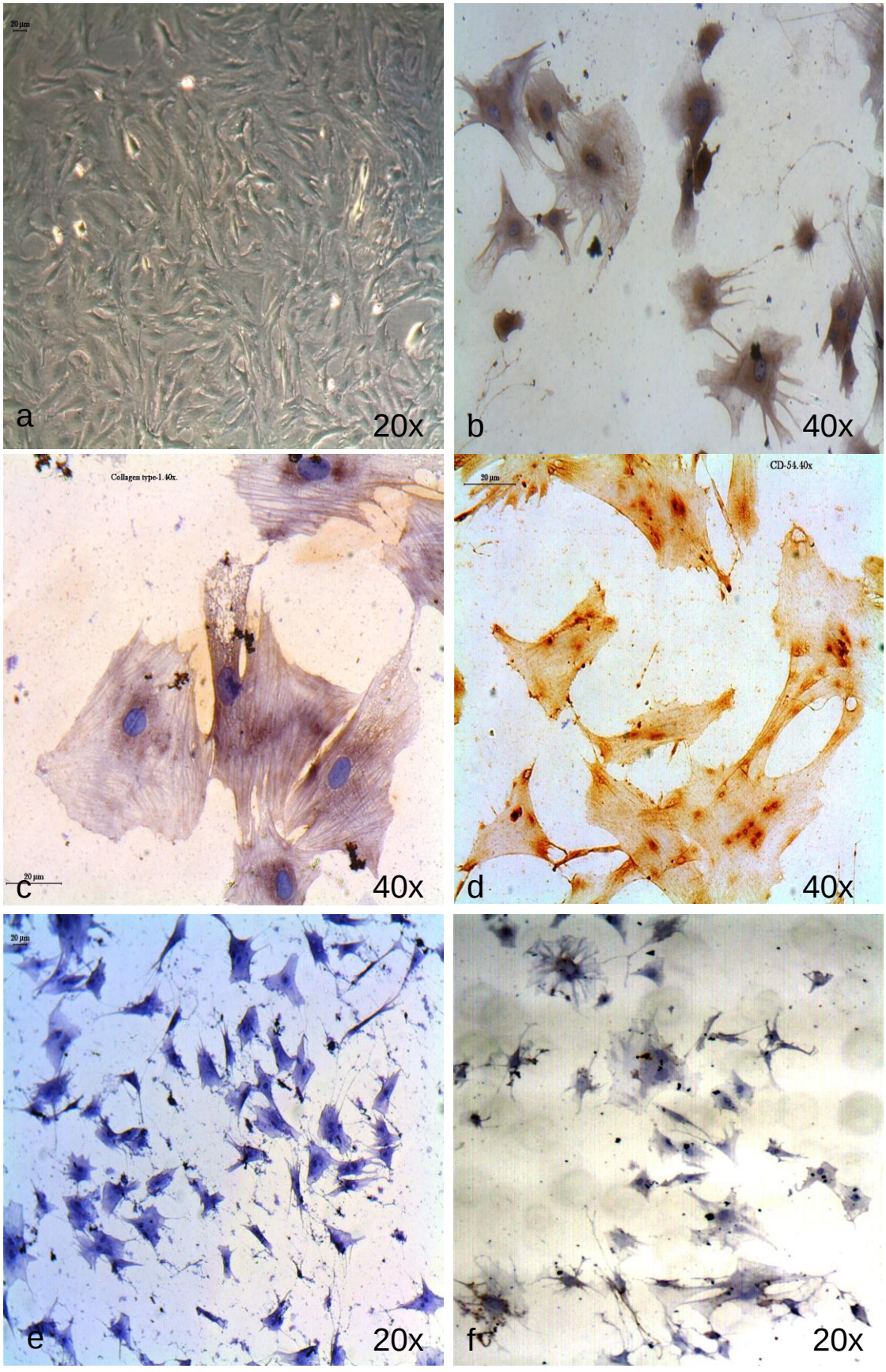
Confluent culture of BMSC:

Within two weeks the cells multipled and assumed confluence as seen in figure below.



Characterization of Mesenchymal stem cell:

Mesenchymal stem cells are characterized by positive cell markers which included antibodies directed against cell-surface molecules (integrin β1 and CD54) present on MSCs along with extracellular matrix molecules (type I collagen) that are synthesized by cultured MSC. As seen in the figure, two specific haematopoietic cell surface markers, (CD14- present on leukocytes and CD45 – present on monocytes and macrophages) are not expressed on mesenchymal stem cells.

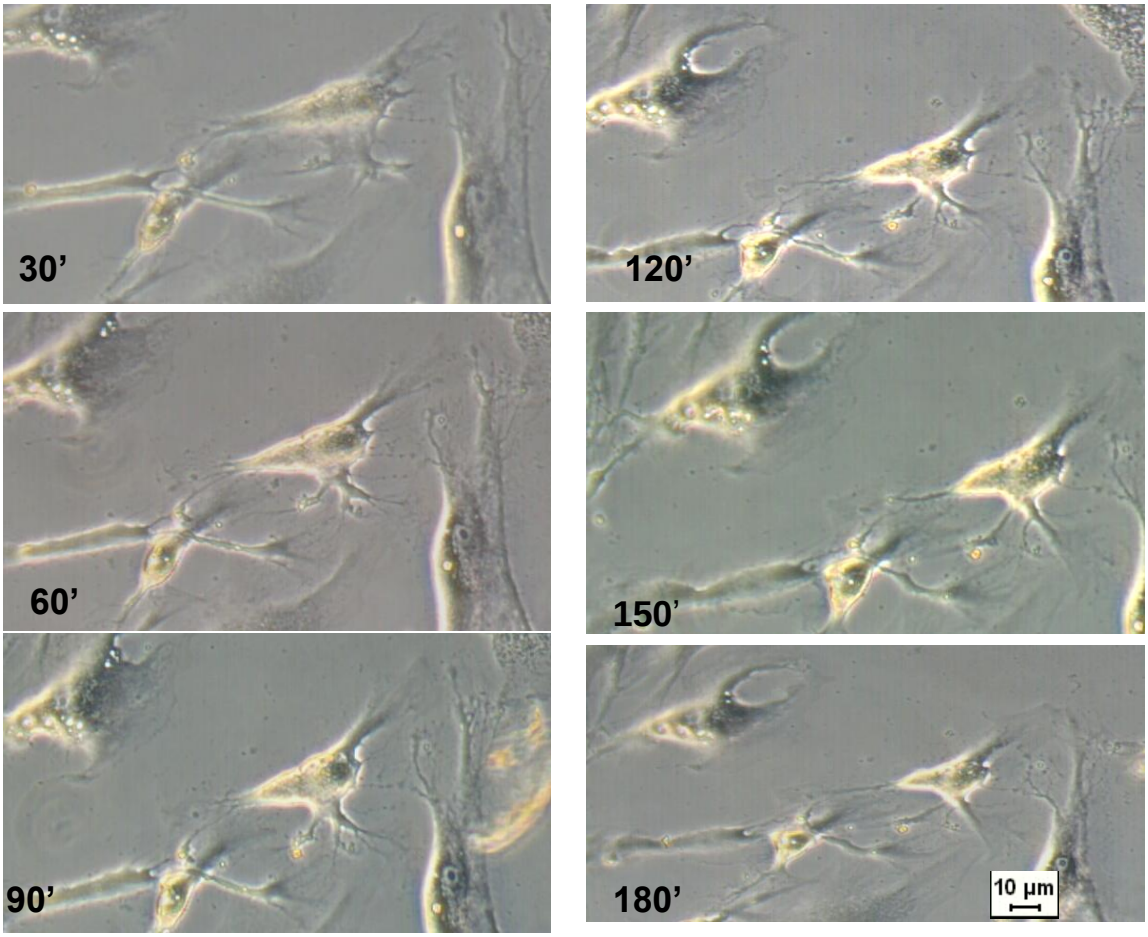


a) Confluent culture **b)** Integrin β1 **c)** Collagen type I **d)** CD54 **e)** CD14 **f)** CD45.

Neuronal induction of Bone marrow Mesenchymal Stem cells:

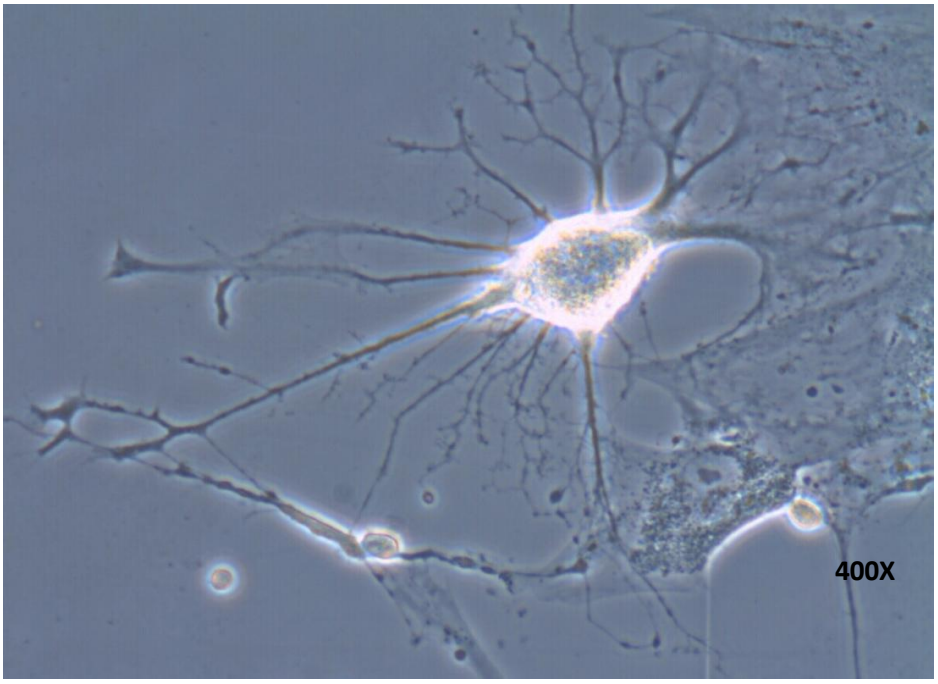
It has been reported that rat and human MSCs retain the capacity to differentiate into non-mesenchymal derivatives, specifically neurons. A simple treatment protocol has been described to induce the stromal cells to exhibit a neuronal phenotype expressing Neuron-specific enolase, Neurofilament, and Tau protein by Woodbury et al[57] which was conducted as a part of this study and the results are shown in the figures below.

To initiate neuronal differentiation, neuronal induction media composed of DMEM, dimethylsulfoxide (DMSO), butylated hydroxyanisole (BHA) was utilized. Long term neuronal induction media consisting of DMEM, DMSO, BHA, KCl, valproic acid, forskolin, hydrocortisone and insulin was used.

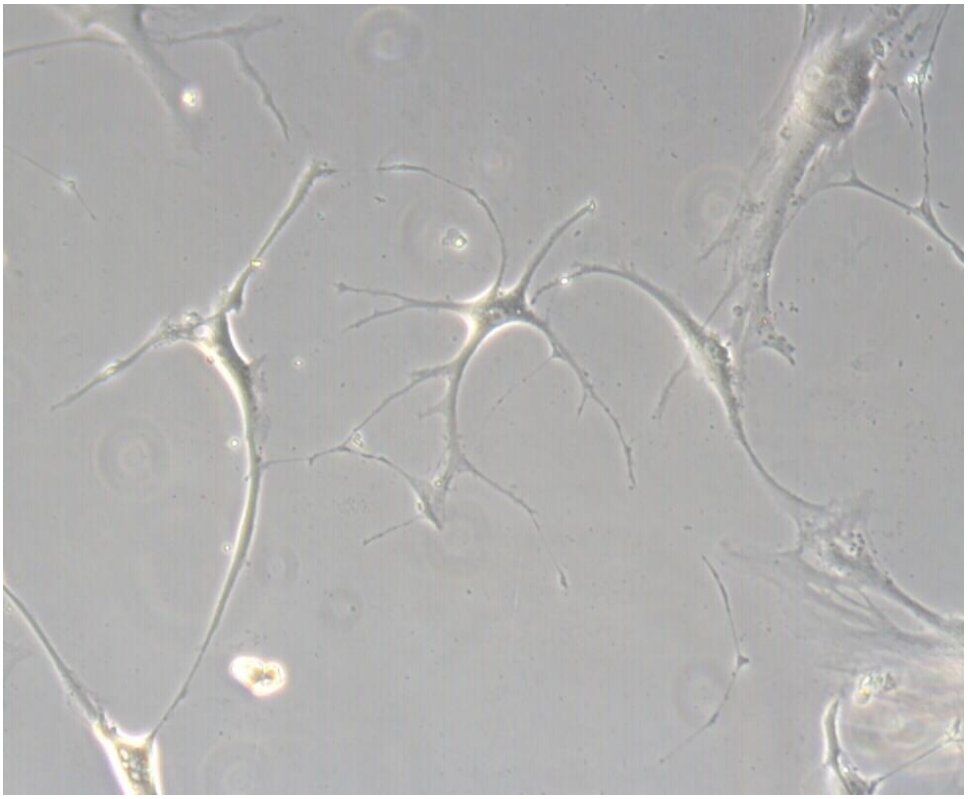


After treatment with the medium for neuronal induction, morphology of cells was observed from 30 min to 180 min. Retraction of cell body and process elaboration is evident with increasing time. Processes continued to elaborate, displaying primary and secondary branches with growth cone-like terminal expansions and filopodial extensions.

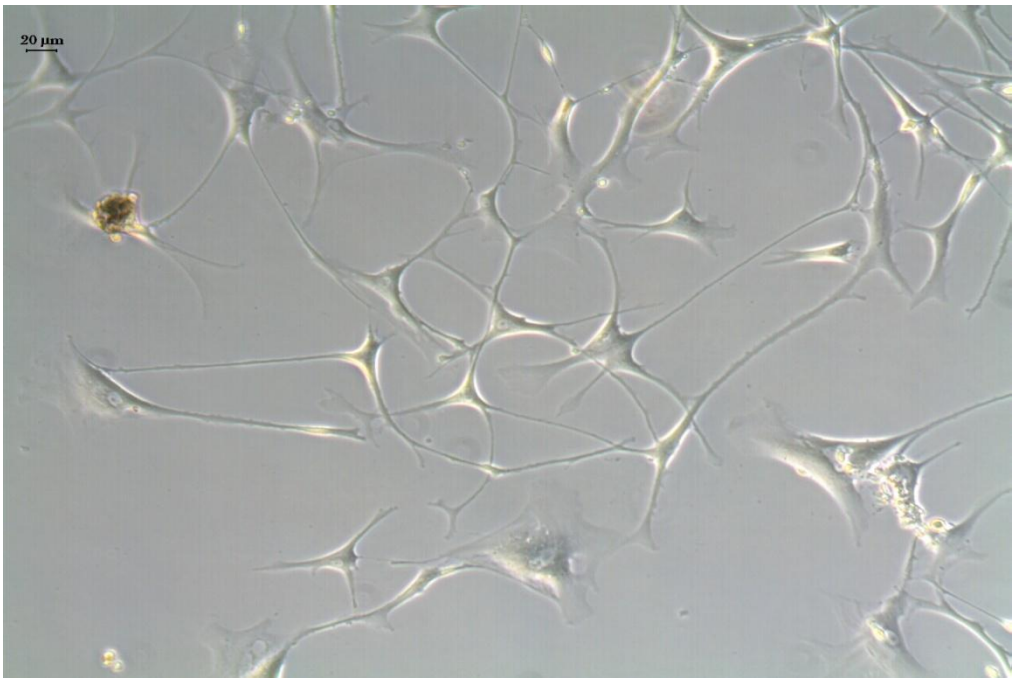
Neuronal induction after 3hrs:



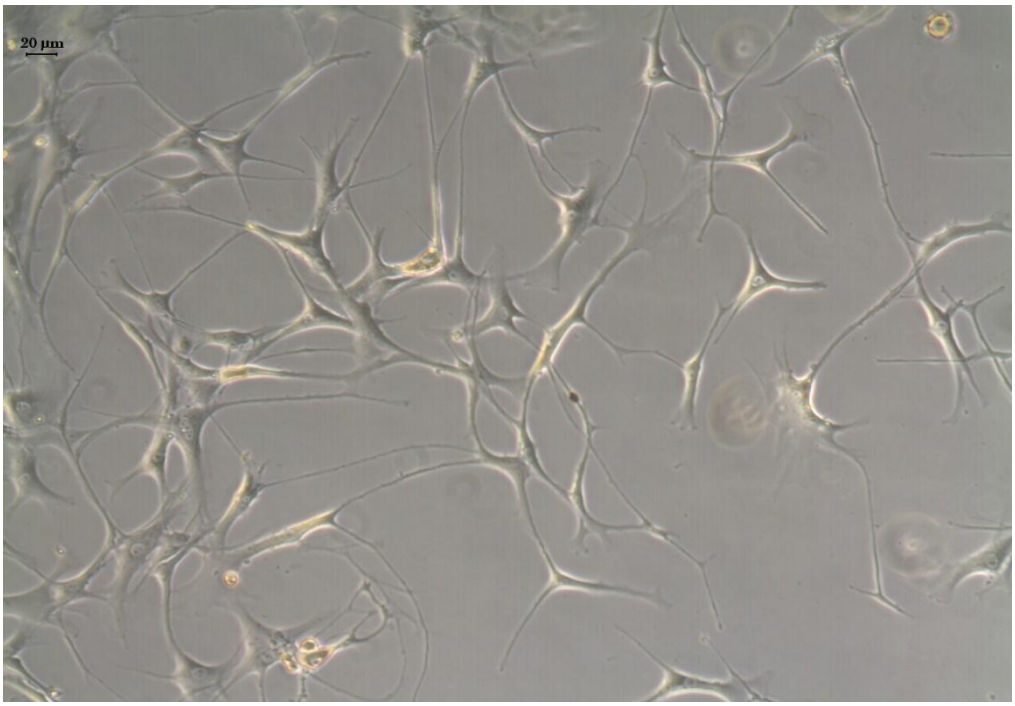
Neuronal induction after 24hrs:



Neuronal induction after 48 hrs:

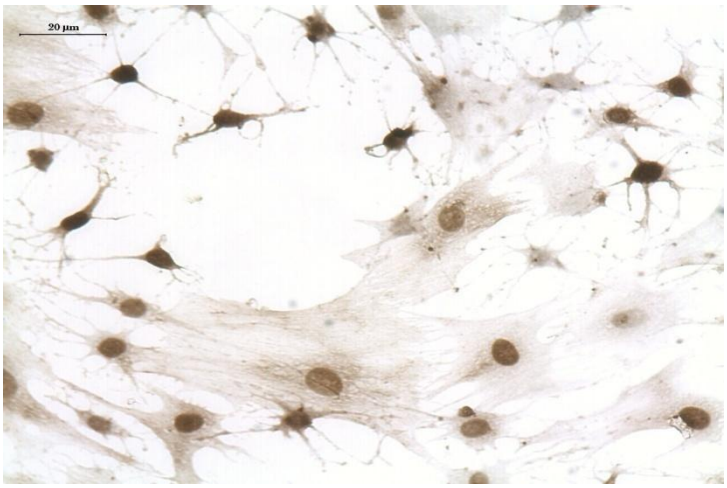


Neuronal induction after 72 hrs:

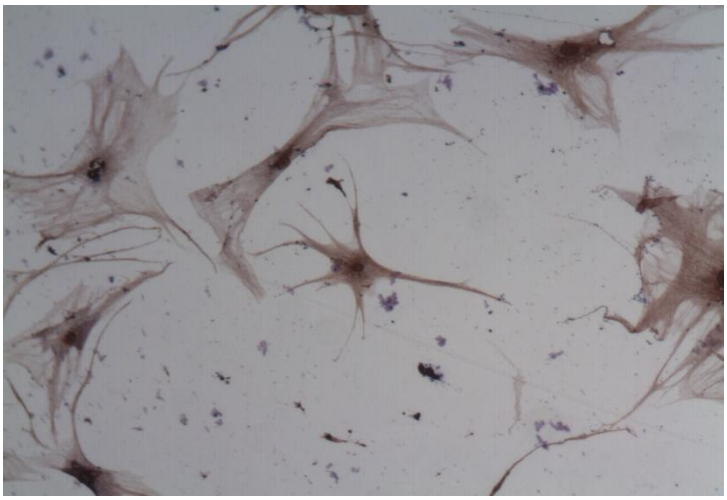


Identification of Neuronal induced cells by neuronal markers:

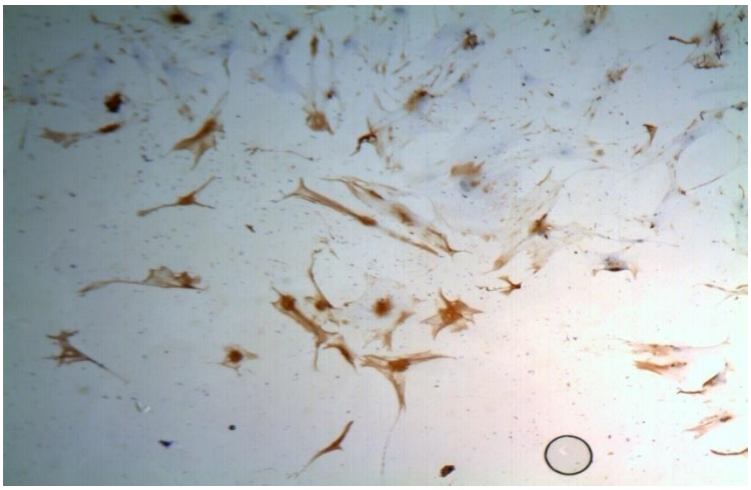
Tau protein immunostained:



Neurofilament marker:



Neuron-specific enolase marker:



BBB motor score:

Hind limb movement of the transplanted rats progressed from 0 to maximum of 8 in BBB rating scale. In comparison of control group, transplant group rats improved in hind limb function, which is statistically significant ($p<0.05$), whereas control group did not show any progress indicating that there is no spontaneous recovery after spinal cord injury. Control group improved in BBB score maximum of 2, though statistically significant ($p=0.035$) in comparison to that of before transplant group.

Graph 1 *BBB score of the transplant group before and after cell transplantation.*

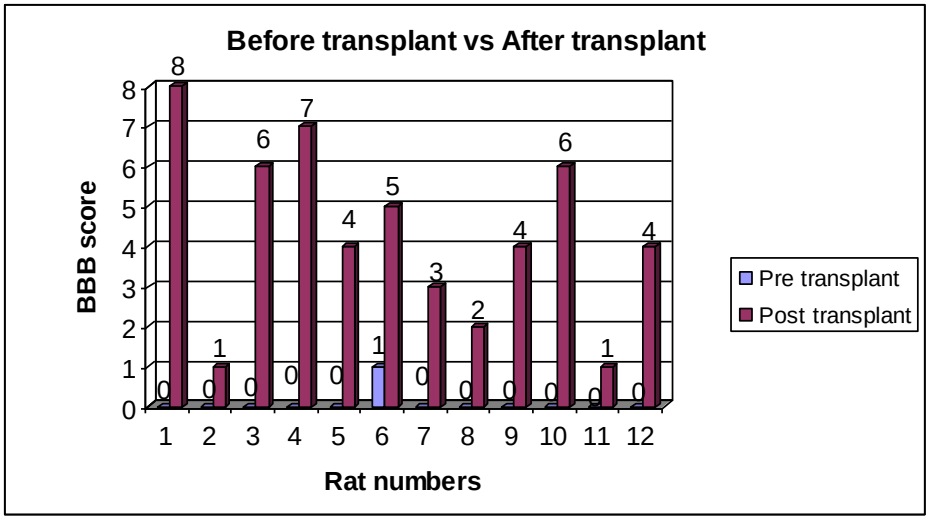


Table 2 BBB

	Transplant group (n=12)				Control group (n=12)		P value
	Before transplant		After transplant				
	Mean	SD	Mean	SD	Mean	SD	
Before transplant Vs control group	0.083	0.288			0.583	0.792	0.108
After transplant Vs Control group			4.250	2.261	0.583	0.792	0.0001
Before transplant Vs After transplant	0.083	0.288	4.250	2.261			0.0001

Cortical evoked potential:

Electromyographic signal was recorded using intra-muscular bipolar electrodes in triceps (forelimb) and soleus muscles (hind limb) by stimulating the motor cortex. On stimulation, the forelimb exhibits normal EMG pattern both in control and transplanted group since forelimbs are not involved in a T10 injury. EMG amplitude in hind limb muscles between control and transplanted group showed significant difference ($p<0.001$).

50% of control rats exhibited no response in soleus on stimulation. There was no onset potential compared to transplanted group ($p=0.053$).This shows transplanted MSCs in the injured cord has a role in functional repair.

Table 3 Electromyography

		Transplant group (n=10)		Control group (n=11)		P value
		Mean	SD	Mean	SD	
Hind limb	Onset time (msec)	0.032	0.021	0.013	0.016	0.052
	Peak time (msec)	0.037	0.023	0.016	0.018	0.059
	Amplitude (mV)	0.926	0.416	0.085	0.104	0.0001

Discussion

The consequences following spinal cord injury are devastating affecting physical and emotional facets of life. The costs encountered by the individual are enormous, to address the medical, surgical and rehabilitative care as well as for social and vocational reintegration. Last decade has seen an aggressive pursuit to mitigate the cascade of problems following spinal cord injury to alleviate some of these difficulties, if not to offer cure. Several intense experimental studies are being conducted across the world, and some of them have found their way into initial clinical trials with promising results.

Following spinal cord injury there is disruption of axons and damage to central gray matter and local haemorrhage. This primary event is followed by a complex cascade of chemical processes resulting in disturbances in local electrolyte homeostasis, high concentration of free radicals, excitotoxicity due to excitatory amino acids, local inflammation, regional hypoxia, and cellular apoptosis. The secondary mechanism damages the tissue that was spared during primary injury. This offers room for neuroprotective agents to reduce secondary damage, which has potential to change course of treatment of spinal cord injury in future. The secondary injury subsides over weeks and months following trauma. At the site of injury cellular reaction is represented by activation of astrocytes, microglia, macrophages, fibroblast and meningeal cell which together constitute the glial scar which is an impediment to neural regeneration. The challenge at this stage is to promote axonal sprouting or remyelinate the preserved axons. Though several experimental approaches have been developed and published, an optimal strategy could be combinatorial treatment which addresses multiple barriers to neuronal regeneration[58].

Exciting reports have been published in literature regarding the use of cell therapy to restore motor function following spinal cord injury. The reports that have attracted wide scientific

attention include transplantation of autologous olfactory ensheathing cells, foetal olfactory ensheathing cells, autologous activated macrophage transplantation, human embryonic cell derived pre-oligodendrocyte transplantation, and autologous bone marrow stromal cells transplantation which have entered initial clinical trials.

As discussed in review of literature, several studies reported favourable outcome following bone marrow stromal cell transplantation. However there are conflicting reports on the efficacy of MSC in spinal cord regeneration in experimental models following transplantation[59]. With these controversial data in literature, it was decided to observe the effect of bone marrow stromal cells for motor recovery following spinal cord injury in this study.

MSC have the advantage of being autologous and thus without ethical controversies. More over it obviates the need for immunosuppression following administration. Further, these cells are readily available as well as accessible, without causing any deficits in the donor tissue. Whole adult bone marrow contains a mixture of haemopoeitic cells including mononuclear cells like macrophages, and non haemopoeitic marrow stromal cells. MSC support the growth and differentiation of HSC (haemopoeitic stem cells). MSC can be isolated by their ability to adhere to the plastic containers during tissue culture. MSC contain a population of cells that are self renewing and are capable of differentiating into mesodermal tissue, like bone, cartilage, fat, and muscle. In this study, bone marrow from femur of adult male albino Wistar rat, were harvested, mesenchymal stem cells separated by ficoll-paque density gradient method, and cultured. The haemopoeitic fraction was separated by rosette cell separation. This was done to improve the population of mesenchymal stromal stem cells. The cultured cells were characterized by immunostaining techniques with specific markers which included beta 1- integrin, collagen1, CD54 positivity and negative for CD14 and 45.

These cells were then injected into experimentally induced spinal cord injured animal models.

Different models of spinal cord injury could be created such as thermal injury, crush injury with aneurism clips, electro fulguration injury or radiation injury. The model of injury chosen in this study was a drop weight contusion model, since this mirrors the type of injury that a human individual sustains following a fall or trauma. Contusion injury by drop weight method in female Albino Wistar rats was created which served as the animal model of spinal cord injury. The injury created was comparable as the force was measurable and the effects on the cord consistent, since a known weight was dropped from a known height and force was measured through a transducer. Cord injury was created at T9 /T 10 cord level following laminectomy as described in literature. However maintaining a paraplegic rat is an intense and delicate task, since the rats are vulnerable for pressure sores, urinary tract infections, bladder calculi, and most of the complications that the human counterpart is also vulnerable to. The bladder needs to be emptied twice a day, and bowel once a day. Antibiotics need to be used judiciously and with caution. Hydration needs to be maintained and adequate supplies of nutrition ensured. The rats have to be nursed on a soft bed to prevent the pressures sores. Female albino wistar rats were chosen for the study, since management of bladder and related complications were easier to address than in the male rats.

Rats were selected as the animal model because, the pathology following spinal cord injury is similar to human subjects. The central area of necrosis and fluid collection surrounded by demyelination of axons is similar to the pathology observed in human subjects; hence, an ideal model for intervention studies. Many experimental studies were conducted to find the optimal time for cell transplantation. To avoid destruction of transplanted cells by inflammatory processes in the acute phase, period between 10 to 14 days is considered

optimal for the transplantation. Animal studies show that significant gliosis is a major hurdle for axonal regeneration, in the chronic stage.

The functional outcomes were measured by BBB score and EMG. BBB is a numerical motor scale, easy to perform, record, and compare. This scale measures the extent of movement of hip, knee and ankle by analysing the gait of the rat. The electrophysiological evidence of motor recovery was measured by transcranially stimulating the motor cortex using electrical stimulators and recording EMG from the gastrosoleus muscle. Eliciting wave forms in the calf muscle on stimulation of the cortex denotes functional continuity of the cortico spinal tract.

The rat models of spinal cord injury was considered in two group of twelve rats each. One group of rats received cell transplantation (transplant group, n=12) and the other did not have cell transplantation (control group, n=12). Only rats with complete spinal cord injury was included in the study. Following the spinal cord injury the rats were observed for atleast for a period of two weeks before transplantation, to confirm completeness of the spinal cord injury. All the rats had the BBB score of 0 or 1. This excludes the possible recovery due to preserved axons. This period also ensured that cells were not transplanted during the acute inflammatory phase, as this could lead to loss of transplanted cells by reactive macrophages. A total cell dosage ranging from 10 to 24 lakhs were injected into the injured spinal cord at and around the site of contusion. Cells were administered at multiple sites as described in literature to ensure adequate cell delivery and minimize loss during injections. Rats were followed up for a period ranging from two weeks to thirteen months. Post transplant BBB score showed improvement ranging from 1 to 8. Within the group which received transplantation, BBB motor score showed significant improvement (Graph 1) after transplantation (P=0.0001). In comparison with control group, transplanted group showed statistically significant improvement (P=0.0001). When the control group is compared with

pre-transplant group there is no significant change in motor score (P=0.108). These observations suggest motor recovery following cell transplantation. The control group rats were followed up for a period of ten months and the BBB score did not reveal any spontaneous recovery in course of time.

In congruence to BBB, electrophysiological response in hindlimb of transplanted group also showed significant amplitude of the cortical evoked potential (P=0.0001) compared to control group. The transcranial electrical stimulation of the motor cortex of anaesthetised rats were performed in control and transplanted group, and response recorded from the gastrosoleus muscles. This also demonstrated statistically significant change in amplitude among rats which received stem cell transplantation, indicating improved conduction as a factor for functional improvement. Remyelination of the demyelinated fibres from transplanted BMSC has been reported which contributes to the functional recovery. It has been observed that transplantation of bone marrow stromal cells can remyelinate the demyelinated spinal cord in animal models and the transplanted cells acted like the myelin forming cells as discussed earlier in the review of literature.

Controversial results in the literature prompted the study to observe the trans-differentiation potential of bone marrow stromal stem cells. Woodbury et.al., reported that bone marrow stromal stem cells on exposure to neuronal induction medium, rapid transdifferentiation of responsive cells occurred within 30 minutes to 180 minutes. Based on this interesting report the cultured bone marrow stromal stem cells was treated with the neuronal induction medium and morphological changes started appearing with in few minutes. The cytoplasm retracted towards the nucleus, forming a contracted multipolar cell body leaving membranous extensions in the periphery. These cells assumed morphological characteristics of neural cells microscopically. Further it was also observed that these cells expressed neuronal markers, like neuron specific enolase, and neurofilament protein, and Tau protein suggesting

neuronal phenotype. Even though these results were similar to that have been described in literature it raises fundamental questions related to transdifferentiation . Mesenchymal stromal cells are mesodermal derivatives whereas neural tissue develops from ectoderm. Thus these findings though in congruence with the reported literature, are controversial. It has been suggested that the inducing medium shocks the cell organelles which retracts and deforms assuming a neural morphology. It needs further corroboration with physiological tests using patch clamp techniques to demonstrate voltage-gated sodium channels on the cell membrane indicating exicitablitiy of the cell purporting neural transdifferentiation. Several studies has since been published refuting this ability of the mesenchymal stromal cell for neural transdifferentiation. It has been suggested that the morphological neuron like changes could be created by rapid disruption of actin cytoskeleton as a result of cellular toxicity. However, it is likely that these cells may differentiate in to neural cells or glia invivo in the spinal cord responding to molecular environment in the spinal cord[60].

Different routes of cell delivery have been described in literature. They include, direct injection to the cord after surgical exposure of the cord, or into the CSF (cerebrospinal fluid) through a lumbar puncture, or intravenous stem cell infusion, or administered by arterial cannualtion towards the anterior spinal artery . In this study the cells were delivered directly into the spinal cord as described in literature. The cord was re exposed and the injured segment received cells at multiple sites to reduce loss of cells during administration and avoid the controversies regarding homing of cells if provided through any other route. The dose of cells administered varied from 10 to 24 lakhs to observe any dose response relationship. However no such direct relationship was seen. This could also be due to small number of rats in the study.

Transplantation of bone marrow stromal stem cells into injured spinal cord may provide therapeutic benefit. Although it has been suggested that differentiation of MSC into neural

lineage may occur in vitro and in vivo, it is unlikely to be a major factor in the functional recovery following spinal cord injury. Other mechanisms include neuroprotection, paracrine effects like expression of growth factors, cytokines, vascular factors, inhibition of apoptosis or remyelination. These may operate in parallel to contribute to the functional recovery. Potential advantages over other types of cell transplants include their ability to be harvested from autologous donors and rapid expansion. Allogenic MSC are non immunogenic and can be readily available for clinical application. MSC lack B7 and co stimulatory molecules CD80 and CD86 and are non immunogenic on allogenic transplantation[61].

The host response to transplanted MSC is of critical importance. It has been demonstrated that MSC are capable of suppressing mixed lymphocytic reactions. Human MSC by virtue of their distinct immune phenotype profile associated with the absence of expression of HLA class II and low expression of co stimulatory molecules are non immunogenic or hypo immunogenic. Allogenic MSC have many advantages in therapeutic application that it can be used even in acute setting. It has been reported that MSC even when delivered by intravenous infusion are capable specific migration into the injury. The mechanisms that guide of implanted cells could be chemoattractive proteins on the injured cells. The undifferentiated cells delivered into injured host migrate to the site of injury and differentiate into the cells of appropriate phenotype under the influence of local signals. Homing is considered to be a coordinated, multistep process, which involves signalling by stromal-derived factor 1 (SDF-1) and stem cell factor (SCF), activation of lymphocyte function-association antigen 1 (LFA-1), very late antigen 4/5 (VLA-4/5) and CD44, cytoskeleton rearrangement, membrane type 1 (MT1)-matrix metalloproteinase (MMP) activation and secretion of MMP2/9. Rolling and firm adhesion of progenitors to endothelial cells in small marrow sinusoids is followed by trans-endothelial migration across the physical endothelium-extracellular matrix (ECM) barrier. Stem cells finalize their homing uniquely, by selective access and anchorage to their

specialized niches in the extravascular space of the endosteum region and in periarterial sites[62].

The findings of this study indicate that bone marrow stromal cells can be cultivated and maintained in culture easily in the culture lab which can be utilised for transplantation for spinal cord injury. The challenges of recovery due to transplantation of cells appears to be, cell survival, axon regeneration, and accurate targeting of the growing axons.

The results demonstrated that the transplantation of bone marrow stromal stem cells in rat models of spinal cord injury at mid thoracic level led to moderate improvement in motor recovery as seen by behavioural analysis as well as by electrophysiological evaluation. Further studies in larger numbers with different dosages and different time since injury as well as combinatorial treatment with other agents will help to enhance the moderate recovery observed in this study.

Conclusions

Spinal cord injury results in permanent paralysis, loss of bowel and bladder control and other autonomic functions. Despite extensive research, there is no treatment available for this condition. This study demonstrated the role of bone marrow mesenchymal stem cells in spinal cord injury in rat models.

1. Bone MarrowMesenchymal Stem Cells can be cultured from the bone marrow sample and can be characterised to be used for transplantation.

2.Bone marrow Mesenchymal Stem Cells can be trans differentiated into neural cells in vitro using appropriate inducing media. However this observation needs further confirmation.

3.Drop weight model of spinal cord injury gives a consistent comparable contusion model for research studies.

4.Transcranial stimulation can be used to elicit motor evoked potential to demonstrate functional continuity of corticospinal tract in rat models of spinal cord injury.

5.Transplantation of bone marrow stromal stem cells can improve the motor out come in rat models of spinal cord injury as measured by BBB motor score and electrophysiological studies.

Recommendation for Future Studies

1. Safety and and efficacy of MSC transplantation needs to be evaluated.
2. The cell dosage of administration needs to be ascertained.
3. The timing of cell transplantation after injury needs to be standardised.
4. Frequency of administration for best response needs to be studied.
5. The mechanism of action of MSC in spinal cord injury needs to be elucidated.
6. The long term survival of MSC and integration into the host tissue needs study.
7. The type of injury (ASIA scale) where this will be most suitable needs confirmation.
8. The homing mechanism of MSC needs further clarification.
9. The transdifferentiation of MSC into neural cells needs further verification.
10. Non invasive methods for tract tracing and cell tracking needs to be developed.

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