SPINAL CORD REGENERATION USING STEM CELL TRANSPLANTATION & OTHER NOVEL TECHNIQUES

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List of abbreviations

aFGF: Acidic fibroblast growth factor

ANS: Autonomic nervous system

BBB score: Basso, Beattie, Bresnahan

bFGF: Basic fibrobast growth factor

BSA: Bovine serum albumin

BDA: Biotin dextran amine

BBB: Blood brain barrier

GBC: Globose basal stem cell

CNS: Central nervous system

CST: Corticospinal tract

CSF: Cerebrospinal fluid

CD: Cluster of differentiation

DMEM/F12: Dulbecco's Modified Eagle Medium, Nutrient mixture F12(Ham)

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

DAPI: 4,6-diamidino-2-Phenylindole

EDTA: Ethylene diamine tetra acetic acid

EGF: Epidermal growth factor

EMG: Electromyography

FBS: Fetal bovine serum

FACS: Fluorescence-activated cell sorting

FB: Fast blue

GFP: Green fluorescent protein

GFAP: Gial fibrillary acidic protein

HBSS: Hank's Balanced salt solution

IHC: Immunohistochemistry

LMN: Lower motor neuron

MSC: Mesenchymal stromal stem cell

MAP2: Microtubule associated protein

NF: Neurofilament

NeuN: Neuronal nuclei

NS: Normal saline

OEC: Olfactory ensheathing cells

ONF: Olfactory nerve fibroblast

PBS: Phosphate buffered saline

PLL: Poly-L-Lysine

PDL: Poly-D-Lysine

PFA: Paraformaldehyde

PI: Propidium iodide

PNS: Peripheral nervous system

SCI: Spinal cord injury

UMN: Upper motor neuron

Introduction

Spinal cord injury inflicts permanent neurological injury and long term consequences in the socio-economic life of affected individuals worldwide. This is because CNS lacks the ability for spontaneous regeneration and there is no medical option to repair neural damage in the central nervous system. Currently, the treatment is focused on comprehensive rehabilitation of affected persons utilizing the spared functional abilities. Nevertheless, recent years have been witnessing serious experimentation to regenerate damaged neural tissue using various methods in animal models. The pathophysiology of spinal cord injury involves mechanical disruption of axons, blood vessels and cell membrane (1,2). This is followed by apoptotic loss of cells around the lesion. The pathology of the lesion evolves over a prolonged period. If pharmacological intervention is made during this window period, the lesion extension from the epicenter may be minimized (3).

Following injury, central nervous system fails to repair spontaneously as the neurons lack the ability to regenerate; in addition, inhibitory factors and molecules present in the vicinity of glial scar provide a hostile environment to the regrowth of damaged axons (4). Regenerative approaches to block inhibitory signals including Nogo and Rho associated kinase pathways have shown promising outcome, but they are in the early stages of clinical evaluation (5). Cell based strategies using bone marrow derived stromal cells (MSC), glial cells such as olfactory ensheathing cells (OEC) and olfactory nerve fibroblasts (ONF), neural stem cell, embryonic stem cell to regenerate neural tissue are the fast emerging approaches in the field (6). The outcome of these approaches,

though awaiting a foolproof clearance for human application, has raised hopes to move further towards human trials in the years to come.

Rationale for the present study

Experimentations on cell based therapy in animal models are generally conducted using stem cells and other potential cells that are thought to have a definite role in CNS repair. Individually, these cells can overcome only few of the many intrinsic and extrinsic inhibitory factors. Hence, it is generally agreed that an optimal therapeutic strategy for patients will require a combinatorial treatment that could address the inhibitory factors to the maximum (7). In this thesis, experiments to observe the effect of cell transplantation with different combinations of cells along with growth factors and enzymes in animal models of spinal cord injury were conducted.

The agents adopted for the spinal cord regeneration in the study were the following:

(i) Olfactory ensheathing cells (OEC) along with olfactory nerve fibroblasts (ONF)

(ii) Bone marrow derived mesenchymal stromal cells (MSC).

(iii)Globose basal stem cells.

(iv) Fibroblast growth factor (FGF).

(v) Chondroitinase enzyme.

Rationale for using olfactory ensheathing cells (OEC)

1. Olfactory mucosa is a privileged zone of nervous system as its receptor neurons are continually replaced in adult life in mammals. This continual regrowth of axons is facilitated by olfactory ensheathing cells (OEC) which are located around the olfactory axons (8,9).
2. OECs are unique in that they possess the quality of Schwann cell(PNS glial cell) and astrocyte (CNS glial cell).
3. OEC can act like Schwann cell by facilitating the regrowth of injured axons (10,11).
4. OEC can act like astrocyte; since it can survive in CNS environment (12–14).
5. OEC can integrate with astrocyte in co-culture (12–14).
6. OEC do not induce astrocyte hypertrophy, nor do they upregulate chondroitin sulphate proteoglycans expression in astrocytes which is one of the glial scar obstacle preventing axonal regrowth following CNS injury (12–14).

These CNS friendly qualities of OEC have been exploited by researchers to experiment and evaluate the suitability of these cells as a candidate for intraspinal implantation and thus ameliorate the functional deficits that follow SCI (15–17).

Rationale for using mesenchymal stem cell (MSC)

1. Non-hematopoietic bone marrow stromal cells (MSC) are easily obtainable, expand quickly, and differentiate into other cell types *in vitro* and *in vivo* (18–20).

2. MSCs can enhance axonal growth and promote functional recovery in animal models (21–27).

3. MSCs are nonimmunogenic upon allogenic transplantation as they lack B7 co-stimulatory molecules CD80 and CD86 (28).

4. MSCs can rescue neuronal cells by neruoprotection, immunomodulation and possibly help for remyelination and neuroregeneration (29,30).

Rationale for using chondroitinase

1. Following spinal cord injury astrocytes proliferate at the site of injury and form dense scar rich in chondroitin sulphate proteoglycans. This astrocyte scar is considered to inhibit regeneration in spinal cord injury.

2. Chondroitinase is a potentially therapeutic enzyme which, when injected into the lesion site will minimize the detrimental effect of proteoglycans secreted in response to injury. This is thought to enhance axonal growth through injured area (31).

3. Combination of cell therapy along with chondroitinase injection has been shown to improve function following spinal cord injury (32,33).

Rationale for using Acidic Fibroblast Growth Factor

Fibroblast growth factor (FGF) is one of the key regulator of CNS development and function. It facilitates properties like mitogenesis, differentiation and neurotrophic effect like enhanced the survival and growth of neurons. It has neuroprotective effect after spinal cord injury (7,34). FGF suppress the inhibitory effect of GFAP and Keratan sulfate proteoglycan (35).

Aim & Objectives

Aim

To study the efficacy of cell transplantation, enzymes and growth factor in spinal cord injury in rat models.

Objectives

1. To harvest OEC and ONF from adult rat olfactory mucosa, culture and characterize them by fluorescence-activated cell sorting (FACS) and immunohistochemistry (IHC).
2. To culture and sort globose basal stem cell (GBC) by fluorescence-activated cell sorting (FACS) and characterized by flow cytometry and immunohistochemistry (IHC).
3. To differentiate globose basal stem cells to neuronal cells and these cells were characterized by neuronal markers.
4. To harvest MSC from femur and tibia of adult rat, culture and characterize the same by FACS and IHC.
5. To transdifferentiation of MSC into neuronal cells, characterize the same by immunohistochemistry (IHC), and patch-clamp techniques.
6. To create spinal cord injury in adult rats.
7. To treat spinal cord injury by different combination of stem/glial cells, enzyme and growth factor.

    a) OEC alone.

b) MSC alone.

c) Chondroitinase alone.

d) FGF alone.

e) OEC+MSC (1:1).

f) OEC + MSC + chondroitinase.

g) FGF (1^{st} day) + OEC (9^{th} day).

h) FGF + OEC (1^{st} day after SCI).

i) Globose basal stem cells (GBC).

8. To assess the recovery by

a) Basso, Beattie, Bresnahan locomotor rating scale(BBB score)

b) EMG study

c) Histology

Review of literature

3.0 Epidemiology of spinal cord injury

The annual worldwide incidence of spinal cord injury (SCI) is between 10.4 and 83 cases per million (36)**.** In India, approximately 1.5 million people live with SCI. Every year10000 new cases were included to this group (37). It is associated with severe physical, social, psychological and economic burdens on patients and their relatives.It predominantly occurs in youngsters of age group around 15 to 25 years. The male is more prone to SCI than female; its ratio is approximately 4 to 1. And often life-threatening complications bladder and bowel incontinences, pressure sores, sexual dysfunction, muscle wasting, osteopenia or osteoporosis, hormone dysregulation, immune deficiency and cardiovascular problems typically encountered by chronic SCI individuals (38–40).

3.1 Anatomy & Physiology of spinal cord

The nervous system is responsible for receiving, sending and monitoring all nerve signals. These chemical and electrical signals are necessary to organize to do everything like walking, thinking etc. Anatomically, the nervous system is divided into two main system: the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS, the main processor of information, includes the brain and spinal cord. The PNS involves those parts of the nervous system outside the brain and spinal cord, and it connects the CNS to the body's organs and extremities. The PNS is responsible for executing commands given by the CNS and relaying information from the body and the surrounding environment to the brain and spinal cord.

The nervous system also has two functional divisions: the somatic and the autonomic nervous systems. These systems are located within the PNS. The somatic nervous system is involved in the control of voluntary activities. The autonomic nervous system (ANS) connects the CNS to the internal organs, glands and is involved in regulating involuntary functions such as heartbeat. The ANS subdivided into sympathetic and the parasympathetic systems. The sympathetic nervous system mobilizes energy during stress, while the parasympathetic conserves energy during relaxed states.

The spinal cord contains bundles of nerves that extend from the brain down the back and serve as sort of a communication cable relaying information to and from the brain and the rest of the body. It is surrounded by series of membranes called meninges. The membrane attached directly to the spinal cord is pia mater, contains the cord's blood supply. Surrounding the pia mater is a liquid called cerebrospinal fluid (CSF), which acts to cushion the cord. The CSF is held in place by a second membrane called arachnoid. The outer membrane, the dura mater, is tough and fibrous.

Spinal cord is relatively small of about 18 inches long, width of our little finger and fragile. To prevent it from being easily damaged, it is housed inside a bony tunnel called vertebral canal. Twenty-nine vertebrae stack on top of each other to make up the vertebral column. The vertebral foramen (orifice) of each spine lines up to form the vertebral or spinal canal through which the spinal cord runs. Between two vertebrae a

spongy cartilage disc that acts as a shock absorber. Ligaments connect all the vertebrae together and move in a coordinated fashion.

The spine has named into four: the first seven bones called cervical vertebrae, make up the neck. The next 12, the thoracic vertebrae, 12 ribs is attached to a thoracic vertebra in the back. In the lower back area are five lumbar vertebrae. Below these is the sacrum, a flat v-shaped bone (made of five fused vertebrae) that anchors the spine to the pelvis. At the end, small tailbone, the coccyx.

There are 31 pairs of spinal nerves. Each pair provides innervetion to the left and right sides of a segment of the body. Like the vertebrae, the spinal nerves are named according to level: 8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal. The spinal cord shorter than the vertebral column and end-up at L1. The long spinal nerves with distinctive in appearance collectively called cauda equina.

Each spinal nerve is attached to the spinal cord by dorsal and ventral roots. On each side, a dorsal root carrying sensory information to the CNS and a ventral root carrying motor information from the CNS. Nerves within the spinal cord involved in controlling movement are called upper motor neurons (UMN), whereas nerves that the spinal cord to connect the muscles are called lower motor neurons (LMN).

3.2 Pathophysiology of spinal cord injury

The pathophysiology of SCI consisting of a primary and secondary phase of injury. The primary phase involves the initial mechanical injury, fracture or dislocation of spine, which impact on inner soft tissue spinal cord, disrupting nerves, blood vessels. This is followed by the delayed onset of a secondary phase of injury involving vascular dysfunction, edema, ischemia, excitotoxicity, free radical production, electrolyte shifts, inflammation and cell death. Neurological deficits occurs immediately following the initial injury.

Primary injury phase

The most common form of acute SCI is a compression/contusive type injury in which displacement of vertebral column, intervertebral discs, exert force on the spinal cord causing immediate traumatic injury and sustained compression (41).

Secondary injury mechanisms

Secondary injury processes are triggered by the primary mechanical injury leading to prolonged phase. The immediate, acute, intermediate and chronic stages of SCI.

Immediate Phase (0 to 2 hours)

The immediate phase begins at the time of injury and last for 2 hours. Traumatic severing and death of neurons and glia, phenomenon of spinal shock (42), Which results in instantaneous loss of function below the level of injury. The first pathological change following injury is swelling of the cord with hemorrhage in the central gray matter, immediately cell undergoes necrotic death due to direct mechanical disruption or ischemia from vascular disruption (43,44). Hemorrhage and swelling leads to cord ischemia that may extend for many spinal segments rostral and caudal to the injury. Instantaneously following injury microglial activation begins with the upregulation of proinflammatory cytokines TNFα and ILβ (45). Extracellular glutamate level increased will leads to excitotoxic within minutes of the injury (46).

Acute phase

In acute phase, secondary injury processes become dominant. In this phase most likely amenable to neuroprotective interventions, where patient arrive earliest to hospital for treatment. It is divided into early acute and subacute stages.

Early acute phase (2 to 48 hours)

This phase last for 2 to 48 hours after SCI, with continuation of hemorrhage, edema, and inflammation. The onset of secondary injury processes of free radical production, glutamate-mediated excitotoxicity, ionic dysregulation, and immune response contribute to further cell death. Vascular disruption, hemorrhage, which results in ischemia are the

major constituents of this secondary injury cascade (47,48). The ischemia results in axonal swelling leading to blockade of action potential (49).

Ionic dysregulation and Excitotoxicity

The loss of appropriate ionic homeostasis is important feature of both necrotic and apoptotic cell death following SCI. Dysregulation of Ca++ ion concentration initiates mitochondrial dysfunction, activation of calpains and production of free radical culminating in cell death (50).

The excessive activation of glutamate receptors leads to influx of Na+ and Ca++ through the NMDA and AMPA receptors. Following SCI, increased level of glutamate in extracellular due to failure of Na+ K+ adenosine triphosphatase membrane transporter (51). Pharmacological interventions to attenuate with antagonists of NMDA and Non-NMDA receptors for excitotoxicity.

Free radicals-Oxidative stress

Free radicals are unstable and highly reactive with other molecules, because of one unpaired electrons present in them. This unpaired electrons capture electrons from DNA, proteins and lipids, which causes damage. Reactive oxygen species (ROS) are produced as by-products of normal metabolism of oxygen in mitochondria. Reactive nitrogen species (RNS) are another free radical and its production is increased by contractile activity (52).

Oxidative stress is a condition where the toxic effects of free radicals are not adequately detoxified by the body's antioxidant defense system. In SCI, oxidative stress is usual with respect to secondary damage after the acute injury. Oxidative stress can lead to muscle fatigue, muscle atrophy, and changes in higher proportion of fast-twitch muscle fibers(Type II) than slow-twitch muscle fibers (Type I) after SCI (53).

Permeability of Blood Brain Barrier (BBB)

Blood Brain Barrier (BBB) functions as a highly selective barrier for the transport of compound in and out of CNS. Following injury, increased permeability of BBB due to mechanical injury and inflammatory response on endothelial cells. Permeability last for 2 hours after injury to 2 weeks. Vascular permeability was increased by two inflammatory cytokines (TNFα and IL-1β) (45,54). Glial and immune cells produces ROS, histamine, nitric oxide, elastase and matrix metalloproteinases, will increases BBB permeability (55).

Inflammatory mediators and cellular immune response

In early acute stage infiltration of inflammatory cells and these cells activate resident microglia. Cells like microglia, astrocyte, T cells, neutrophils and monocytes, and cytokines like TNFα, interferons and IL has tremendous complexity in contribution of deleterious neuroinflammation in secondary injury (55–57). Removal of debris by providing favorable environment for the regeneration of axons(56).

Cell death and demyelination

Cell death after SCI may be due to necrosis or apoptosis. Oligodendrocytes are highly sensitive to ischemic injury (1) and readily undergo apoptosis after SCI. Oligodendrocytes is activated through Fas receptor by microglia Fas ligand. The interaction of Fas receptor and Fas ligand initiates apoptosis through caspase pathway (58,59). Oligodendrocyte also express NMDA receptors are more susceptible to excitotoxic cell death following SCI (60). The loss of oligodendrocytes results in demyelination of axons, and it is upregulated approximately 24 hours following injury. Axonal injury (Such as traumatic, ischemic swelling, lipid peroxidation) and persistent demyelination are associated with atrophy and death of associated cell bodies (61–63). Animal studies has evidence that spared demyelinated axons represent an important therapeutic target for SCI treatments that either improve axonal conduction in demyelinated axons by 4-aminopyridine (64–66) or remyelinate (cell transplants) (67).

Subacute phase (2days to 2 weeks)

This phase last for approximately 2 days to 2 weeks following injury. This time period is important for cell-based therapeutic strategies application. Transplantation of adult neural precursor cells at 2 weeks after SCI in rat model promotes remyelination and functional recovery (68), whereas chronic phase of cell transplantation fails to survive, migrate and differentiate to promote functional recovery. Keirstead transplanted human ESC-derived oligodendrocyte progenitors immediate after SCI or after the subacute phase found non-survival of transplanted cells (69).

The cell debris and growth-inhibitory components of myelin debris are removed by phagocytosis at subacute period. This removal of debris from the lesion area may promote axon growth (55). Following SCI, delayed astrocyte response begins in the subacute phase. Astrocyte proliferate, hypertrophic and increased expression of glial fibrillary acidic protein at the lesion periphery. This is called “glial scar”. This glial scar is a barrier for axonal regeneration.

Intermediate phase (2 weeks to 6 months)

In this phase maturation of glial scar and regeneration of corticospinal tract axons sprouting from 3 weeks to months following injury, whereas reticulospinal fibers sprout from 3 to 8 months post injury (70).

Chronic phase(>6 months)

The chronic phase begins approximately 6 months following SCI. Stabilization of lesion, continued scar formation, wallerian degeneration of injured axons and cystic cavitation takes place (71–73).

3.3 Current strategies for spinal cord injury repair

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

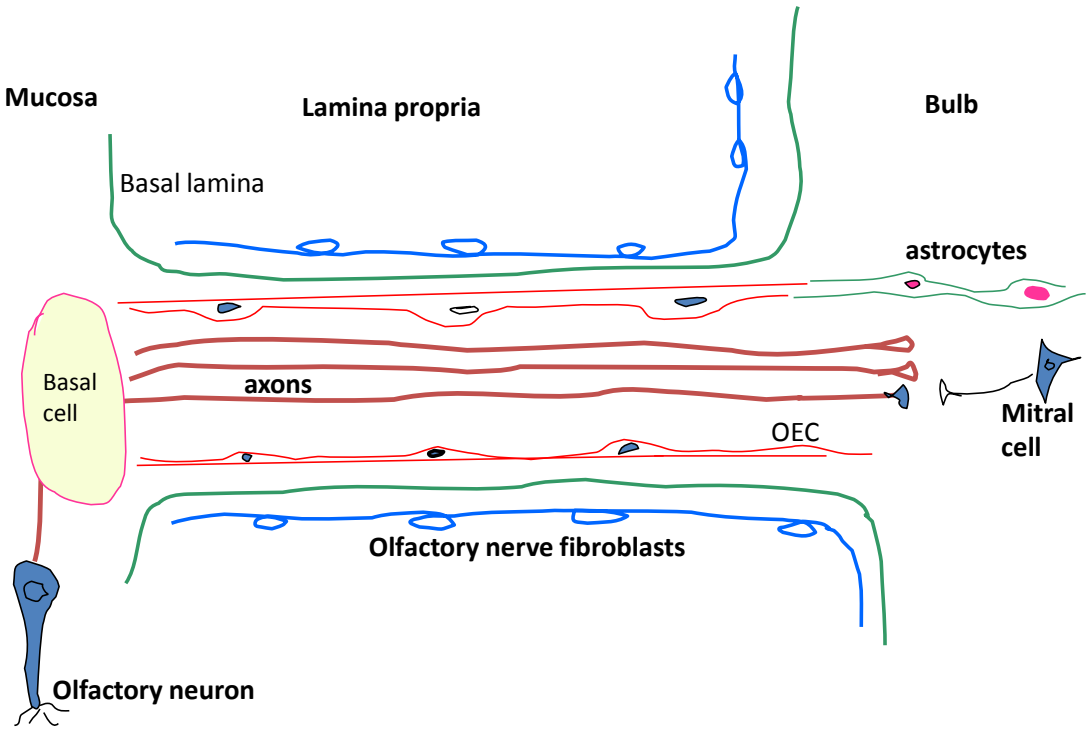
olfactory ensheathing cells, foetal olfactory ensheathing cells, autologous activated macrophage transplantation, human embryonic cell derived pre-oligodendrocyte transplantation, and autologous bone marrow stromal cells transplantation which have entered initial clinical trials.

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 model 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, form compact myelin and promote recovery of motion. This study has now entered into Phase I clinical trial. Remyelination restores saltatory conduction in axon and locomotor deficits (69,74,75).

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 (76,77).

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 (78). 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 (79). The olfactory system is an unusual tissue in that it can support neurogenesis throughout life; permitting the in-growth and synapse formation of olfactory receptor axons into the central nervous system (CNS) environment

of the olfactory bulb. It is thought that this unusual property is in part due to the olfactory glial cells, termed olfactory ensheathing cells (OECs), but also due to neuronal stem cells. These glial cells originate from the olfactory placode and possess many properties in common with the glial cells from the peripheral nervous system (PNS), Schwann cells. Recent data has suggested that olfactory ensheathing cells are a distinct glial cell type and possess properties, which might make them more suitable for transplant-mediated repair of central nervous system injury models. This paper reviews the biological properties of these cells and illustrates their use in central nervous system repair. 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 (80). 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 (81).

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 (82).

4. Schwann cells

It has been observed 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 or 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 (83–86).

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 sensitize 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 (87). 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 (88).

6. Neural stem cells

Recent observations have raised the possibility that the brain has inherent capacity for self –repair in response 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 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 could 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 (89). 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 (90,91).

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 (2).

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 (92).

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 (93).

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 4 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 (94).

Because MSC are easy to isolate, and have abroad 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. 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.

It has been observed that 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 (95). 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 (96).

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 neurite growth (97–99).

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 of harvest, 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 (100–102).

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 statistical significant improvement in reduction of spasticity and a positive trend towards improvement (103).

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 see the effect on spinal cord injury both on walking function and on spasticity.

GM-1 ganglioside

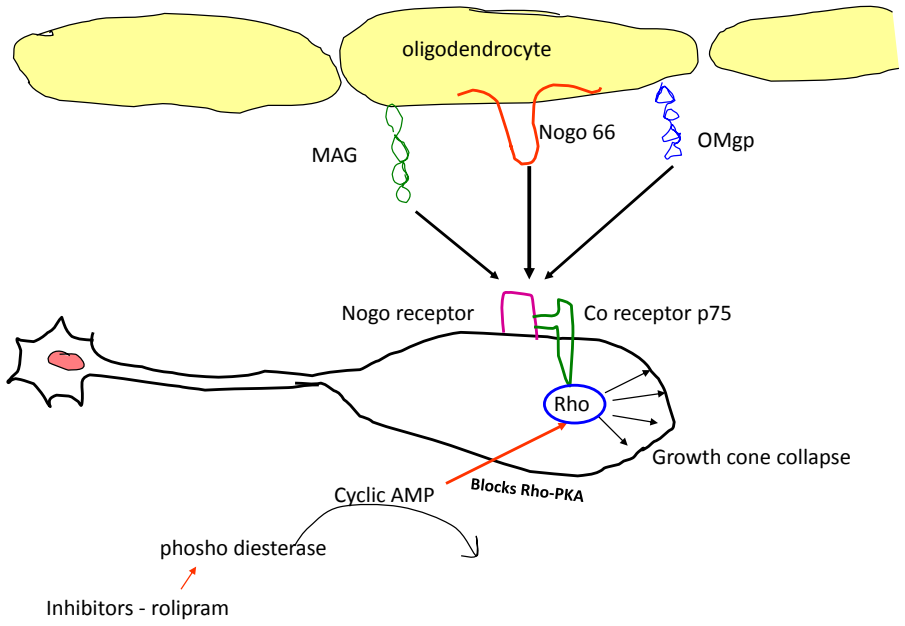
Mono sialo tetra hexosylganglioside GM-1 sodium salt is a naturally occurring compound which is located in the cell membrane of the neural cells. Preclinical studies demonstrating neuro protective and neuroregenerative actions by GM-1 in experimental models have been reported. Clinical trial showed variable outcome with no definitive conclusions (104).

Minocycline

Minocycline is a broad spectrum of antibiotic, a lipophilic derivative of tetracycline that has demonstrated ability to provide neuroprotection. Mechanism attribution to protective actions elicited by minocycline include ability to overcome glutamate mediated exicitotoxicity anti-inflammatory effects through 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 (105).

Nogo

The non-permissive environment for axonal growth are 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 neurite growth produced by oligodendrocytes. Efforts develop antagonist to target Nogo receptor is being studied (106 & 107). It has been demonstrated that oligodendrocytes myelin membrane act as major inhibitor for axons growth. The inhibitory elements which had been suggest to block axonal growth include chondroitinase sulphate proteoglycan,

myelin associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (Omgp). Nogo receptor is part of a receptor complex on the axonal membrane, which act through Rho growth cone. Growth cone cytoskeleton is composed of filamentous actin fibrous 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

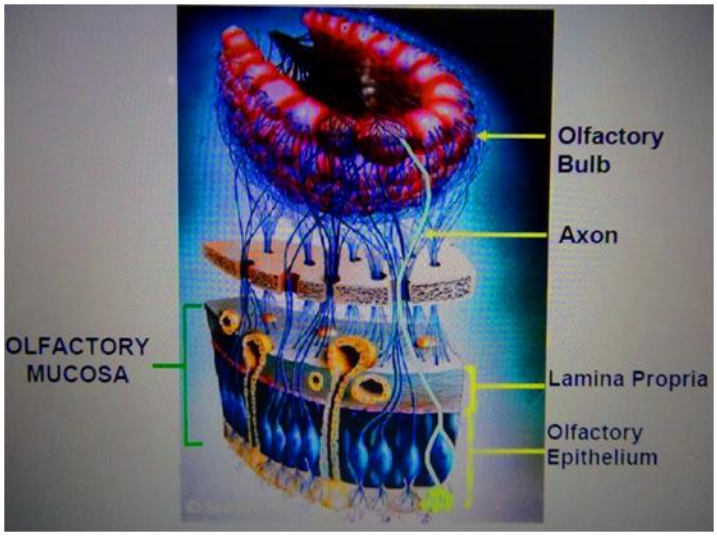
functional outcome reduced the enthusiasm for administration methylprednisolone in acute spinal cord injury (108,109).

3.4 Spinal cord injury model

The commonly used animal models of spinal cord injury are transection, compression and contusion. Transection involves removing of spinous process and laminae to expose the cord and cut the spinal cord partially or completely. Complete transection is perhaps the only technique to prove anatomical and functional demonstration of nerve regeneration in repair methods (110). But, most common human spinal cord injuries are compression (111) and contusion models (112,113).

3.5 Olfactory mucosa

The olfactory mucosa is the organ of the sense of smell. In nasal mucosa, odorants bind to the odorant receptors of olfactory receptor neurons and activate the signal transduction pathway. The impulse propagate and terminates in glomerulus of the olfactory bulb. The olfactory mucosa is comprised of two distinct layers, the olfactory epithelium and the lamina propria.



Structure of the olfactory system. The olfactory mucosa is made up of the lamina propria and olfactory epithelium. Olfactory neurons extent from nasal cavity to olfactory bulb through the lamina propria where they form nerve bundles (114). (Adapted from Morice, 1990).

Olfactory lamina propria

The olfactory lamina propria is the tissue through which olfactory sensory axons pass through to synapse with olfactory bulb. This tissue is composed of blood vessels, nerve bundles, bowman's gland, connective tissue and fibroblast. Consequently, the lamina propria is thicker than the epithelium. In addition, specialized glial olfactory ensheathing cells ensheath the newly developing axons as they enter the lamina propria and secrete some neurotrophic factors, extracellular matrix molecules to guide the axons to grow and connect to the olfactory bulb.

3.6 Olfactory epithelium

The olfactory epithelium is a pseudo-stratified, columnar epithelial cells. It contains different types of cells: sensory neurons at different stages of differentiation, sustentacular cells and basal cells. There are two types of basal cells: horizontal basal cells (HBC) and globose basal cells (GBC). Horizontal basal cells are electron-dense, flat shape lie on the basal lamina and shows cytokeratin positive, whereas globose basal cell are electron-lucent, spherical shape resides superficial on the top of the HBC and does not express cytokeratin. Sustentacular cells are columnar cells situated throughout the epithelium and as various functions like transporting molecules across the epithelium, secreting some factors to mucus to prevent from adverse effects.

Neurogenesis in olfactory system

Vigorous neurogenesis takes place throughout adult life in olfactory system by replacing olfactory sensory neurons (115–118). This olfactory receptor neuron (ORN) lifespan is 4-

6 weeks, after that sensory neurons are vulnerable to die by environmental insult in the nasal cavity, which has direct contact with the environment. This neurons are regenerated by a process of neurogenesis occurs in basal cells of the olfactory epithelium (115 and 119–121). The basal cell proliferate and differentiate into fully mature neurons(122). The capacity to replace the damaged or dead olfactory sensory neuron by olfactory epithelium is well established in several studies (123–127). Neurogenesis in this system is highly regulated by growth factors (128).

Stem cells as a ability to self-renewal and multilineage differentiation. Stem cells resides in the adult tissue designated according to the tissue type like neural stem cells, hematopoietic stem cell etc. Pluripotent stem cell give rise to all body cells and multipotent will give rise to all types of cell in a tissue. Neural stem cells from brain as a invitro property to grow as “neurospheres” (129). Olfactory mucosa derived neurosphere can give rise to neuronal cells (130). Basal cells in the olfactory epithelium as a potency to give non-neural cells in olfactory mucosa (131). Adult stem cells from olfactory mucosa exhibit similarities like bone marrow mesenchymal stem cells (BM-MSC), they named as olfactory ecto-mesenchymal stem cells (OE-MSC) (132).

Precursor of the olfactory receptor neuron located in the olfactory epithelium as a small population of transit amplifying cells called globose basal cells (116,133). It is generally agreed that GBCs are the immediate neuronal progenitor cells. Invitro studies shown that “olfactory neurons” are defined by neural cell adhesion molecule (NCAM) expression

(117,134,135), OMP-immunoreactive cells (136–138) . Among the basal cells, a group of GBC expresses certain early stages of differentiation marker like GBC-1 (139), m-musashi (140), and MASHI (133,141). Stem cells express cytokeratin 14, β1-integrin, intercellular adhesion molecule-1(ICAM-1) (142). NCAM expressed in immature neurons (143) and fully differentiated cells express olfactory marker protein (OMP), gal-NCAM (144).

Invitro studies shown that growth factors used for proliferation or differentiation, FGF-2 as proliferation-promoting growth factors (118), EGF (117), LIF (145) and NGF (146). To promote differentiation of stem or neuronal precursor cells, BDNF (147), dopamine (148), NT3 (149), TGF-β2 (117), IGF-1 (150). These factors play a key role on regulating neurogenesis during central nervous system development. Some of the growth factors and their receptors expression continues throughout the adulthood in the subventricular zone, hippocampus and the olfactory epithelium (151). EGF and TGF-α receptors expression was predominant in these three zones (hippocampus, sub-ventricular zone, olfactory epithelium), where active neurogenesis takes place. Both in adult and neonatal rats, neuronal precursor are stimulated to proliferate by EGF in the hippocampus and in the SVZ (152).

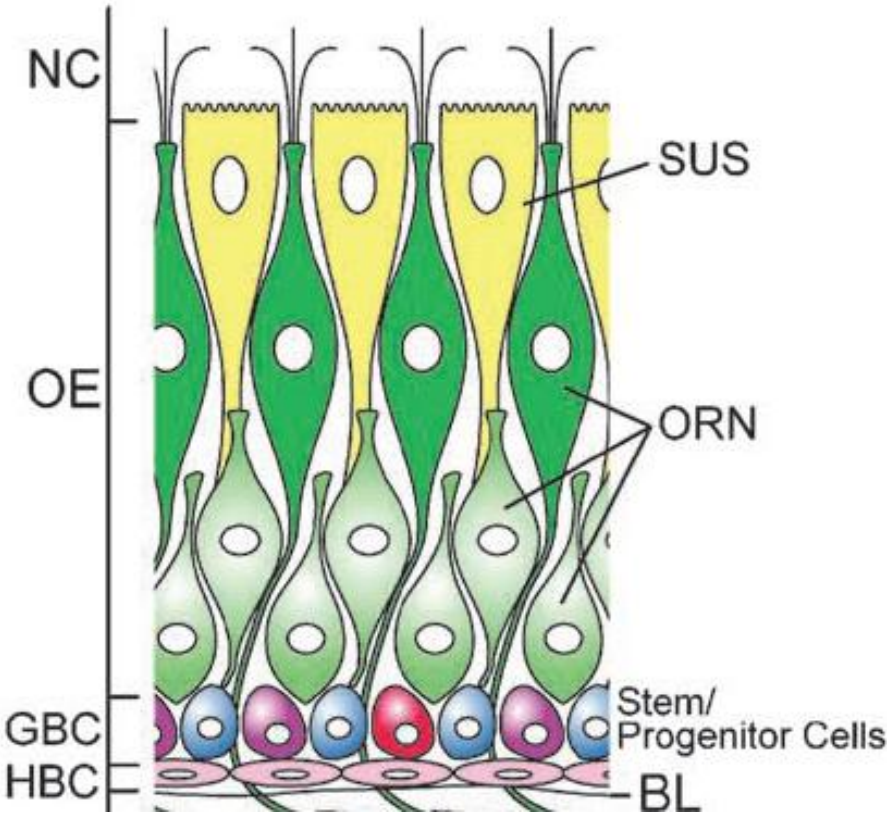
Olfactory stem and progenitor cells are isolated by using marker NCAM-1^{+} and ICAM-1/β1 $integrin^{+}$, and GBC-2 (131,153). Olfactory epithelial monolayer culture consists of

spindle, flat and globose cells. This heterogenous population are ORN,glial, fibroblast, supporting cells, stem and progenitor cells, but only 5-10% are stem cells (130).

Human OE cells aggregates i.e neurospheres are detached from the culture, and this single neurosphere consist of about 1000 cells (154). These spheres express predominantly nestin (neural stem cell marker), β-tubulin III and glial fibrillary acidic protein (GFAP). Differentiating cells also express GFAP, O4GalC (oligodendrocyte marker) and β-tubulin III, this indicates multipotent nature of these cells after transplantation. In another study neurosphere characterized immunocytochemically express nestin, β-tubulin III, NCAM, MAP2ab, Peripherin (protein of cellular intermediate filaments neural crest derivatives) and Trk A,B and also glial marker GFAP, A2b5 (155–157).

Brain stem cells isolated and cultured forms neurosphere in a week. These cells were passaged upto 200 passages and their mitotic cycle were 18-20 hours of each passage, it still retains same telomerase activity (158). GFP transgenic mice globose basal stem cells isolated using GBC-2 antibody. Isolated globose basal stem cells transplanted into the nasal cavity of mice, which olfactory epithelium was pre-destroyed. Transplanted GFP cells proliferate and differentiate into mature ORN and supporting cells (159). Another type of multipotent stem cell is horizontal basal cells, isolated by NCAM-1 and ICAM-1/β1integrin. These cells have high proliferative potential, when cultured in NGF, TGF-α,

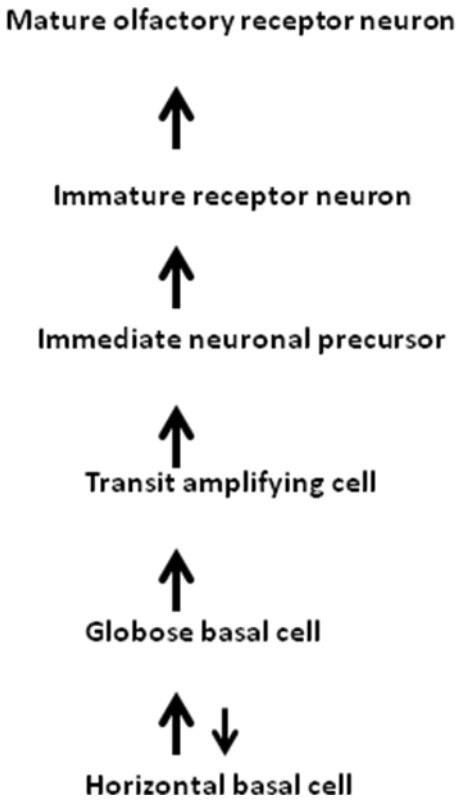
EGF media, which gives rise to globose stem cell, glial cells and horizontal NCAM-1^{+} progenitor cells (142).



Types of cells in olfactory epithelium (OE) and lamina propria (LP). Horizontal basal cells (HBC), globose basal cells (GBC) are located on the basal lamina (BL). Globose basal cell (GBC) proliferate and differentiate to give rise to olfactory receptor neurons (ORN). One end of ORN extend cilia into the nasal cavity (NC) and other end of ORN extend through LP where they combine to form olfactory nerves (ON) and are encompassed by glial olfactory ensheathing cells (OEC) (160). (Taken from: Beites et al., 2005).

Globose basal cell

Globose basal cell has a property of self-renewal and differentiation; hence it is termed as multipotent stem cell. When transplanted in vivo it differentiates and give rise to neural and non-neural cells.



A proposed model in olfactory epithelium of the neuronal lineage.

(Adapated from: Illing et al 2002) (161).

3.7 Acidic fibroblast growth factor (FGF acidic)

FGF acidic also known as FGF-1, is a 17 KDa nonglycosylated member of the FGF family of mitogenic peptides. It is produced by multiple cell types, stimulates proliferation of mesodermal origin and many cells of neuroectodermal, ectodermal and endodermal origin (162–164). It plays a various roles in development,regeneration and angiogenesis (165). Human FGF acidic shares 54% amino acid sequence identity with FGF basic. FGF acidic shares 92%, 96%, 96% and 96% amino acid sequence identity with bovine, mouse, porcine and rat FGF acidic respectively. FGF acidic associates with heparin sulfate and interacts with FGF receptors. Ligation triggers receptors dimerization, transphosphorylation, and internalization of FGF-receptor complexes. Internalized FGF acidic translocate to nucleus and functions as a survival factor by inhibiting p53 activity (166).

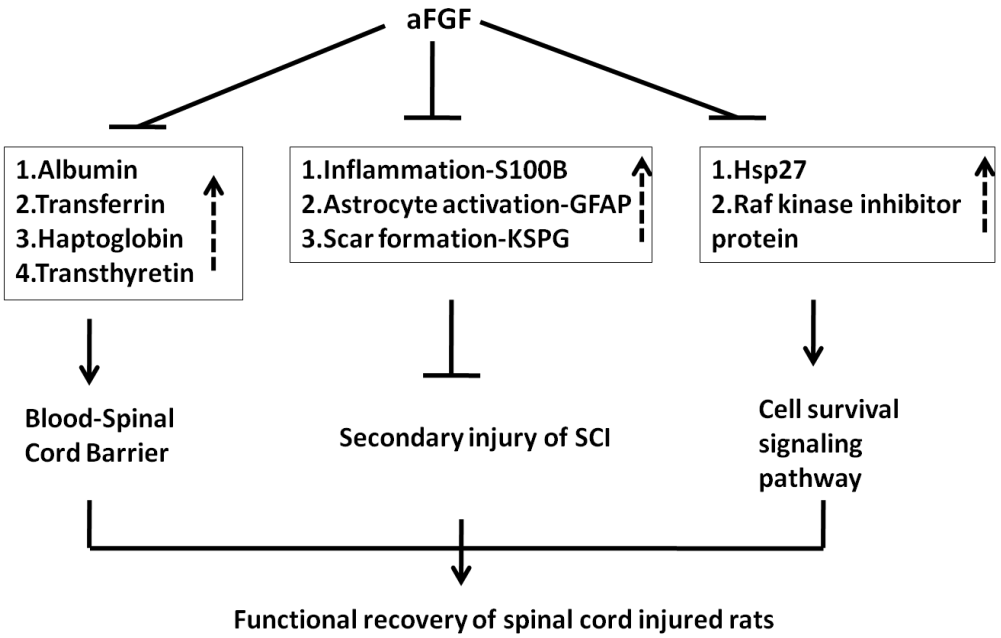
Minimising the initial damage- Neuroprotection

In most spinal injuries some spared axons remain intact. After spinal cord injury neurons die, they send signals that cause neighbouring other injured neurons to die.Which enlarges the damaged area within first few hours after injury. Which causes the scar tissue to accumulate at the site of injury. In order to reduce the spread of secondary damage at an early stage of injury, FGF acidic was administered.

FGF is potent neurotrophic factor synthesized in limited quantity in the adult central nerves system after injury, exogenous delivery of these factors shows neuronal survival and regeneration of injured spinal cord (167–169). aFGF is synthesized within cells, and

lack of amino-terminal signal peptide to be functional. It is released when cell membrane lysed after injury (170). It has shown that , aFGF rescue neuronal cell from death by preventing secondary inflammatory cascade and regrowth, sprouting of tract by functional recovery in animal model (171–173). By using recombinant aFGF enhances the intrinsic ability of mature neurons to survive and regrowth in paraplegic rats (174). Previous study illustrated glutamate-induced toxicity in cell line through phosphatidylinositol 3-kinase /Akt kinase signaling pathways (175).

aFGF attenuate secondary injury damage by suppressing the upregulation of inflammation (S100β), glial scar (KSPG) and Raf Kinase inhibitor protein(RKIP). Maintaining blood-spinal cord barrier, initiation of the ERK signaling pathways for survival and regeneration of injured spinal cord. So that it favored the functional recovery after SCI in rats (176) as described in the chart below.



3.8 Chondroitinase

Chondroitinase ABC protease free from Proteus vulgaris catalyzes the degradation of polysaccharides containing (1→4)-β-D-hexosaminyland (1→3)-D-glucuronosyl or (1→3)-α-L-iduronosyl linkages to disaccharides containing 4-deoxy-β-D-gluc-4-enuronosyl groups. Chondroitinase acts on chondroitin 6-Sulfate, Chondroitin 4-Sulfate and dermatan sulfate and also acts slowly on hyaluronate. The enzyme is highly specific target for the galactosaminoglycan chains and without activity on core proteins, keratin sulfate chains, and heparin/heparin sulfate chains even in the absence of inhibitors for proteases, keratanases, and heparitinases. Initial rates of degradation of Chondroitin, Chondroitin Sulfate B, and hyaluronic acid were 20%, 40% and 2% respectively (177).

After spinal cord injury ascending sensory and descending motor axons fail to regenerate because of glial scar at the site of injury. This scar is composed of hypertrophic glial cells and extracellular matrix (ECM). ECM is composed of chondroitin sulfate proteoglycans (CSPG) and it is expressed by glial cells (178). CSPG is a proteoglycans composed of a core protein and sulfated glycosaminoglycans which is covalently linked. The polysaccharide chains are cleaved by many enzymes, including chondroitinases. There are four members in this enzyme family: chondroitinase ABCI, ABCII, AC and B. Chondroitinase ABCI is exolyase and functionally cleaves both chondroitin and dermatan sulfates (179).

Extensive studies was done in invitro to treat spinal cord injury (180–183) and invivo studies in central nerves system injury (184–187). CSPG is a molecular barrier, regeneration of neuron ceases abruptly when neuron enters the proteoglycan-rich glial scar. Earlier studies demonstrate that chondroitinase treatment in SCI models, cat spinal cord hemisection (188), rat dorsal column lesion (184) highlight regeneration and functional recovery.

Scope and Plan of work

Scope

Spinal cord injury results in permanent loss of motor and sensory function below the injury level. There is no treatment available to cure spinal cord injury. To address this issue, OEC, MSC, Chondroitinase, FGF and globose basal stem cells were tried in spinal cord injury in rat model to restore the lost function. If the results are promising then this treatment strategies could be tried in human spinal cord injured patients.

Plan of work

Plan of work in this thesis is to culture, characterize and transplantation of OEC, MSC and globose basal stem cells in varing cell doses in rat model of spinal cord injury. In addition, effect of administration of FGF and chondroitinase will also be observed. Long term observation will be assessed by hind limb motor recovery scale (BBB score), transcranial electrical motor evoked potential studies, and by histological studies. The number of rats in each group and different cells with different combination / dose shown in table .1 below.

Table.1 The number of rats in each group which received different cell dosages and different cell/enzyme/ growth factors combinations.

Cell type	Cell dosage and number of animals				Total sample
	2 lakhs	5 lakhs	10 lakhs	>10 lakhs	
OEC	6	6	6	6	24
MSC	6	6	6	6	24
OEC+MSC	6	6	6	6	24
OEC+MSC+ Chondroitinase	6	6	6	6	24
GBC		6			6
Control (without cell transplant)	11				11
Chondroitinase	6				6
FGF	6				6
FGF(1^{st} day)+OEC(9^{th} day)	6				6
FGF+OEC(1^{st} day)	6				6

Materials & Methods

Materials

1. Ketamine Hydrochloride injection (Aneket), Neon Laboratories Limited, Mumbai, India.

2. Xyazine injection (Xylaxin), Indian Immunologicals Limited, India.

3. Enrofloxacin injection 10% w/v (Quin Intas),Intas Pharmaceuticals Ltd, India.

4. Meloxicam injection (MELONE X), Intas Pharmaceuticals Ltd, India.

5. Neomycin and Polymyxin B sulfates and Bacitracin zinc Ophthalmic ointment USP (Neosporin), GlaxoSmithkline Pharmaceuticals Limited, Bangaloree,India.

6. Povidone-Iodine solution IP, wokadine-10%, Wockhardt Limited, Mumbai,India.

7. Surgical spirit BP, Kakatiya Pharma, Hyderabad, India.

8. Sodium Chloride(NS) injection IP, 0.9% w/v, Fresenius Kabi India Pvt Limited, Goa,India.

9. Ringer-Lactate (RL) solution for injection, Claris Lifesciences Ltd, Ahmedabad, India.

10. 3M Tegaderm Film, 10cm×12cm (1626W),USA.

11. Absorbable surgical suture (synthetic), Vicryl, 3-0, 20cm ½ circle round bodied, 70cm (NW2437), Ethicon, Johnson&Johnson Limited, India.

12. Absorbable surgical suture USP, synthetic, Vicryl, 3-0, 22mm ½ circle cutting, 90cm, (NW2472), Ethicon, Johnson &Johnson Limited, India.

13. Surgeon's Mask (3 layered), Mediklin Healthcare Ltd, India.

14. Sterile surgical gloves, Armour, Safeshield India Rubber Products Pvt. Ltd, India.

15. Sterile surgeon blade, size:22, Kehr surgical Pvt. Ltd, Kanpur, India.

16. Sterile surgeon blade, size:15, Kehr surgical Pvt. Ltd, Kanpur, India.

17. Needle, 26×1/2 (0.45×13mm) Dispovan, Hindustan syringes & Medical devices Ltd, Faridabad, India.

18. Syringe, 5ml, Becton Dickinson India (p) Ltd, Haryana, India.

19. Top three-way stopcock (Type:R1-FL-3CAPS), Meditop corporation (M) Sdn.Bhd, Malaysia.

20. I.V catheter with wings, 24GA0.75IN,0.7×19mm, BD Insyte-W, Singapore.

21. Vented Infusion set, Romsons juniors India, Agra, India.

22. Dulbecco's Modified Eagle Medium, Nutrient mixture F12(Ham) (DMEM/F12), Catalog: 11320082, Invitrogen-Gibco.

23. Fetal Bovine Serum (FBS), Heat inactivated, Catalog:10082147, Invitrogen-Gibco.

24. L-Glutamine 200mM (100x), Catalog:25030081, Invitrogen-Gibco.

25. Collagenase type II, Catalog:K01-040-2, PAA Laboratories Gmbh.

26. Dispase II (neutral protease), Catalog:04942078001 from Bacillus polymyxa, Roche, Japan.

27. 0.25%Trypsin-EDTA (1x), catalog:25200-056, Invitrogen-Gibco.

28. Poly-L-Lysine hydrobromide(PLL), Mol.wt 30,000-70,000, catalog:P9155-5MG, Sigma.

29. Phosphate Buffered Saline(PBS) PH 7.4 , without calcium chloride and Magnesium chloride, Catalog:10010049, Invitrogen-Gibco.

30. Hank's Balanced salt solution(HBSS) 1x, without calcium chloride, Magnesium chloride and Magnesium sulfate, catalog:14170161, Invitrogen-Gibco.

31. Rosette Sep,Human Mesenchymal cell enrichment cocktail, catalog:15168, Stem cell Technologies Inc.

32. Ficoll-Paque, catalog:17-1440-03, GE Healthcare Bio-Sciences AB, Sweden.

33. Dulbecco's Modified Eagle Medium, catalog:11995-073, Invitrogen-Gibco.

34. B27 supplement (50x), catalog:17504-044, Invitrogen-Gibco.

35. Retinoic acid, catalog:R2625, Sigma.

36. bFGF(Human recombinant), catalog:13256-029, Invitrogen-Gibco.

37. T25-Tissue culture flask,50ml, 25cm2, sterile, Greiner bio-one.

38. T75-Tissue culture flask,250ml, 75cm2, sterile, Greiner bio-one.

39. Centrifuge tube conical 15ml (PS) sterile,catalog:546010, Tarsons, India.

40. Conical tube with stand 50ml, Greiner bio-one.

41. 12mm coverslip-Blue star.

42. Triton X-100 (Polyethyleneglycol 4-tert-octylphenolether), catalog:2020130, SRL.

43. Bovine Serum Albumin(BSA), IgG-free, protease free, catalog:001-000-161, Jackson-ImmunoResearch Laboratories Inc.

44. aFGF (Recombinant human FGF acidic, Catalog:231-BC-025/CF, R&D.

45. Chondroitinase ABC protease free(Proteus vulgaris), catalog:100332, Seikagaku BioBusiness corporation, Japan.

46. Paraformaldehyde, catalog:23995, Qualigens.

47. Poly-D-Lysine hydrobromide (PDL), Mol wt 70,000-150,000, Catalog:P6407, Sigma.

48. EGF (Recombinant human EGF), catalog:PHG0311, Invitrogen, Gibco.

49. N2 supplement (100x), catalog:17502-048, Invitrogen-Gibco.

50. DMSO, catalog:C6295, Sigma.

51. NeuroTrace BDA-10,000 Neuronal tracer Kit, catalog:N7167, Molecular Probes, Life technologies, USA.

52. Syringe filter, PES membrane, 0.22µm, 33mm, Millipore.

53. Hyaluronidase from bovine testes, catalog:H3506, Sigma.

54. Collagenase from clostridium histolyticum, catalog:C9722, Sigma.

55. Cell strainer, 40µm nylon,catalog:352340, BD Falcon, USA.

56. Streptavidin- Alexa Fluor 568 conjugate, catalog:S11226, Invitrogen.

57. Vectashield with DAPI, catalog:H-1200, Vector Laboratories Inc.

58. Fast blue, catalog:F0125-5G, Sigma.

59. GFP Lentiviral particle, catalog:LVP001, Gentarget.

60. 6 well plate, nunc, Denmark.

61.24 well-plate.

Method

The experiments done were approved by Institutional Review Board (Approval No. 7022 dated 16.12.2009) and the Institutional Animal Ethics Committee (IAEC) and were in accordance with the guidelines of the Committee for the purpose of Control and Supervision of Experimentation of Animals (CPCSEA), Government of India.

5.1 Olfactory ensheathing cells (OEC)

5.1.1 Collection of rat olfactory mucosa

Adult male Albino Wistar rats, were used for the study. The animal was anesthetised with intraperitoneal injection of ketamine and xylazine and the mid-sagittal skin incision was made from nasal to scalp. The nasal turbinates were revealed by removal of the lower jaw and surrounding musculature, the lateral and medial checkbones. The salivary glands, nasal turbinates, and cartilage on both sides of the nasal septum were removed to reveal the olfactory mucosa, which were dissected and immediately placed in Dulbeccos modified Eagle medium/ Hams F12 (DMEM/F12; Gibco-Invitrogen). The olfactory mucosa was readily identified by the yellowish colour, striation of the lamina propria, and its posterior position on the nasal septum. Care was taken to avoid the anterior edge of the olfactory mucosa, which could be contaminated with respiratory epithelium. The excised olfactory mucosa were placed in an ice-cold culture medium mixture (1:1, v:v) of Dulbecco's modified Eagle medium and Ham's F12 (DMEM/F12; Gibco) supplemented with 100U/mL penicillin, 100µg/ml streptomycin and 25ng/ml of Amphotericin-B and

then transported to cell culture laboratory with in 30 minutes and processed for cell culture.

5.1.2 Culture of olfactory ensheathing cells from lamina propria

The olfactory mucosa was washed twice in Hank's balanced salt solution (HBSS). OM was incubated for 30 minutes at 37ºC in 1ml of 2.4U/ml Dispase II from Bacillus polymyxa (Roche). Digestion was stopped by adding Hanks Balanced Salt Solution (HBSS, calcium- and magnesium- free; Invitrogen). The olfactory epithelium was carefully peeled away from the lamina propria with a microspatula under a dissection microscope. Lamina propria looks brownish appearance, whereas epithelium as whitish. The lamina propria was washed in HBSS and finely chopped into pieces with scalpel blade. Then the tissue was incubated at 37ºC/5%CO2 for 15 minutes in 0.05% Collagenase type-II (PAA Laboratories). Collagenase activity was stopped using 9ml of HBSS. Transferred the content into 15ml centrifuge tube, mix by tilting the tube twice and allow the cells to settle down for 5 min. Discard the supernatant and treat with 0.1% trypsin-EDTA for 5 minutes at 37°C to get tissue fragmented. Triturate in DFF10 (DMEM+F12+ 10%FBS) to stop the trypsin action. Centrifuged the content at 1200rpm for 5 minutes. Supernatant was discarded and the cell pellet was resuspended in 1ml of DFF10. 10µl of the cell suspension was taken and mixed with 10µl of 0.4% trypan blue stain (Gibco). Cells were counted using haemocytometer under phase-contrast microscope for cell viability and total number of cells. Cells were plated approximately at a concentration of 8000/cm^2 onto polystyrene culture dishes (Greiner bio-one) coated

with poly-L-lysine hydrobromide (PLL, Sigma) (2µg/cm^2) 0.1mg/ml 30- to 70kDa.Culture dishes with these cells in culture media (DMEM/F12 (1:1) Gibco, 10% FBS, 2mM L-Glutamine, 100U/mL penicillin, 100µg/ml streptomycin and 25ng/ml of Amphotericin-B) were incubated at 37°C in 5% carbon dioxide in air, at 95% humidity. The cells were fed every second day by replacing half of the complete culture medium. Once the cell attains 80 to 90 % confluency, then the cells were passaged.

Passage procedure

Growth media was removed and the cells monolayer washed three times with HBSS. Entire monolayer of cells was treated with pre-warmed 0.1% trypsin-EDTA (Gibco) at 37°c for 5 minutes. Observed the cells detached from the surface and look round in morphology under phase contrast microscope. To stop trypsin action, add 5ml of OEC culture media and the content transferred to 15ml test tube for centrifugation at 1200 rpm for 5 minutes. Supernatant was discarded, and the cell pellet dissolved in culture media was split into three flasks. Required volume of growth medium was added and kept at 37^{O}C and 5% CO_2 incubator until confluency. The cultured cells from rat olfactory mucosa was characterised by FACS method and by using specific markers namely p75 and fibronectin.

5.1.3 Characterization of OEC & ONF by Immunohistochemistry

Cells were cultured on 12mm round coverslip at a cell density of 8000cells/cm2. Cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature. Then the cells

were washed with PBS (Invitrogen, Gibco) for three times. Blocking and permeabilization in 2% goat serum/2% Bovine serum albumin (BSA) with 0.1% Triton X-100\PBS. Cells were incubated with primary antibody at 4°C overnight. Cells were washed with PBS and incubated with secondary antibodies for 2 hours at room temperature. They were then washed and mounted with DAPI (Vectashield mounting medium with DAPI). Coverslips were immediately transferred to glass slides and examined in fluorescent microscope.

List of Primary and Secondary antibodies used :

P75NTR-FITC conjugate(1:200); Millipore.

Mouse anti-fibronectin (1:100); Santa cruz Biotech.
SEC. Goat anti-mouse IgGi-PE conjugated; Southern Biotech.

Mouse monoclonal IgG1 anti-S100(β subunit)(1:100); Sigma.
Sec.Goat anti-mouse IgG(H+L) Alexa Fluor 594(1:100).

Mouse monoclonal IgG1 anti-Glial fibrillary acid protein (GFAP) Alexa Fluor 488 conjugated(1:50); eBioscience.

Mouse IgG anti-GalC(1:100);Millipore.
Sec. Goat anti-mouse IgG(H+L)Alexa Fluor 594(1:100).

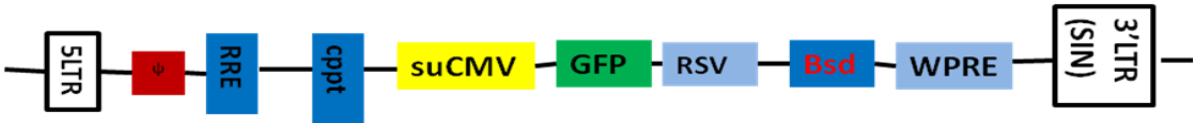
5.1.4 Characterization of OEC & ONF by Flow cytometry

Cultured cells were trypsinized and washed with PBS. Two to five lakhs of cell suspension were stained with primary antibody with or without fluorescent if it is extracellular marker. For intra-cellular protein markers, cells were fixed with 4% Paraformaldehyde for 15 minutes at room temperature. This waas washed with PBS

following blocking and permeabilization in 2% goat serum/2% Bovine serum albumin (BSA) with 0.1% Triton X-100\PBS. 5 to 10 µl of primary antibody incubated for 20 minutes on ice. Excess unbound antibodies washed with PBS and removed. Appropriate secondary antibody which is fluorescent tagged 5 to 10 µl were incubated for 20 minutes. Finally unbound secondary antibody washed with PBS. The cell suspension was aspirated and analyzed in flow cytometry.

5.1.5 GFP labeling of cells

0.5×10^5/ml of cells were grown in complete medium overnight. At the time of transduction 50% to 75% confluent, and then add 50µl of 1×10^7 IFU/ml of pre-made lentiviral particles for fluorescent proteins.(catalog number:LVP001), GenTarget Inc.,USA. After 72 hours of transduction, the transduction rate was checked in fluorescent microscope. Green fluorescent protein gene expresses by CMV promoter and fused with antibiotic resistance gene Bsd. Labelled cells are selected by adding blasticidin, these cells survive because of Bsd resistance gene and unlabelled cells may not survive. Only labeled cells were used for transplantation.



5.1.6 Transplantation of cells to SCI rat model

For transplantation the fresh cultured second passage cells were trypsinized and washed with HBSS. The cell suspension with trypan blue stain was added at appropriate ratio and charged on Neubauer counting chamber for cell counting and cell viability. Total cells were calculated by using the formula

Total cells per ml=average count per square×dilution factor×10^4.

Total cells = ……cells/ml.

Cell viability was assessed by using the formula

Cell viability (%)=total viable cells(unstained)/total cells(stained and unstained) ×100.

OEC harvested were kept on ice for 2 hours and stained for propidium iodide - a red fluorescent DNA counterstain. Dead cells stain with PI and live cells don't.

The number of olfactory ensheathing cells transplanted 9^{th} day following spinal cord injury was summarized in the table. OEC alone with different dosage 2 Lakh, 5 Lakh, 10 Lakh and more than 10 Lakh in six rat in each group (n=6). OEC combined with MSC (1:1) with different dosage 2 Lakh (1 Lakh OEC +1 Lakh MSC), 5 Lakh(2.5 Lakh OEC+ 2.5 Lakh MSC), 10 Lakh(5 Lakh OEC +5 Lakh MSC) and more than 10 Lakh(>5 Lakh OEC +>5 Lakh MSC) with six rats in each group (n=6). OEC combined with MSC and Chondroitinase(0.2U) in four group, six rats in each group(n=6).

Chondroitinase ABC protease free (from Proteus vulgaris) Catalog number:100332, Seikagaku Biobusiness Corporation, Japan. 0.2 Units/2µl dissolved in 0.1% protease free BSA solution. 0.1% BSA was prepared 1mg/ml water. OEC +MSC with ratio 1:1 with different dosage as mentioned above with constant dose (0.2U) of Chondroitinase. 2 Lakh (1 Lakh OEC +1 Lakh MSC + 0.2U chondroitinase), 5 Lakh(2.5 Lakh OEC+ 2.5 Lakh MSC +0.2U chondroitinase), 10 Lakh(5 Lakh OEC +5 Lakh MSC+0.2U chondroitinase) and more than 10 Lakh(>5 Lakh OEC +>5 Lakh MSC+0.2U chondroitinase).

In one group (n=6), immediate after drop-weight spinal cord injury 2µg (1µg/µl dissolved in sterile PBS) of recombinant aFGF(R&D System) with 10 Lakh olfactory ensheathing cells combined and injected into the dorsal column of the injured spinal cord. In another group(n=6), aFGF(2µg/2µl) injected immediate after spinal injury and 10 Lakh of OEC on 9th day following SCI.

Cells/Enzyme Dose/number of rats	**2 Lakh**	**5 Lakh**	**10 Lakh**	**>10 Lakh**
OEC	2 Lakh OEC (n=6)	5 Lakh OEC (n=6)	10 Lakh OEC (n=6)	>10 Lakh OEC (n=6)
OEC+MSC (1:1)	1L OEC+1L MSC (n=6)	2.5L OEC+2.5L MSC (n=6)	5L OEC+5L MSC (n=6)	>5L OEC+>5L MSC (n=6)
OEC+MSC+Chondroitinase	1L OEC+1L MSC +0.2U Chondroitinase (n=6)	2.5L OEC+2.5L MSC +0.2U Chondroitinase (n=6)	5L OEC+5L MSC +0.2U Chondroitinase (n=6)	>5L OEC+>5L MSC +0.2U Chondroitinase (n=6)
OEC +a FGF			FGF(1st day after SCI) +10 Lakh OEC on 9th day. (n=6)	
OEC+aFGF			FGF+10 Lakh OEC (1st day after SCI) (n=6)	

2. Olfactory globose basal stem cells

5.2.1 Isolation of olfactory epithelium

Albino wistar rat olfactory mucosa removed from the posterior regions of nasal septum and placed in ice cold DMEM/F12 (Gibco) supplemented with penicillin, streptomycin and amphotercin. The olfactory mucosa was incubated for 30 minutes at $37^{O}C$ in 2.4 units/ml dispase II. The olfactory epithelium is carefully separated from the underlying lamina propria under the dissection microscope.

5.2.2 Culture of epithelial stem cells

The olfactory epithelium is incubated with 0.05%trypsin-EDTA (Gibco) in low calcium ringer solution for 5-10 minutes at $37^{O}C$, followed by dissociation enzyme cocktail (collagenase/hyaluronidase/trypsin inhibitor; 1mg/ml, 1.5mg/ml, 0.1mg/ml respectively) in ringer's solution for 15 minutes at $37^{O}C$ with trituration. The olfactory epithelium is gently triturated for about 10-20 times to separate the cells. Dissociated cells were subsequently transferred to a 15ml conical tube and the enzymes were inactivated by adding 10ml of DMEM/F12. The cell suspension was centrifuged at 200g for 10 minutes. The supernatant was aspirated and the cell pellet was resuspended in culture media and then plated in culture flask coated with Poly-D-Lysine at a density of $4\text{-}5\times10^{4}/cm^{2}$. Cultures were incubated at $37^{O}C$ in 5% CO_2 and medium changed every alternate days. Expansion medium composed of DMEM/F12(1:1), 2%FBS(Gibco), N2 supplement (Gibco) and EGF (25 ng/ml).

5.2.3 Characterization of globose basal stem cell by IHC

Cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature. Then the cells were washed with PBS (Invitrogen, Gibco) for three times. Blocking and permeabilization in 2% goat serum/2% Bovine serum albumin (BSA) with 0.1% Triton X-100\PBS. Cells were incubated with primary antibody at 4°C overnight. Cells were washed with PBS and incubated with secondary antibodies for 2 hours at room temperature. They were then washed and mounted with DAPI (Vectashield mounting medium with DAPI). Coverslips were immediately transferred to glass slides and examined in fluorescent microscope.

GBC-III mouse monoclonal IgM **(**gift from James E. Schwob, Woochan Jang; Tufts University School of Medicine, Department of Anatomy and Cellular Biology, Boston, USA).

Seconday antibody:Donkey anti-mouse IgM-Cy3 conjugated.

Primary: Monoclonal anti-NCAM mouse IgG1, Sigma, catalog number:C9672.

Secondary:Goat anti-mouse IgG1-PE conjugated.

Anti-SOX2 clone 6G1.2-FITC conjugated, Catalog number:FCMAB112F, Millipore.

Primary: Anti-Nestin mouse IgG, catalog number:MAB353, Millipore.

Secondary: Goat anti-mouse IgG1-PE conjugated.

5.2.4 Fluorescence-activated cell sorting of GBC by GBC III antibody

Globose basal cells were sorted by using GBC-3 antibody (gift from James E. Schwob; Tufts University School of Medicine, Department of Anatomy and Cellular Biology, Boston, USA). 80% to 90% confluent cultures were trypsinized and washed with HBSS. Cell pellet was incubated with primary antibody (GBC-3) for 20 minutes on ice. Further washed with PBS by centrifugation and secondary antibody (Donkey anti-mouse IgM-Cy3 conjugated) was incubated for 20 minutes. Finally washed with PBS and cells were sorted by ARIA-BD. Sorted cells were plated in culture media for further studies and transplanted on 9^{th} day following spinal cord injury in rat model at a dose of 5 lakh cells.

5.2.5 Flow cytometry of globose basal stem cells

Cultured globose basal stem cells were trypsinized and washed with PBS. Two to five lakh cells were fixed with 4% Paraformaldehyde for 15 minutes at room temperature. Then washed with PBS following blocking and permeabilization in 2% goat serum/2% Bovine serum albumin(BSA) with 0.1% Triton X-100/PBS. 5 to 10 µl of primary antibody incubated for 20 minutes on ice. Excess unbound antibodies washed with PBS and removed. Appropriate secondary antibody, which is fluorescent tagged 5 to 10 µl were incubated for 20 minutes. Finally unbound secondary antibody washed with PBS. The cell suspension was aspirated and analyzed in flow cytometry for neural stem cell marker (Neural cell adhesion molecule, Nestin, SOX2), mesenchymal stem marker(CD54,CD90,CD73,CD29,CD105) and haematopoietic marker (CD45, CD34).

5.2.6 Sphere formation-globose basal stem cells

Sorted globose basal stem cells were plated on Poly-D-Lysine coated dishes at a density of 16000cells/cm^2. To form neurosphere, globose basal cells were cultured in neurosphere medium composed of DMEM/F12(1:1), N2 supplement, EGF (50ng/ml) and bFGF (50ng/ml).

5.2.7 Neuronal Induction of globose basal stem cell

Neuronal induction media composed of DMEM/F12(1:1) supplemented with 2%FBS (Invitrogen,Gibco), B27 supplement (Gibco), 20mM Retionic acid and 12.5ng/ml bFGF. Cells were maintained in neuronal induction media for 12 days(189). After 12 days these cells stained for neuronal marker (β III tubulin, MAP2, NeuN, Neurofilament and O4).

List of primary and secondary antibodies used:

CD54-FITC: Mouse anti-rat CD54-FITC(1:50); BD Pharmingen.

CD29: Monoclonal mouse anti-beta 1 integrin(1:50); Millipore.
Sec: Goat anti-mouse IgG2b-RPE (1:50); Southern Biotech.

CD90: Monoclonal mouse anti-rat CD90-FITC conjugated(1:100); Millipore.

CD73: Monoclonal mouse anti-rat CD73 (1:50); BD Pharmingen.
Sec: Goat anti-mouse IgG1-PE conjugated (1:50); Southern Biotech.

CD105: Goat polyclonal IgG (1:25); Santa cruz Biotechnology.
Sec:Donkey anti-goat IgG-perCp conjugated (1:100).

Mouse monoclonal IgG1 anti-CD34-FITC conjugated (1:100); santa cruz Biotechnology.

Mouse monoclonal anti-rat CD45-PE conjugated (1:100); BD Pharmingen.

CD14(1:50); Goat anti-rabbit IgG-RPE(1:50); Jackson Immunoresearch)

Mouse monoclonal anti-BetaIII tubulin(1:50); Millipore.

Sec: Goat anti-mouse Rhodamine(1:50); Millipore.

Mouse monoclonal anti-MAP2 IgG1(1:100);Millipore.
Sec:Goat anti-mouse IgG1-FITC (1:50).

Mouse monoclonal anti-NeuN IgG1(1:50); Millipore.
Sec: Goat anti-mouse IgG1-FITC(1:50)

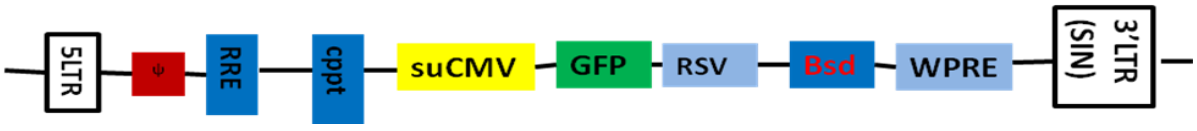
Mouse monoclonal anti-Neurofilament IgG1(1:100); Millipore.
Sec: Goat anti-mouse IgG1-FITC(1:50).

Mouse monoclonal IgG1 anti-Glial fibrillary acid protein (GFAP) Alexa Fluor 488 conjugated(1:50); eBioscience.

Mouse monoclonal IgM anti-Oligodendrocyte(O4) (1:50); Sigma.
Sec:Donkey anti-mouse IgM-CY3 conjugate (1:50): Jackson Immunoresearch.

5.2.8 GFP labeling of cells

0.5×10^5/ml of cells were grown in complete medium overnight. At the time of transduction 50% to 75% confluent, and then add 50µl of 1×10^7 IFU/ml of pre-made lentiviral particles for fluorescent proteins.(catalog number:LVP001), GenTarget Inc.,USA. After 72 hours of transduction, the transduction rate was checked in fluorescent microscope. Green fluorescent protein gene expresses by CMV promoter and fused with antibiotic resistance gene Bsd. Labelled cells are selected by adding blasticidin, these cells survive because of Bsd resistance gene and unlabelled cells may not survive. Only labeled cells used for transplantation.



5.2.9 Transplantation of cells in SCI rat model

For transplantation, GBC sorted fresh cultured cells were trypsinized and washed with HBSS. The cell suspension with trypan blue stain was added at appropriate ratio and charged on Neubauer counting chamber for cell counting and cell viability.

Total cells were calculated by using the formula

Total cells per ml=average count per square×dilution factor×10^4.

Total cells= ……cells/ml.

Cell viability was assessed by using the formula

Cell viability(%)=total viable cells(unstained)/total cells(stained and unstained) ×100.

Five lakh globose basal stem cells were transplanted into the injured spinal cord on the 9^{th} day following dropweight spinal cord injury in six rats(n=6).

3.Bone marrow mesenchymal stem cells (MSC)

5.3.1 Collection of Bone marrow

Male Albino wistar rat was weighed and anaesthetized with over dose of Ketamine and Xylazine by intraperitoneal injection using 31-gauge 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 and tibia 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.

5.3.2 Isolation of MSC

The mesenchymal stromal cells were separated from hematopoietic cells by using the Rosette Sep antibody cocktail (Stemcell Technologies Inc). Bone marrow cell suspension was incubated with Rosette Sep cocktail for 20 minutes at room temperature. This cocktail cross links undesired cells in bone marrow forming immune rosettes. This increases the density of the unwanted (rosetted) cells, such that they pellet along with the free RBCs when centrifuged at 1200g for 20 minutes over a buoyant density medium such as Ficoll-Paque(GE Healthcare). Desired cells which are not bound to the antibody were easily collected as a highly enriched population at the interface (buffy coat) between the plasma and the buoyant density medium.

5.3.3 Culture of MSC

The cell culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM) (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. When the cells attain 80 -90% confluency, they are trypsinized and passaged upto second passage. Second passage cells were used for immunostaining, flow cytometry and patch-clamp characterization.

5.3.4 Characterization of MSC by IHC

Second passage cells were cultured on 12mm round coverslip at a cell density of 8000cells/cm2. Cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature. Then the cells were washed with PBS (Invitrogen, Gibco) for three times. Blocking and permeabilization in 2% goat serum/2% Bovine serum albumin(BSA) with 0.1% Triton X-100\PBS. Cells were incubated with primary antibody at 4°C overnight. Cells were washed with PBS and incubated with secondary antibodies for 2 hours at room temperature. They were then washed and mounted with DAPI (Vectashield mounting medium with DAPI). Coverslips were immediately transferred to glass slides and examined in fluorescent microscope. The following mesenchymal stem cell marker(CD54,CD90,CD73,CD29,CD105), haematopoietic marker (CD45, CD34, CD14) and neuron markers (NeuN, Neurofilament, MAP2) were used.

5.3.5 Characterization of MSC by flow cytometry

Second passage cells were trypsinized and washed with PBS. Two to five lakh cells were used for each antibody in a separate test tube. 5 to 10 µl of primary antibody incubated for 20 minutes on ice. Excess unbound antibodies washed with PBS and removed. Appropriate secondary antibody, which is fluorescent tagged 5 to 10 µl were incubated for 20 minutes. Finally unbound secondary antibody washed with PBS. The cell suspension was aspirated and analyzed in flow cytometry for mesenchymal stem cell marker (CD54,CD90,CD73,CD29,CD105) and haematopoietic marker (CD45, CD34, CD14).

5.3.6 Neuronal induction of MSC

Second passage MSC was induced to neuronal cells by using neuronal induction medium consisting of DMEM/F12, 2% FBS, B27 supplement, 20mM Retinoic acid, and 12.5ng/ml bFGF.(189) Cells were maintained in neuronal induction media for 12 days. After 12 days these cells stained for neuronal marker.

5.3.7 Characterization of neuronal induced cells

After 12 days these cells stained for neuronal marker. (Beta III tubulin, MAP2, NeuN, Neurofilament, O4, Nav1.1) by IHC method as described above.

List of primary and secondary antibodies used:

CD54-FITC: Mouse anti-rat CD54-FITC(1:50); BD Pharmingen.

CD29: Monoclonal mouse anti-beta 1 integrin(1:50); Millipore.
Sec: Goat anti-mouse IgG2b-RPE (1:50); Southern Biotech.

CD90: Monoclonal mouse anti-rat CD90-FITC conjugated(1:100); Millipore.

CD73: Monoclonal mouse anti-rat CD73 (1:50); BD Pharmingen.
Sec: Goat anti-mouse IgG1-PE conjugated (1:50); Southern Biotech.

CD105: Goat polyclonal IgG (1:25); Santa cruz Biotechnology.
Sec:Donkey anti-goat IgG-perCp conjugated (1:100).

Mouse monoclonal IgG1 anti-CD34-FITC conjugated (1:100); santa cruz Biotechnology.

Mouse monoclonal anti-rat CD45-PE conjugated (1:100); BD Pharmingen.

CD14(1:50); Goat anti-rabbit IgG-RPE(1:50); Jackson Immunoresearch)

Mouse monoclonal anti-BetaIII tubulin(1:50); Millipore.
Sec: Goat anti-mouse Rhodamine(1:50); Millipore.

Mouse monoclonal anti-MAP2 IgG1(1:100);Millipore.
Sec:Goat anti-mouse IgG1-FITC (1:50).

Mouse monoclonal anti-NeuN IgG1(1:50); Millipore.
Sec: Goat anti-mouse IgG1-FITC(1:50)

Mouse monoclonal anti-Neurofilament IgG1(1:100); Millipore.
Sec: Goat anti-mouse IgG1-FITC(1:50).

Mouse monoclonal IgG1 anti-Glial fibrillary acid protein (GFAP) Alexa Fluor 488 conjugated(1:50); eBioscience.

Mouse monoclonal IgM anti-Oligodendrocyte(O4) (1:50); Sigma.
Sec:Donkey anti-mouse IgM-CY3 conjugate (1:50): Jackson Immunoresearch.

Rabbit polyclonal anti-Nav1.1 (1:100); alomone labs.
Sec: Goat anti-rabbit IgG-PE (1:50).

Mouse IgG2A Neuron specific β-III tubulin PerCp conjugated (1:100); R&D systems.

5.3.8 Electrophysiology- Patch clamp studies

The mesenchymal stem cells at second passage were placed on a 35mm petri-dish with 2 ml external solution, the composition of which is given below. Patch pipettes were fabricated using borosilicate glass capillaries by using a gravity assisted two-stage pipette puller. The pipette tips were heat polished and coated with Sylgard. When filled with internal solution (composition given in the following table) the pipette resistance ranged between 2 and 4 megaohms. The bath and pipette solutions were designed to elicit sodium, potassium and chloride currents.

Solutions used

	External (Bath) solution	Internal (Pipette solution)
Chemical	**Concentration (mM)**	**Concentration (mM)**
NaCl	135	
NaH_2PO_4	1	
KCl	4	140
$CaCl_2$	1.2	
$MgCl_2$	0.5	1
EGTA		1
HEPES	10	10
Glucose	10	10
	pH titrated to 7.4 with NaOH	pH titrated to 7.2 with KOH
	Osmolality adjusted to ~300mOsm/Kg	Osmolality adjusted to ~300mOsm/Kg

Once a giga-ohm seal was obtained between the patch pipette and the cell membrane, whole cell configuration was established by applying sharp suction. Cell capacitance was cancelled.The series resistance before compensation was below 15 megaohms, and was compensated 60-70%.

Voltage protocol

The holding voltage was -50mV or -80mV. (At more negative holding voltages a significant inward leak current was seen, and hence the choice of -50mV as the holding potential more recently). A prepulse to -40mV was given to elicit the voltage-gated sodium current, if it is present. The prepulse would also serve to inactivate the sodium channels (if they are present), before the test pulse, so that the potassium and chloride currents are not contaminated with sodium currents (if they are present). The test pulses ranged from -110 to +180mV at +10mV increments for 2 cells, while in 2 two other cells, the depolarizations ended at + 60 mV.

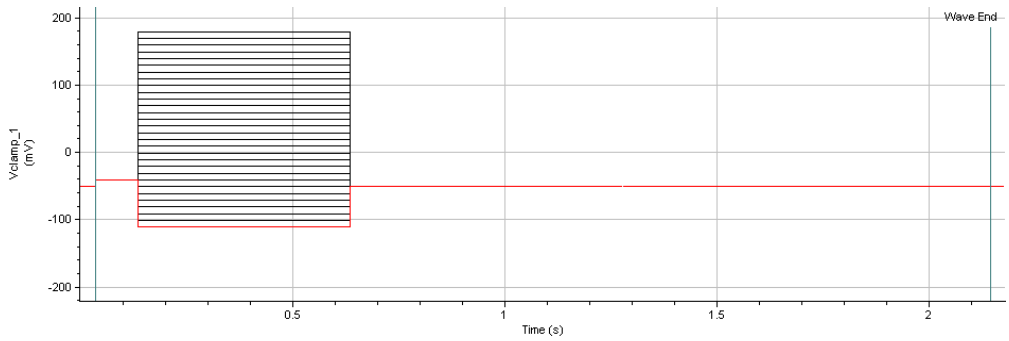


Figure:2 Voltage-protocol. V_{Hold} = -50mV; Prepulse = -40mV (to produce depolarization block of voltage gated sodium channels, if they exist); test pulses ranging from -110mV to 180mV, at 10mV increments.

The data during voltage-clamp were acquired using Axopatch 200B patch-clamp amplifier and digitized with Axon Instruments Digidata 1322A analogue-digital converter. The data were filtered using online 10 kHz low pass Bessel filter and off-line filtering was done during analysis whenever required. The data were sampled at a rate of 100 kHz. P-clamp software was used for acquisition and analysis of the data.

5.3.9 GFP labeling of cells

0.5×10^5/ml of cells were grown in complete medium overnight. At the time of transduction 50% to 75% confluent, and then add 50µl of 1×10^7 IFU/ml of pre-made lentiviral particles for fluorescent proteins.(catalog number:LVP001), GenTarget Inc.,USA. After 72 hours of transduction, the transduction rate was checked in fluorescent microscope. Green fluorescent protein gene expresses by CMV promoter and

fused with antibiotic resistance gene Bsd. Labelled cells are selected by adding blasticidin, these cells survive because of Bsd resistance gene and unlabelled cells may not survive. Only labeled cells used for transplantation.

5.4.0 Transplantation of cells in SCI rat model

Total cells were calculated by using the formula

Total cells per ml=average count per square×dilution factor×10^4.

Total cells= ……cells/ml.

Cell viability was assessed by using the formula

Cell viability (%)=total viable cells(unstained)/total cells(stained and unstained) ×100.

Cell harvesting to cell transplant, it takes approximately one hour, during this time cells were kept on ice. To assure that viable cells were transplanted into SCI rat.MSC trypsinized and kept on ice for 2 hours, then the cells were stained with propidium iodide for cell viability.

Fresh second passage MSC were used for transplant with different dosage like 2 lakh, 5 lakh, 10 lakh and more than 10 lakh individually as well as combination with olfactory ensheathing cells and chondroitinase as shown in table below.

Cells/Enzyme Dose/number of rats	**2 Lakh**	**5 Lakh**	**10 Lakh**	**>10 Lakh**
MSC	2 Lakh MSC (n=6)	5 Lakh MSC (n=6)	10 Lakh MSC (n=6)	>10 Lakh MSC (n=6)
OEC+MSC (1:1)	1L OEC+1L MSC (n=6)	2.5L OEC+2.5L MSC (n=6)	5L OEC+5L MSC (n=6)	>5L OEC+>5L MSC (n=6)
OEC+MSC+Chondroitinase (1:1)+0.2U	1L OEC+1L MSC +0.2U Chondroitinase (n=6)	2.5L OEC+2.5L MSC +0.2U Chondroitinase (n=6)	5L OEC+5L MSC +0.2U Chondroitinase (n=6)	>5L OEC+>5L MSC +0.2U Chondroitinase (n=6)

Bone marrow mesenchymal stem cell transplanted into the injured cord on 9^{th} day following spinal injury with different dosage 2 Lakh, 5 Lakh, 10 Lakh and more than 10 Lakh, in each group six rats were used(n=6). Mesenchymal stem cell (MSC) combined with OEC at a ratio (1:1) with different dosage 2 lakh (1 lakh MSC+1 lakh OEC), 5 lakh(2.5 lakh MSC+2.5 lakh OEC),10 lakh(5 lakh MSC+5 lakh OEC), more than 10 lakh(>5 lakh MSC+>5 lakh OEC).

MSC combined with OEC and Chondroitinase, chondroitinase treated as a constant dose of 0.2Units in all groups. But different dosage of cells 2 lakh(1 lakh MSC+1 lakh OEC+0.2U chondroitinase), 5 lakh(2.5 lakh MSC+2.5 lakh OEC+ 0.2U chondroitinase), 10 lakh(5 lakh MSC+5 lakh OEC+ 0.2U chondroitinase), more than 10 lakh(>5 lakh MSC+>5 lakh OEC+0.2U chondroitinase). In each group sample size (n=6) is maintained.

5.4 Chondroitinase

Chondroitinase ABC protease free from Proteus vulgaris catalyzes the degradation of polysaccharides containing (1→4)-β-D-hexosaminyland (1→3)-D-glucuronosyl or (1→3)-α-L-iduronosyl linkages to disaccharides containing 4-deoxy-β-D-gluc-4-enuronosyl groups. Chondroitinase acts on chondroitin 6-Sulfate, Chondroitin 4-Sulfate and dermatan sulfate and also acts slowly on hyaluronate. The enzyme is highly specific target for the galactosaminoglycan chains and without activity on core proteins, keratin sulfate chains, and heparin/heparin sulfate chains even in the absence of inhibitors for proteases, keratanases, and heparitinases. Initial rates of degradation of Chondroitin, Chondroitin Sulfate B, and hyaluronic acid were 20%, 40% and 2% respectively.

Unit definition

One unit of enzyme that catalyzes the formation of 1.0μmole of unsaturated disaccharide from chondroitin sulfate C per minute at $37^{O}C$.

2Units/vial lyophilized powder is dissolved in 20μl of 0.1% protease free BSA solution (water) and its molecular weight is 80,000.

Each rat was dosed with 0.2 Units into the spinal cord following spinal cord injury on 9^{th} day as described in the table below. In one group(n=6), 0.2U/2μl of chondroitinase alone was treated and in another group, chondroitinase as constant dose with different dose of OEC+MSC (i.e) 2Lakh, 5Lakh, 10Lakh and more than 10 Lakh n=6 in each group.

Cells/Enzyme Dose/number of rats	2 Lakh	5 Lakh	10 Lakh	>10 Lakh
OEC+MSC+Chondroitinase (1:1)+0.2U	1L OEC+1L MSC +0.2U Chondroitinase (n=6)	2.5L OEC+2.5L MSC +0.2U Chondroitinase (n=6)	5L OEC+5L MSC +0.2U Chondroitinase (n=6)	>5L OEC+>5L MSC +0.2U Chondroitinase (n=6)
Chondroitinase alone	0.2U chondroitinase alone without cells(n=6)			

5.5 Acidic fibroblast growth factor (FGF acidic)

FGF acidic also known as FGF-1, is a 17 KDa nonglycosylated member of the FGF family of mitogenic peptides. It is produced by multiple cell types, stimulates proliferation of mesodermal origin and many cells of neuroectodermal, ectodermal and endodermal origin. It plays a various roles in development, regeneration and angiogenesis Human FGF acidic shares 54% amino acid sequence identity with FGF basic. FGF acidic shares 92%, 96%, 96% and 96% amino acid sequence identity with bovine, mouse, porcine and rat FGF acidic respectively. FGF acidic associates with heparin sulfate and interacts with FGF receptors. Ligation triggers receptors dimerization, transphosphorylation, and internalization of FGF-receptor complexes. Internalized FGF acidic translocate to nucleus and functions as a survival factor by inhibiting p53 activity.

Recombinant human FGF acidic amino acid 2-155, catalog number: 231-BC/CF, R&D systems, USA was used. FGF dissolved in PBS at 1µg/µl. So, therefore 2µg/2µl of FGF injected into each spinal injured rats as described in the table below. Immediate after drop-weight injury, FGF alone injected into injured spinal cord in one group (n=6). In

another group (n=6), FGF on the first day (i.e) after injury and 10 lakh of olfactory ensheathing cells(OEC) on 9th day after SCI. Third group(n=6), both FGF and 10 lakh of OEC treated immediate after SCI.

Cells/Growth factor	**Dose/Sample size**
aFGF	FGF alone (1st day after SCI) (n=6)
OEC +a FGF	FGF(1st day after SCI) +10 Lakh OEC on 9th day. (n=6)
OEC+aFGF	FGF+10 Lakh OEC (1st day after SCI) (n=6)

5.6 Animal experiments

5.6.1 Spinal cord injury impactor

This device was fabricated to produce a drop weight contusion injury to the cord where a weight of 10 grams was allowed to fall onto the exposed cord. The drop-weight 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. The forces that usually correspond to a drop of a weight of 10 gms from a height of 25cm, producing a mild to severe injury. 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.

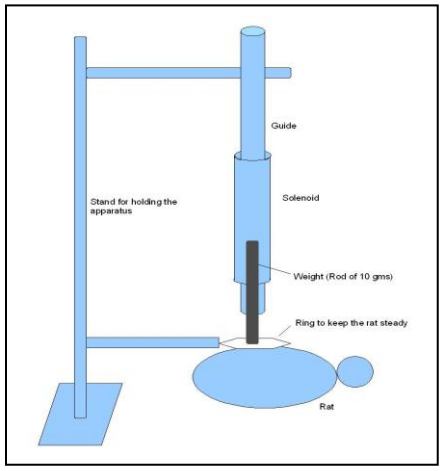


Figure:3 Schematic diagram of the apparatus used for drop-weight method to injure the spinal cord.

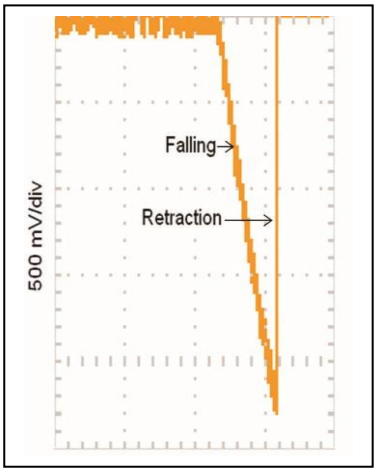


Figure: 4 A typical curve as given by the position sensor when the rod is dropped and retracted

5.6.2 Laminectomy and spinal cord injury by drop-weight method

Female Albino wistar rat, 100-250g in body weight, were anaesthetised with ketamine and xylazine (90:10 mg/kg) administered intraperitoneally. Ophthalmic ointment was applied to the eyes to prevent drying during the operation. 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. 2.0cm incision was made over the lower thoracic area, and 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, 10g weight rod falling from 25cm height on the exposed spinal cord. Absorbable suture (vicryl, Johnson-Johnson Ltd) 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 as mentioned below.

5.6.3 Postoperative care

Following the surgery, rats were placed in cage and monitored until they recovered from anesthesia. Rats were monitored twice a day throughout the post-injury survival period for general health, mobility within the cage. Bladder was manually expressed twice daily. Ringer lactate 5ml/100g was administered subcutaneously twice daily after each bladder expression on the first 7 postoperative days. Meloxicam 1mg/kg as analgesic, Enrofloxacin 2.5mg/kg as antibiotic, were administered for first seven post-operative days. Animals were monitored for urinary tract infections (UTI) for the entire period of the experiment. If indicative of a UTI, they were treated with the antibiotics (Enrofloxacin 2.5mg/kg). Inspection for skin ulcers or evidence of autophagia, was carried out daily. Bedding (paddy husk) was changed every alternate days.

5.6.4 Cell Transplantation

Cell transplantation was done on 9^{th} day following the drop-weight injury. Behavioural assessment (BBB) were conducted prior to the cell transplantation as described below. 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, the wound was explored and the injured spinal

cord segment as well as a few millimeter above and below normal spinal cord was exposed. On the day of transplantation the olfactory ensheathing cells, MSC, GBC were harvested by trypsinisation after which the enzymatic activity was stopped by adding DMEM with Fetal bovine serum. Cells were pelleted, transferred into 25µl Hamilton syringe (approximately 100,000 cells/µl). A Hamilton syringe, mounted on a injection device with 3D stabilizer. All injections were made with the aid of a sterile Hamilton syringe. 2-50µl of cell suspensions was injected at multiple sites, in and around the injured spinal cord. Following the cell transplantation, the surgical wound was closed and routine post-operative care was given.

For transplantation five things are considered:

1.Dose (number of cells).

2.When to transplant.

3.Route of administration.

4.Cell viability.

5.Graft versus host disease (GVHD).

1. DOSE

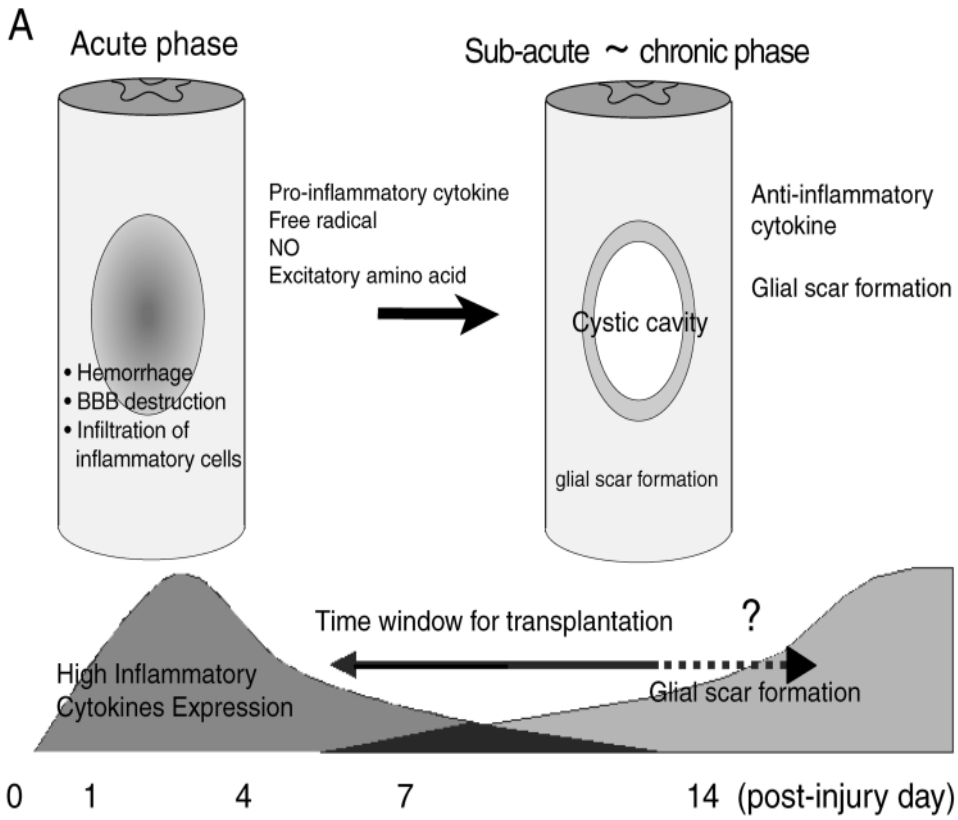
Olfactory ensheathing cells, Bone marrow mesenchymal stem cells transplanted with different dosage's like 2 lakh, 5 lakh, 10 lakh and more than 10 lakh individually. Both OEC and MSC transplanted as a combined therapy with different dosage 2 lakh, 5 lakh, 10 lakh and more than 10 lakh at a ratio of 1:1. Chondroitinase alone 0.2U were treated

in one group of SCI rats. Combination of all three OEC, MSC and Chondroitinase were done with different dosage 2 lakh, 5 lakh, 10 lakh and more than 10 lakh cells at a ratio 1:1 with constant dose of chondroitinase (0.2U). Globose basal stem cells were transplanted on 9^{th} day following SCI with same dose of 5 lakh cells in one group of rats. aFGF alone was injected into the injured spinal cord immediate after spinal injury in one group of rats(n=6). In another group, aFGF injected immediate after spinal injury(1^{st} day) and 10 lakhs of OEC injected on 9^{th} day. Both aFGF and 10 lakh of OEC injected into the injured spinal cord immediate after injury, that is on the first day. Control rats re-opened the injured site and injected DMEM alone on the 9^{th} day. Routine post-operative care was given.

The number of rats in each group which received different cell dosages and different cell combinations/Enzyme/Growth factor are shown below:

Cell type	Cell dosage and number of animals				Total sample
	2 lakhs	5 lakhs	10 lakhs	>10 lakhs	
OEC	6	6	6	6	24
MSC	6	6	6	6	24
OEC+MSC	6	6	6	6	24
OEC+MSC+ Chondroitinase	6	6	6	6	24
GBC		6			6
Control (without cell transplant)	11				11
Chondroitinase	6				6
FGF	6				6
FGF(1st day)+OEC(9th day)	6				6
FGF+OEC(1st day)	6				6

2. Transplantation window



Picture adopted from H.Okano. Stem cell Biology of the central nervous system. Journal of Neuroscience Research 69:698-707 (2002).

For transplantation 9th day has preferred after SCI, because acute to injury inflammatory process up-regulated and at the 9th day inflammatory response declined. After 10th day glial scar persist and have great impact on regeneration. Single doses was given in all rats, but did not tried multiple dose in this study due to constraint of time.

3. Route of administration

Bone marrow mesenchymal stem cell can be administered intravenous, but bioavailability may not be 100% at the desired site. So, injected the cell into injured spinal cord.

4. Cell viability

To address, whether the cells are healthy for transplantation. Freshly cultured second passage cell were used for transplant. Before transplant cell viability was done as described in Figure 6.1.6 and Figure 6.4.4

5. Graft versus host disease (GVHD)

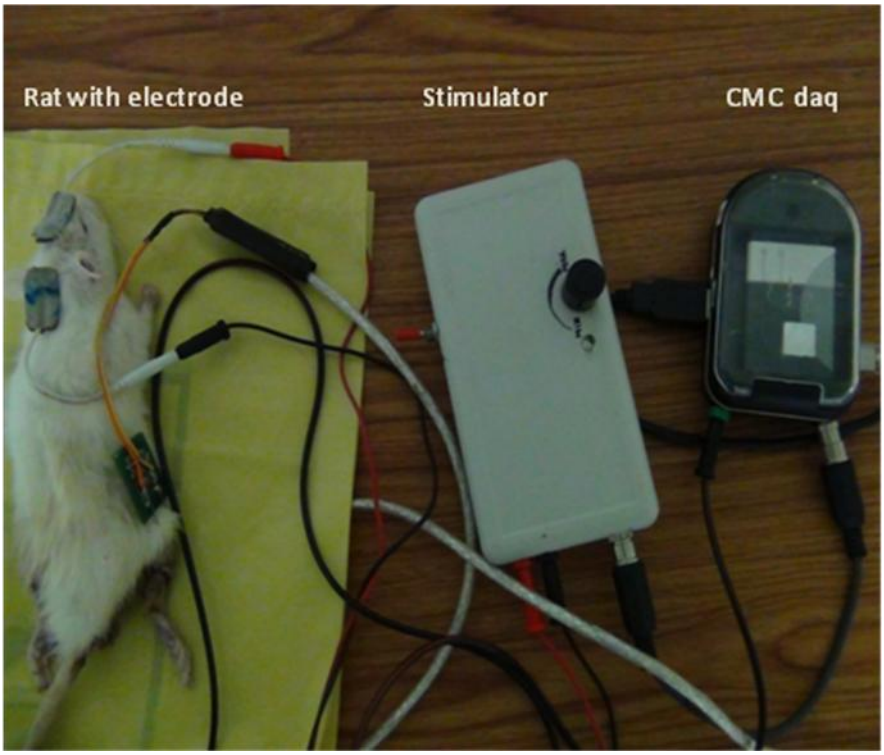
MSC has a immunomodulatory properties and lack CD80 and CD86, they are not immunogenic upon allogenic transplant. So, exploited the advantage of MSC. OEC is also a neurogenic glial cells and can able to reside in CNS like astrocyte. So, GVHD was not addressed and encountered in this study. Allogenic transplantation was done, both olfactory mucosa cells and bone marrow mesenchymal stem cells from male rat and transplanted into female spinal cord injured rat. All albino wistar rat are inbred, so graft rejection are not encountered.

5.6.5 Behavioural assessment- BBB score

The BBB (Basso,Beattie,Bresnahan)scale (190) is an operationally defined 21 point scale, designed to assess hindlimb locomotor recovery after impact injury to the spinal cord in rats. This locomotor scale categories 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. The motor assessment will be done upto 8 to 10 weeks after injury/transplant. Open-field observations were made on rats. All rats received bladder expression before open field testing to eliminate behaviors due to bladder fullness. Rats were allowed to walk in the open field (45cm×60cm rectangular tray) and videographed for assessment. All rats assessed for BBB before transplant (i.e) on 9^{th} day after SCI and every week post-transplant onwards up to 8 to 10 weeks. BBB score was done with the aid of BBB scale as shown in appendix.

5.6.6 Motor evoked potential studies

Transcranial stimulation of motor cortex were done in the anesthetized rats and the EMG signals were recorded from the lower limb muscles to indicate the functional integrity of the spinal cord. Motor cortex was stimulated and the responses were recorded from the gastrosoleus muscles. Recording was done from control as well as cell/enzyme/growth factor transplanted rats at 8 to 10 weeks post spinal injury/transplantation. Recorded EMG signals was analysed for onset time, peak time and amplitude.



Set-up used for motor evoked potential study

5.7 Histological evaluation of the outcome of experiments

To evaluate the morphological restoration of the neural tract in experimental animal the following tract tracing method was used.

5.7.1 Retrograde tract tracing

To investigate the ascending regenerated fibres in the injured spinal cord(i.e. across the injury epicenter), a retrograde tracer Fast blue (Sigma) was injected into the dorsal column of the proximal stump, 2-4 mm caudal from the site of spinal cord injury. Injected at 0.5mm depth and 2.0mm caudal to the injury epicenter, 0.5 mm lateral, 1.0 mm lateral of midline, 1.5 mm lateral of midline. 3.5mm caudal to the injury; 0.5 mm lateral, 1.0mm lateral of midline, 1.5mm lateral of midline. FB was administered at about 8 to 10 weeks after SCI/transplant. All injection was made with the pulled glass

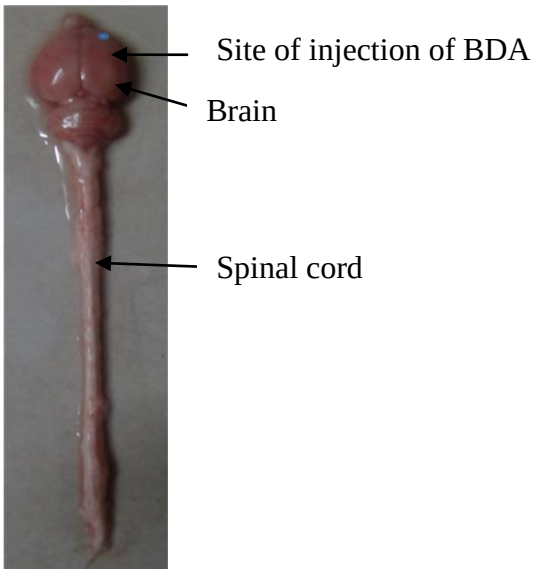
micropipette needle. 5% aqueous FB was injected at about 0.7µl/site at different sites. So total volume of 5.6µl was injected. Paraspinal muscle and skin was sutured and routine post-operative care was given. After seven days, rat was transcardially perfused with 4% paraformaldehyde solution. Spinal cord was removed and post-fixed in 30% sucrose/phosphate-buffered saline at 4^0 C overnight. From dorsal to ventral side of the spinal cord, twenty micrometer (µm) thick longitudinal cryo sections were cut and mounted on poly-L-Lysine coated slides. The blue fluorescence of fast blue labeled neurons and axons was visualized with confocal microscopy. FB-labeled neurons were counted 1.2mm caudal to the injury epicenter, and 1.2mm cranial to the injury epicenter of both control and FGF/OEC treated rats. Neurons are identified by axons and visible cell body. Visible cell bodies were counted for analysis.

5.7.2 Anterograde tract tracting

The descending corticospinal tract fibres were tracked by biotinylated dextran amine 10% (BDA), MW 10000, was injected into motor cortex. The rats were anaesthetized with ketamine and xylazine(90:10 mg/kg) by intraperitoneal injection. The scalp fur was shaved and cleaned with surgical spirit. Mid-saggital incision was made on the skull. The hind-limb corresponding area of skull on the right contralateral side burr hole made 1cm×1cm. Coordinates from bregma: 0mm anterior/1.0mm lateral, 0.5mm anterior/1.0mm lateral, 0.5mm anterior/1.5 mm lateral, 0mm posterior/1.0mm lateral, 0.5mm posterior/1.0mm lateral, 0.5mm posterior/1.5 mm lateral, three weeks after the injection, rats were transcardially perfused with 4% paraformaldehyde solution. Spinal

cord was removed and post-fixed in 30% sucrose/phosphate-buffered saline at 4^0 C overnight. Twenty micrometer (µm) thick cryo sections were cut and mounted on poly-L-Lysine coated slides. Tissue was incubated with 0.3% Triton X-100 in phosphate-buffered saline for overnight at 4ºC. Tissue was washed with PBS and incubated with Streptavidin-Alexa fluor 568 conjugate (1:400) for 2 hours at room temperature.

The sections were then washed with PBS, and visualized in confocal microscope.



Dorsal view of brain and spinal cord of normal rat

5.8 Statistical analysis

Statistical analysis done by using SPSS 16 version ANOVA Post Hoc Tukey to compare significances with different groups. P value < 0.05 is considered as significant. Wilcoxon paired test done to compare within a group and Mann-Whitney test done to compare two groups.

Results & Analysis

The nasal septum (Figure 6.1.1) of rat indicates the locations of olfactory mucosa (OM) and the repiratory mucos (RM). Semicircular line indicates the bony septum, where olfactory mucosa was isolated from the posterior region of septum. Respiratory mucosa lies anterior to the olfactory mucosa.

On either side of nasal septum (NS), olfactory mucosa is located as indicated in picture (b). Lamina propria shows nerve fascicle (NF) and to its periphery olfactory epithelium (E) is present. Lamina propria is enriched in olfactory ensheathing cells and olfactory nerve fibroblast, whereas olfactory epithelium contains HBC, GBC and immature neurons which is responsible for neurogenesis.

Lamina propria shows single nerve fascicle, where the olfactory receptor neurons, nuclei of olfactory ensheathing cells and olfactory nerve fibroblast. Electron microscopy of lamina propria indicates the olfactory ensheathing cells (OEC), which surround the bundle of olfactory sensory axons and olfactory nerve fibroblast (ONF) lies in the periphery of nerve fascicle. OEC is a flattened, curved cell and outer surface connected by basal lamina, and inner surface that encloses the numerous axons.

Rat olfactory ensheathing cells (OEC), olfactory nerve fibroblast (ONF) were characterized by p75NTR-FITC and Fibronectin-PE respectively by immunohistochemically. Olfactory ensheathing cells culture express for S100β (Schwann cell marker), GFAP (Astrocyte marker) and shows negative for Galc a marker for oligodendrocytes (Figure 6.1.2). Flow cytometry analysis P75NTR-FITC shows 40%

positive of OEC and shows 35% positive of anti-fibronectin, marker of ONF (Figure 6.1.3).

OEC exhibit morphologic and antigenic characteristics of astrocytes, schwann cells and oligodendrocytes and express an array of trophic factors, and extracellular matrix molecules (191,192). Previous study demonstrate that, mixed culture of OEC/ONF contains elongated, spindle-shaped cells co-expressed p75NTR and S100β , either diffusely or intensely expressed GFAP are populated as OEC. The ONF population consisted of flattened fibroblast-like morphology immunoreactive for fibronectin and GFAP (193). The OEC/ONF found similar expression of S100β and GFAP of about 75% and 83% respectively (Figure 6.1.4). This shows OEC shares the properties of Schwann cell (S100β) and astrocyte (GFAP). It can able to reside in CNS environment like astrocyte, and can remyelinate axons like Schwann cell pattern (194).

To tract the transplanted cells surviving in the cord, the cells were labeled with lentiviral GFP and then transplanted on the 9^{th} day following spinal cord injury.The labeled cells express GFP (Figure 6.1.5). The viability of cells was assessed by propidium iodide- a red fluorescent DNA counterstain, which shows only 2% of cells were dead cells, remaining 98% viable cells (Figure 6.1.6).

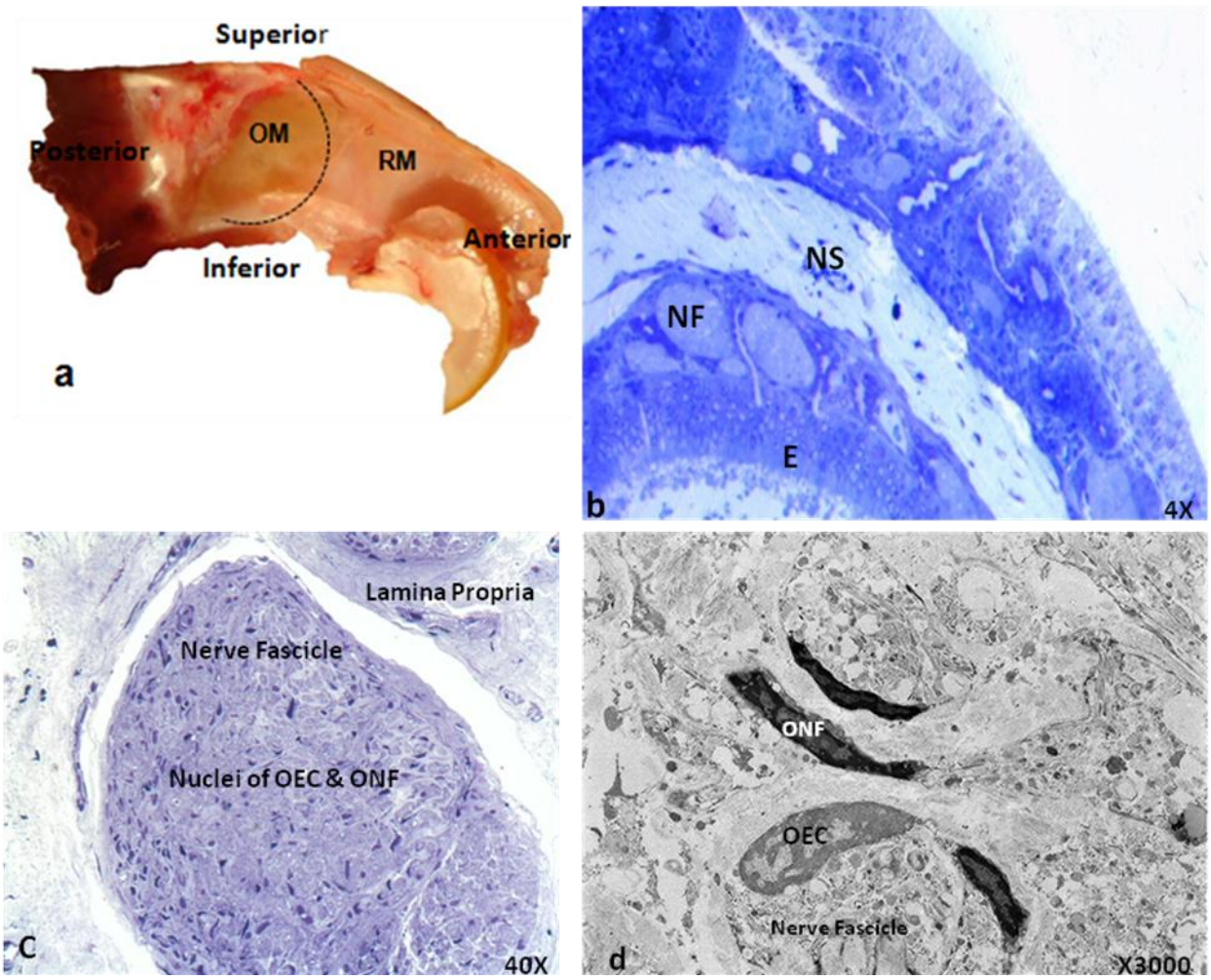


Figure 6.1.1 Olfactory mucosa – light and electron microscopic features

a. Rat septum showing the position of the olfactory mucosa. Note the yellow color of the mucosa and the line of demarcation between the respiratory (RM) and the olfactory mucosa (OM).

b. Rat olfactory mucosa semi-thin section stained with toluidine blue. Nasal septum (NS) and olfactory mucosa showing olfactory nerves

c. Single nerve fascicle showing nuclei of OEC (olfactory ensheathing cells) and ONF (olfactory nerve fibroblasts).

d. EM showing nerve fascicle with OEC and ONF.

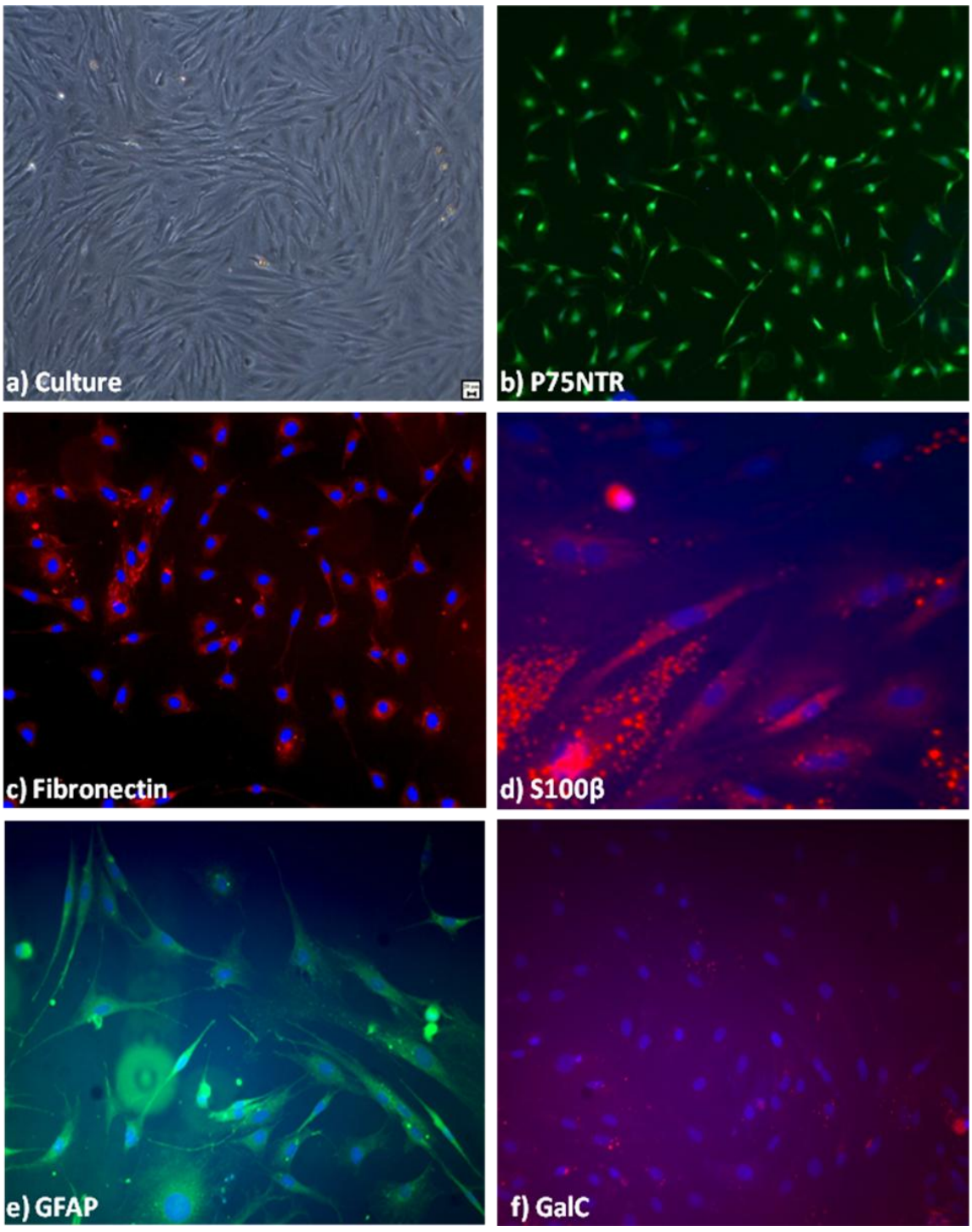

a) Culture
b) P75NTR
c) Fibronectin
d) S100β
e) GFAP
f) GalC

Figure 6.1.2 Immunocytochemical characterization of olfactory ensheathing cells and olfactory nerve fibroblasts.

a. OEC and ONF in culture – phase contrast picture

b. OEC stained with P75NTR-FITC. Cells are positive for the marker

c. ONF stained with anti-Fibronectin-PE. Cells are positive for the marker

d. OEC stained with S100β-PE. Cells are positive for the marker

e. OEC stained with GFAP-Alexa Fluor 488. Cells are positive for the marker

f. OEC stained with Galc-PE Cells are negative for the marker

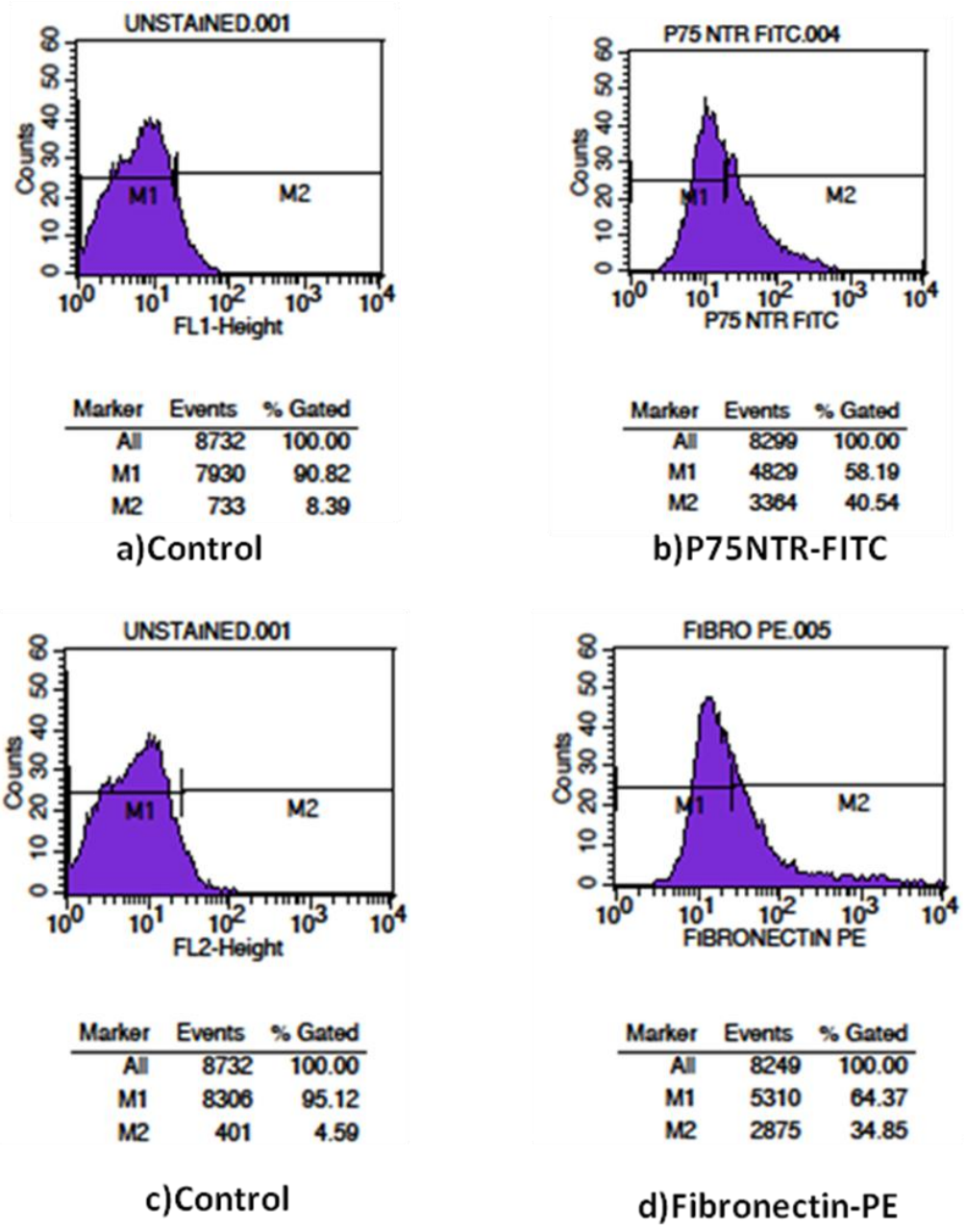


Figure 6.1.3 Characterization of OEC and ONF by Flow cytometry

(a). and (c). shows control. (b).Flow cytometry analysis P75NTR-FITC shows 40% of positive OEC. (d). Flow cytometry analysis shows 35% of positive ONF

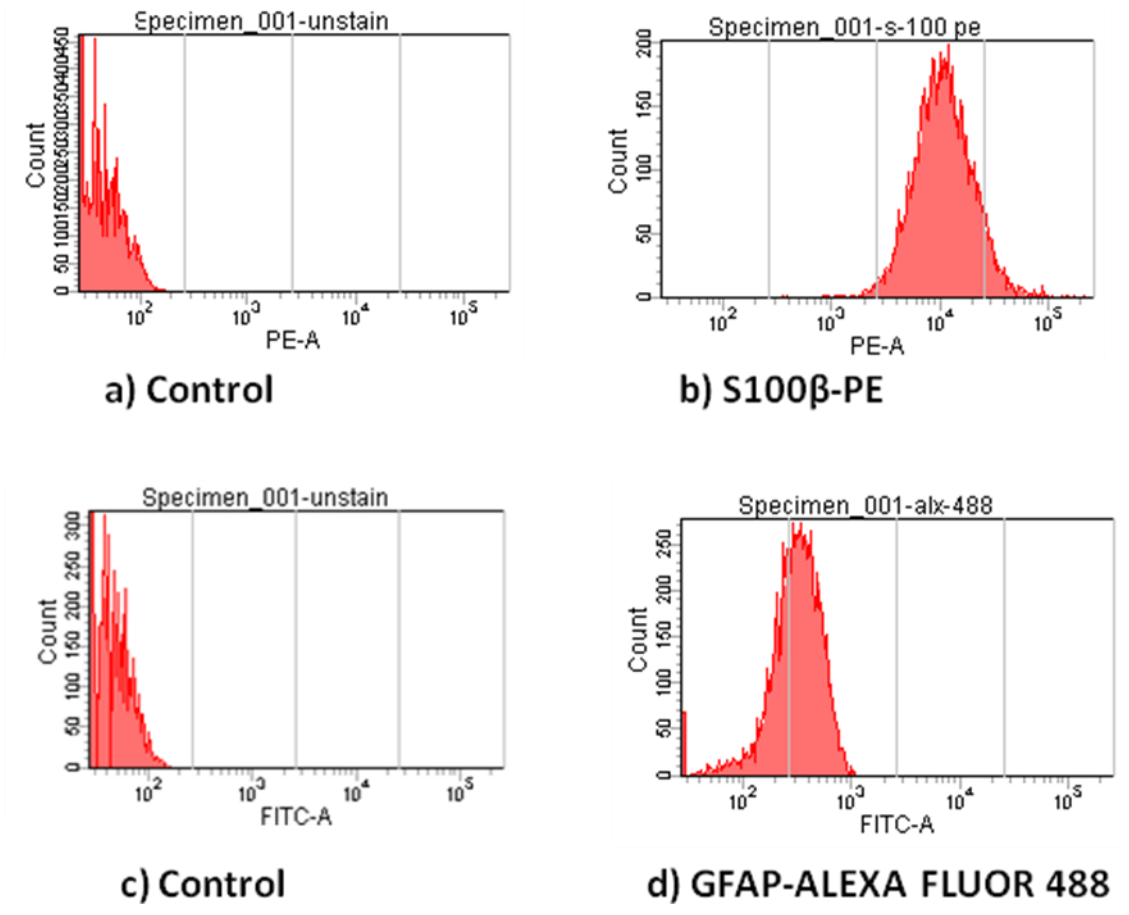


Figure 6.1.4 Characterization of OEC and ONF by Flow cytometry

(a) and (c) control of OEC. (b)75% positive for S100β-PE (d) 83% positive forGFAP-Alexa fluor 488

The culture contains two populations of cells- OEC and ONF. OEC expresses p75NTR of 40% and olfactory nerve fibroblast express 35% of fibronectin which is the marker of OEC and ONF. Similarly express S100β and GFAP of about 75% and 83% respectively. This shows OEC shares the properties of Schwann cell (S100β) and astrocyte (GFAP). It can able to reside in CNS environment like astrocyte, and can remyelinate axons like Schwann cell pattern (194).

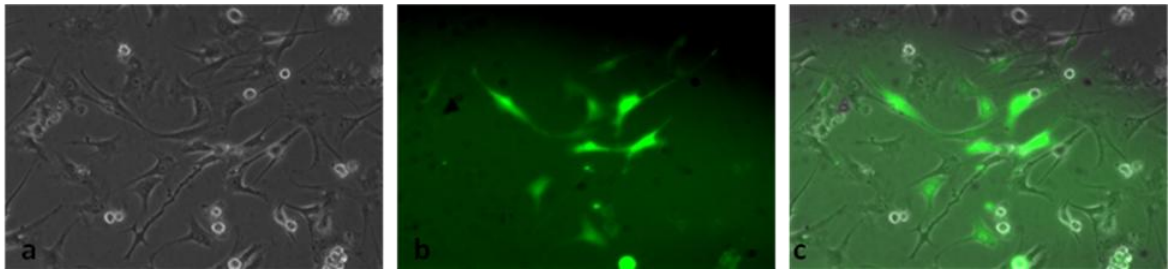


Figure 6.1.5 OEC labeled with lentiviral-GFP before transplantation.

a.culture phase contrast picture.

b.GFP labelled cells.

c. a and b are merged.

To tract the transplanted cells surviving in the cord, the cells were labeled with lentiviral GFP and then transplanted on the 9th day following spinal cord injury.The labeled cells express GFP.

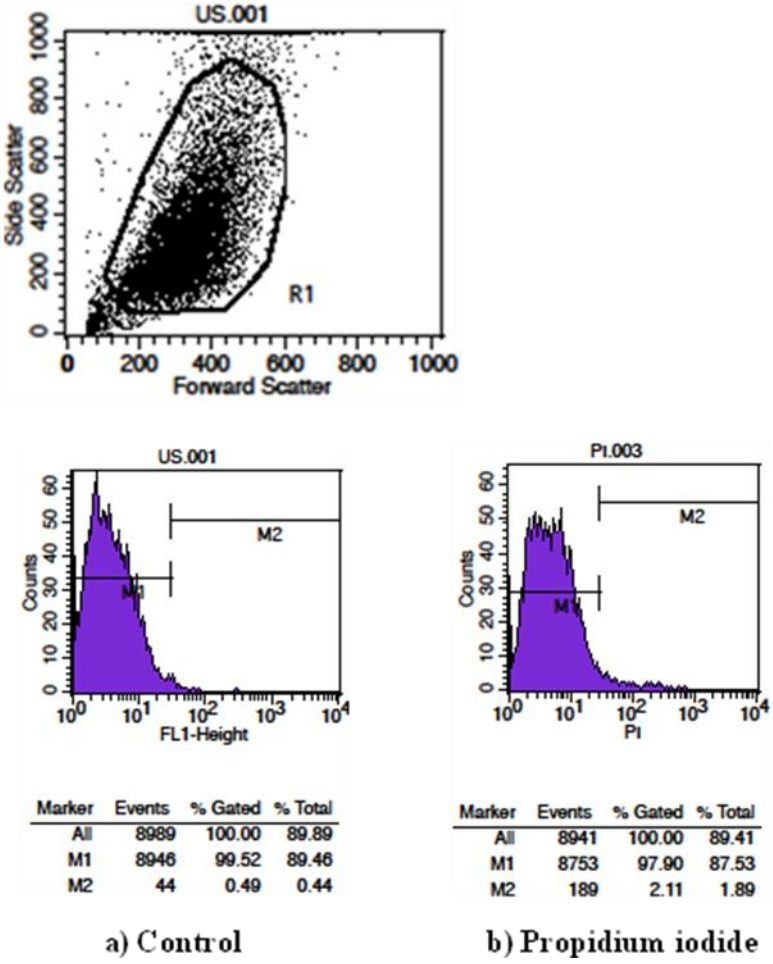


Figure 6.1.6 Quantification of viabile OEC after harvest from culture

a. Control

b.Propidium iodide shows positive of 2%

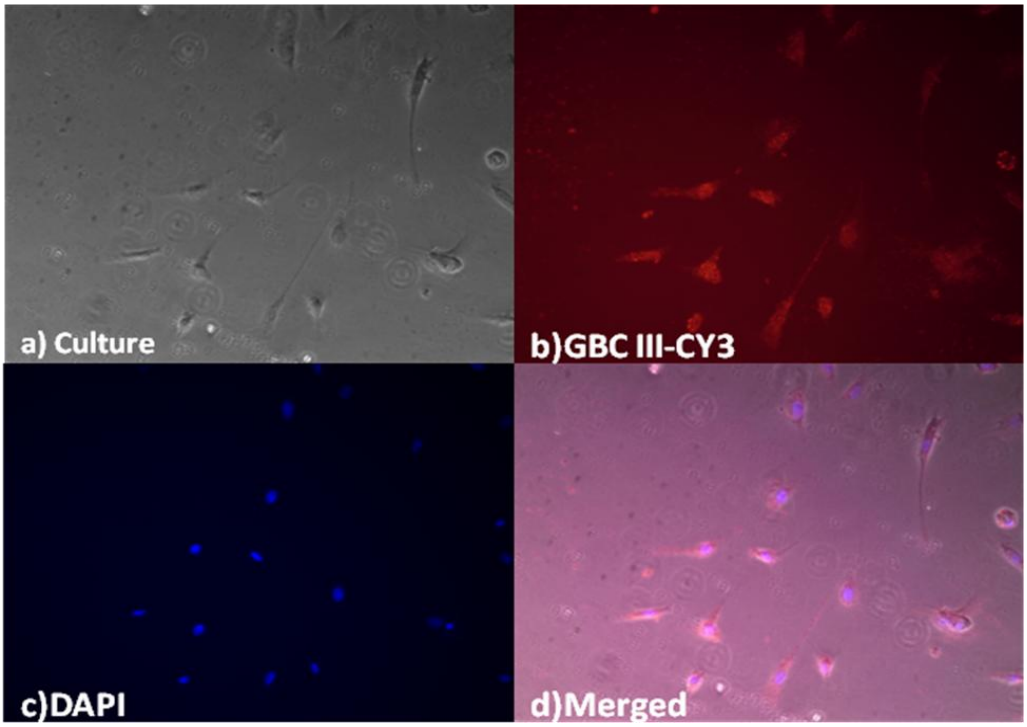


Figure 6.1.7 Characterization of GBC using GBC III by IHC

(a) Cells in culture – phase picture

(b) GBC III-CY3

(c) DAPI

(d) Merged picture of a, b and c.

Olfactory epithelial cells were cultured and stained for GBC III antibody. The cells are positive for GBC III (Globose basal stem cell marker).

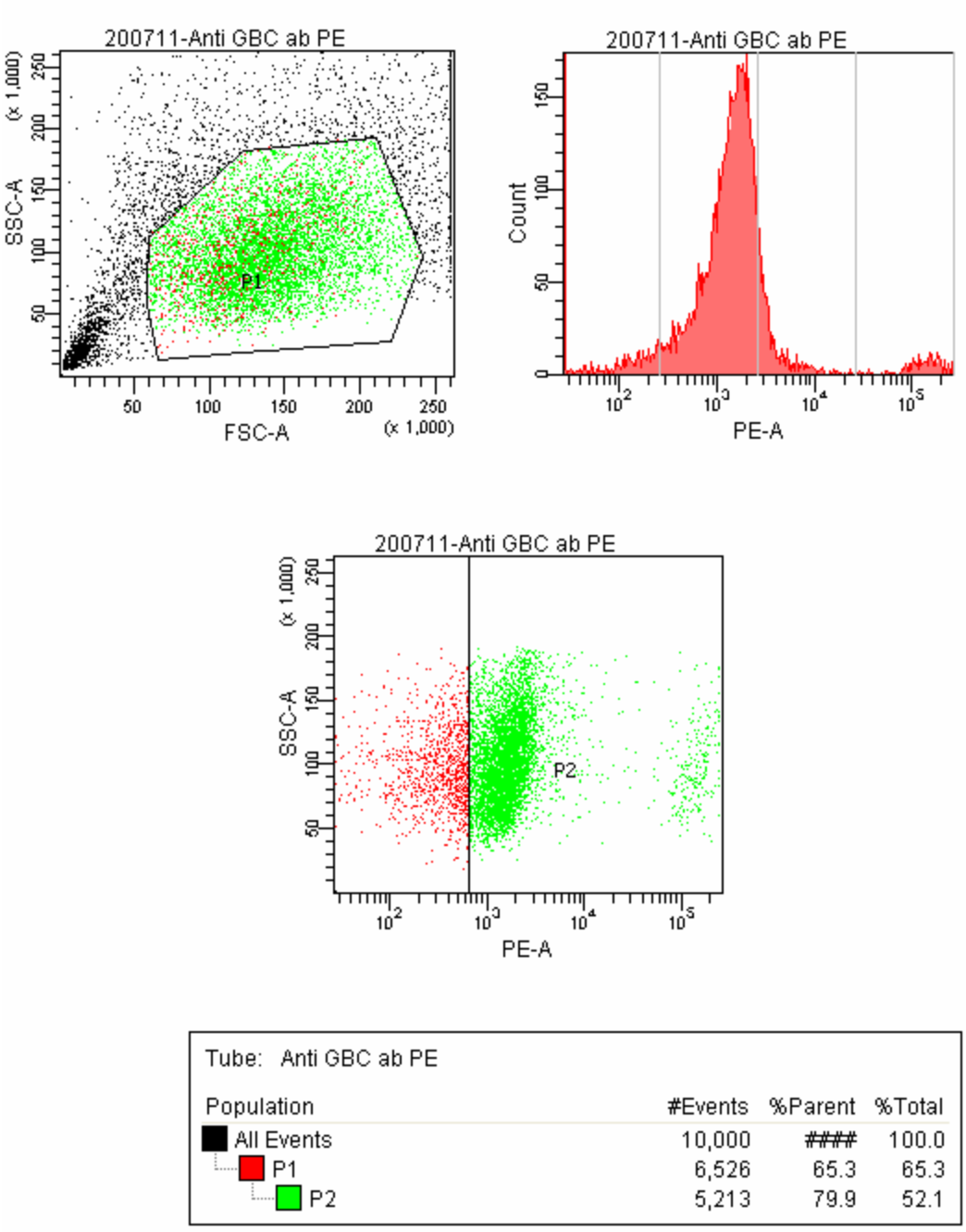


Figure 6.1.8 Fluorescence activated cell sorting (FACS) of GBC III cells

P1 shows negative population of cells.

P2 shows positive for GBC III antibody of 52%.

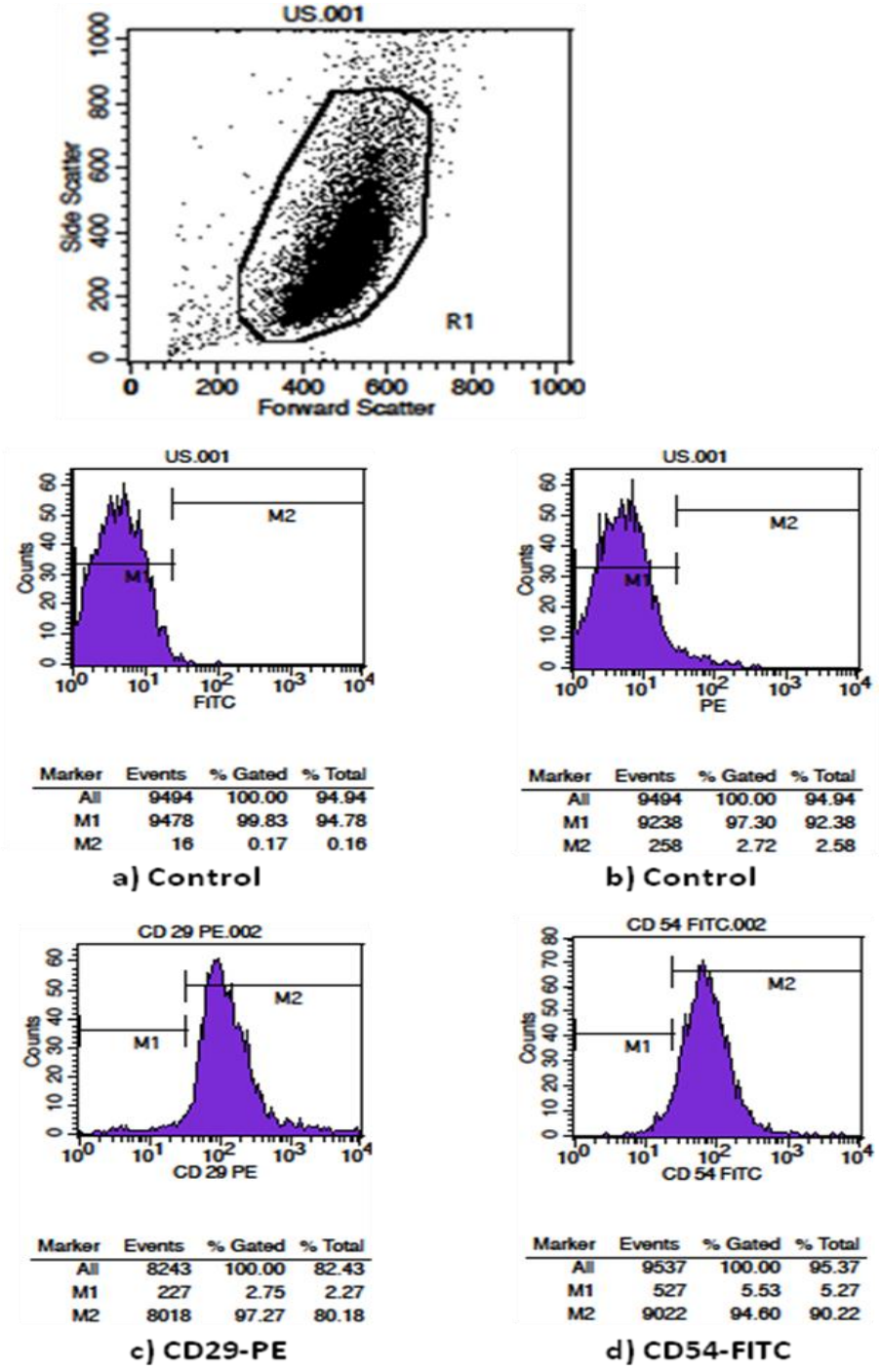


Figure 6.1.9 Characterization of GBC by Flow cytometry

(a) and (b) contol, (c)CD29-PE positive of 80%, (d)CD54-FITC positive of 90%.

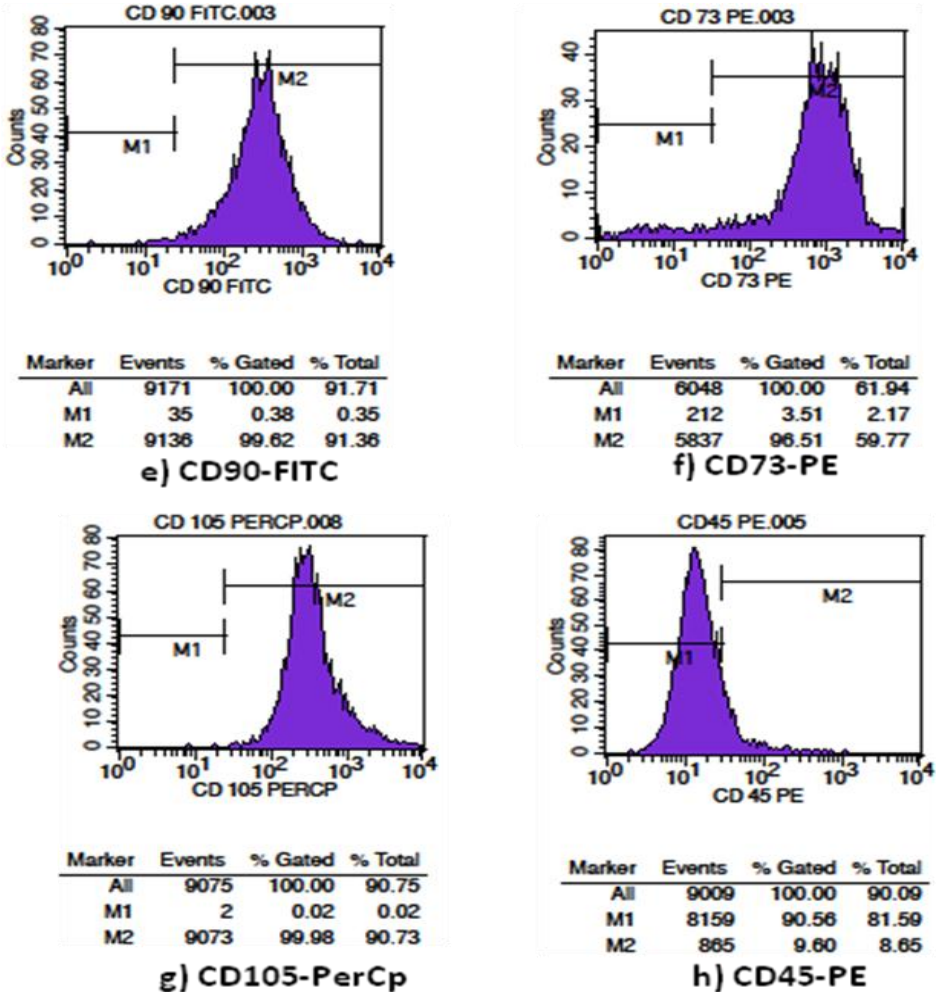


Figure 6.2.0 Characterization of GBC by Flow cytometry

(e)CD90-FITC positive of 91%, (f) CD73-PE positive of 60%, (g)CD105-PerCP positive of 90%, and (h) CD45-PE positive of 8%.

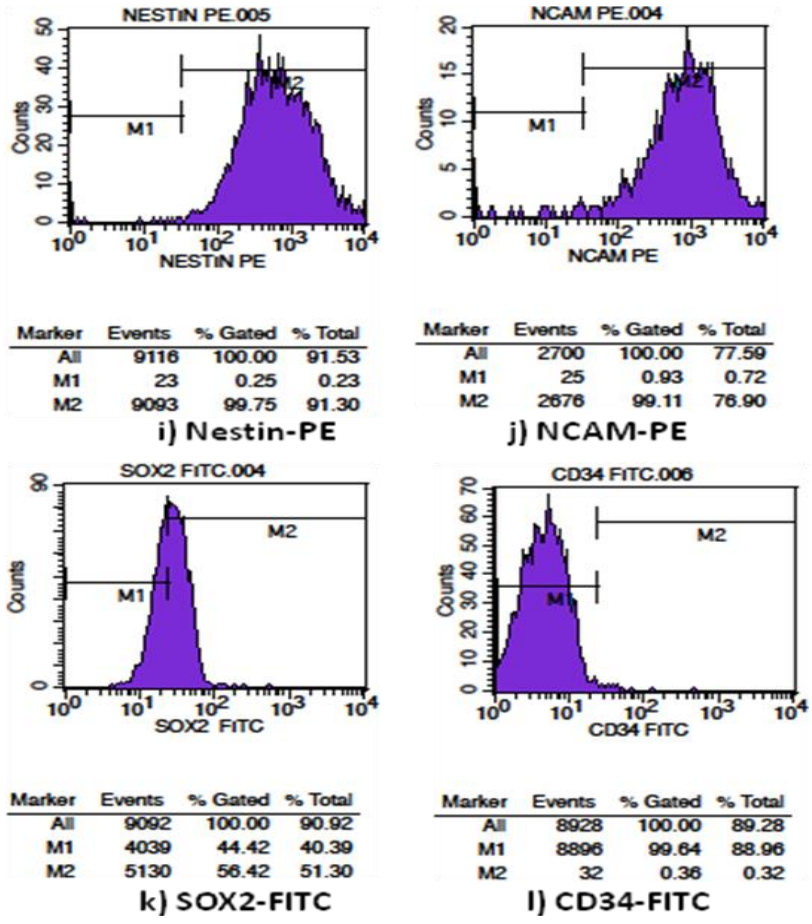


Figure 6.2.1 Characterization of GBC by Flow cytometry

FACS globose basal stem cell (GBC) cultured and further analysed for expression of MSC markers CD29, CD54, CD90, CD105, CD73 and showed 80%, 90% 91%, 90%, 59% positivity in order. Negative of CD45, CD34 (haematopoietic marker). Neural stem cells markers expressed as follows nestin, NCAM, SOX2 positive of 91%, 76%, 51% respectively.Globose basal stem cell has the properties of bone marrow mesenchymal stem cell (MSC) by expressing the MSC markers and neural stem cell markers as well.

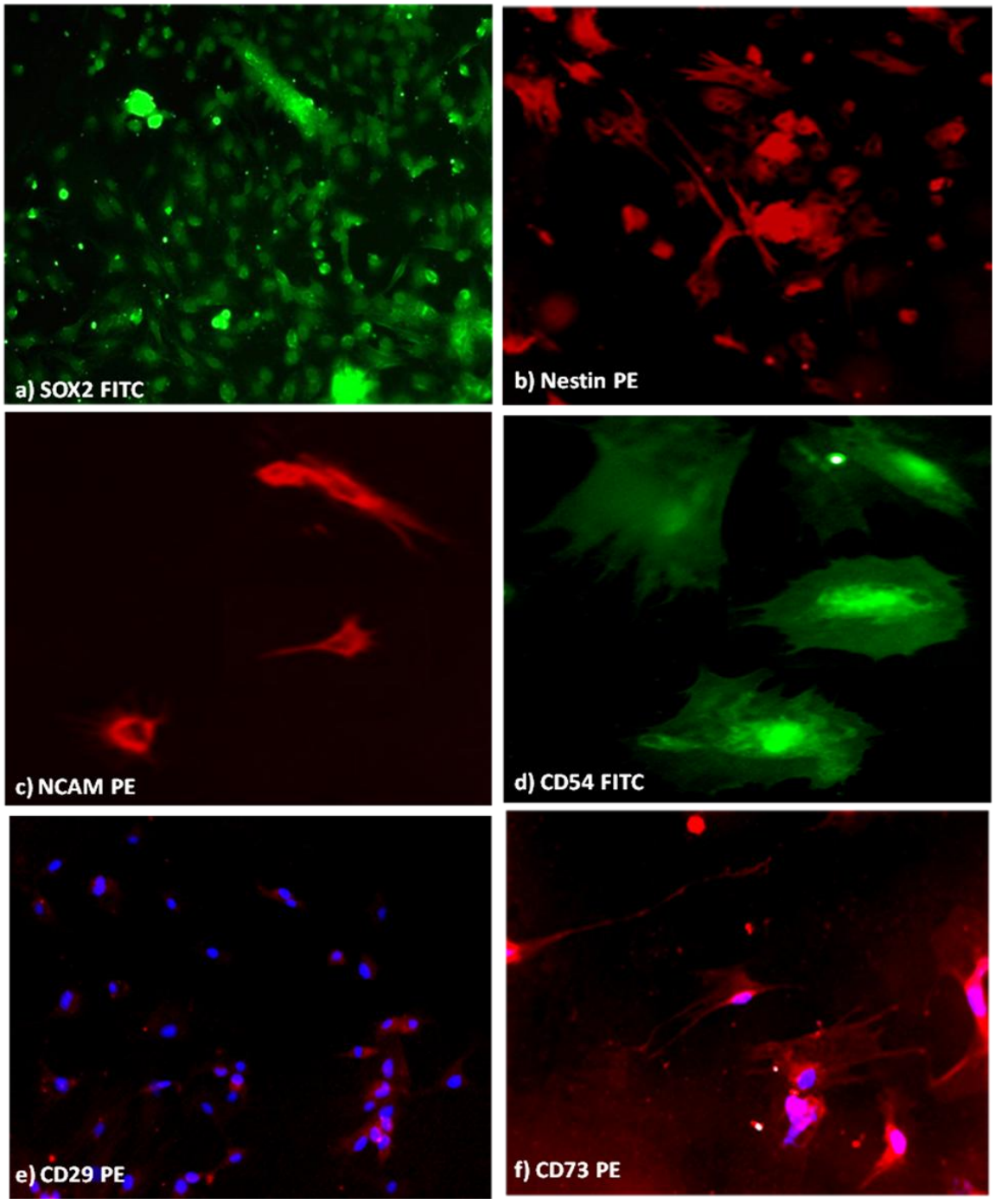


Figure 6.2.2 Characterization of GBC by IHC shows positive for SOX2-FITC, Nestin-PE, NCAM-PE, CD54-FITC, CD29-PE, CD73-PE

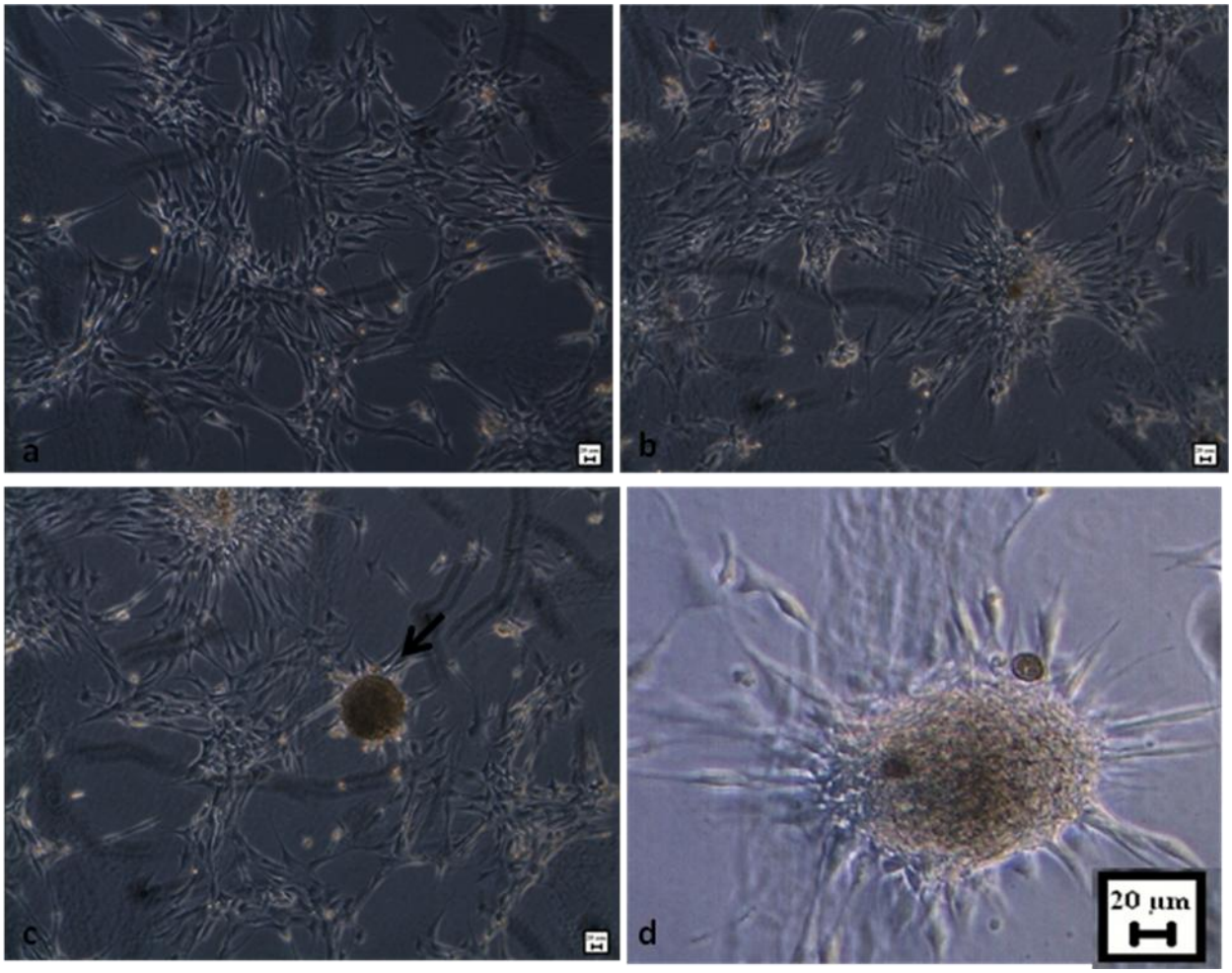


Figure 6.2.3 Neurosphere formation
(a) GBC 1st day in neurosphere medium.
(b) GBC 2nd day in neurosphere medium.
(c) GBC 3rd day in neurosphere medium forms neurosphere (black arrow).
(d) Neurosphere magnified.

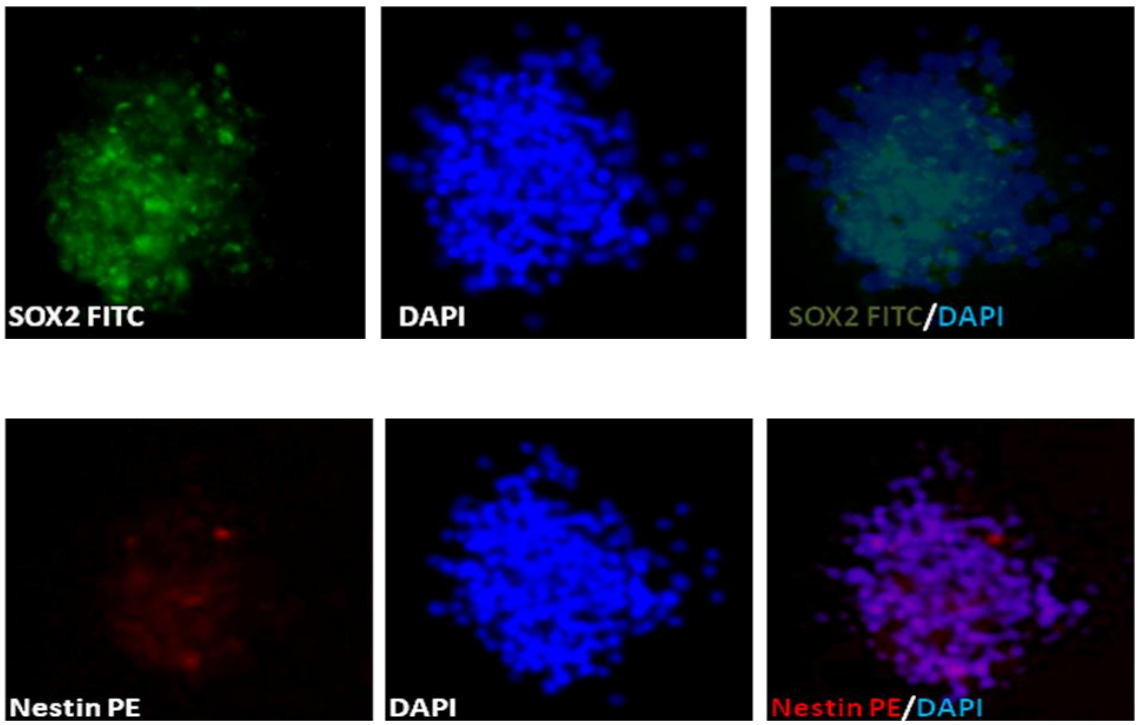


Figure 6.2.4 Neurosphere characterization

Globose basal stem cell forms neurosphere, and these neurosphere expressed neural stem cell marker Sox2 and nestin.

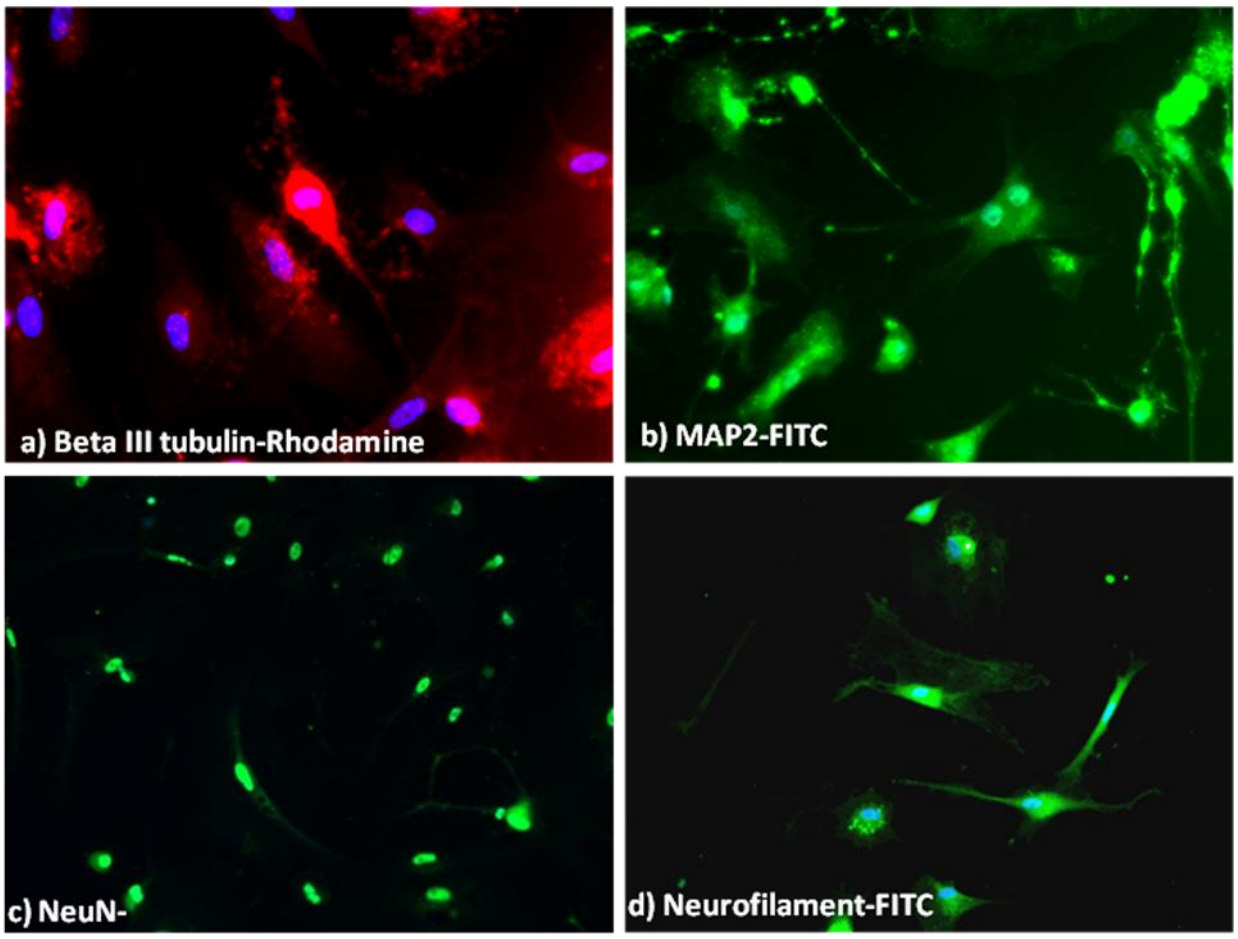


Figure 6.2.5 Characterization of neuronal induced GBC by IHC

The neuronal induced globose basal stem cells express neuronal marker β III-tubulin, MAP2, NeuN and Neurofilament. This shows that GBC can differentiate in invitro condition, proving the multipotent characteristic of GBC.

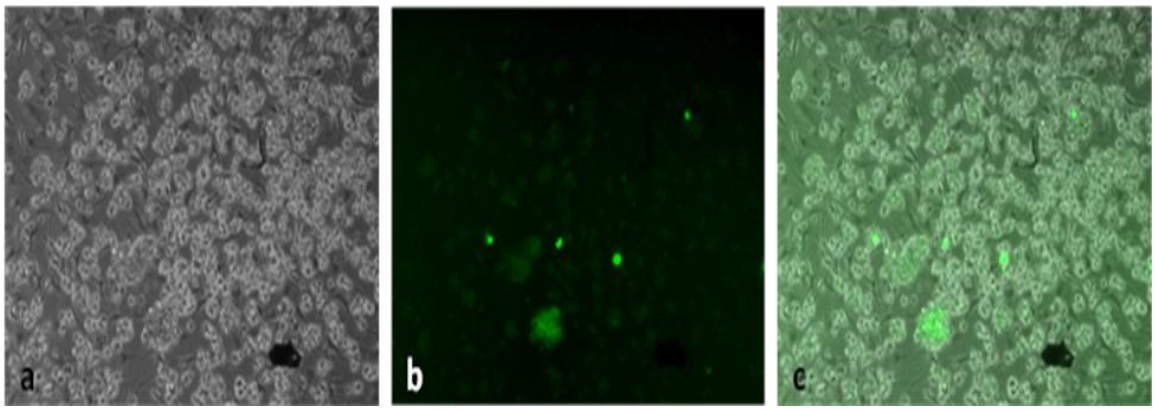


Figure 6.2.6 GBC labelled with lentiviral-GFP for transplantation

(a) Culture

(b) GFP-labeled GBC

(c) Merged (a and b).

To tract the transplanted cells surviving in the cord, the cells were labeled with lentiviral GFP and then transplanted on the 9^{th} day following spinal cord injury.The labeled cells express GFP.

Olfactory epithelial cells were cultured and stained with GBC III antibody, which specifically target globose basal stem cell (GBC) of rodent olfactory epithelium as shown in (figure 6.1.7). GBC III antigen is the 40 KDa laminin receptor protein on GBC (195). 52% of GBC III cells were fluorescent activated cell sorted (FACS) for further studies as shown in (Figure 6.1.8). Cultured (GBC) analysed for MSC marker CD29, CD54, CD90, CD105, CD73 which shows positive of 80%, 90%, 91%, 90%, 59% respectively and shows negative for CD45, CD34 (haematopoietic marker). Neural stem marker shows Nestin, NCAM, SOX2 positive of 91%, 76%, 51% respectively, as shown in (Figure 6.1.9, Figure 6.2.0, Figure 6.2.1 and Figure 6.2.2). Globose basal stem cell has the properties of bone marrow mesenchymal stem cell (MSC) by expressing the marker and also expresses the neural stem cell marker. The expression of neural stem or progenitor cell markers and formation of neurospheres is the potential properties of stem cell in neural tissue. When globose basal stem cell (GBC) plated in neurosphere media, similarly GBC has a capacity to form neurosphere as an invitro characteristic features as shown in (Figure 6.2.3) and these neurosphere expressed neural stem cell marker Sox2 and nestin as shown in (Figure 6.2.4). This GBC could be an alternative source of neural stem cell from olfactory epithelium for neurotransplantation. The neuronal induced globose basal stem cells express the neuronal marker β III-tubulin, MAP2, NeuN and Neurofilament as shown in (Figure 6.2.5). This shows that GBC can be differentiated in invitro and proves multipotent characteristics. To tract the transplanted cells surviving in the cord, which is responsible for transplant mediated repair, the cells were labeled with lentiviral GFP and then transplanted on the 9^{th} day following spinal cord injury.The labeled cells express

GFP as shown in (Figure 6.2.6). Ectodermal origin GBC converted into ectoderm neuronal lineage both in invitro and invivo, concluding same germ layer conversion.

Bone marrow mesenchymal stem cell (MSC) are multipotent and express the panel of markers CD54, CD29, CD73, CD90, CD105 of about 40%, 82%, 85%, 99%, 13% respectively, and shows negative for haematopoietic marker CD14, CD45, CD34, of about 0%, 0%, 2% respectively, which indicates the purity of MSC (Figure 6.2.7, Figure 6.2.8, Figure 6.2.9, Figure 6.3.0, Figure 6.3.1). Stem cell has the plasticity in morphological changes in invitro condition. On providing neuronal culture condition, the morphology changes from fibroblast-like to spindle shaped network. Morphology of spindle shaped cells taken on 12^{th} day post neuronal induction (Figure 6.3.2) Mesenchymal stem cells shows negative for neuronal marker MAP2, NeuN, and Neurofilament, but after neuronal induction express the differentiated marker of neuron and glia (MAP2, Neurofilament, Neuronal neuclei, βIII-tubulin, GFAP and O4). This shows that MSC capable of transdifferentation, mesodermal origin MSC to ectodermal neuronal lineages (Figure 6.3.3, Figure 6.3.4, Figure 6.3.5). Differentiated neuron express the voltage-gated sodium channel is essential for generation and propagation of action potential. This is the hallmark for the excitable cell neuron. The results shows the expression of voltage-gated sodium channel type1 (Nav1.1) by immunohistochemically (Figure 6.3.6), but did not have functional expression of voltage-gated sodium by patch-clamp studies. MSC did not exhibit voltage-gated sodium channels at the prepulse potential of -40mV. Small outward currents activating at about -40 mV were seen (Figure 6.3.7). Neuronal transdifferentiated cells exhibit outwardly rectifying k^+ current, absence

of inward current at -40 and further depolarization. So, there is no Na^+ current seen (Figure 6.4.1). Second passage cells were labeled with lentiviral GFP (Figure 6.4.3) and then transplanted into injured spinal cord rat model. Before transplantation the cell viability was assessed by propidium iodide, only 3% of dead cells were quantified (Figure 6.4.4).

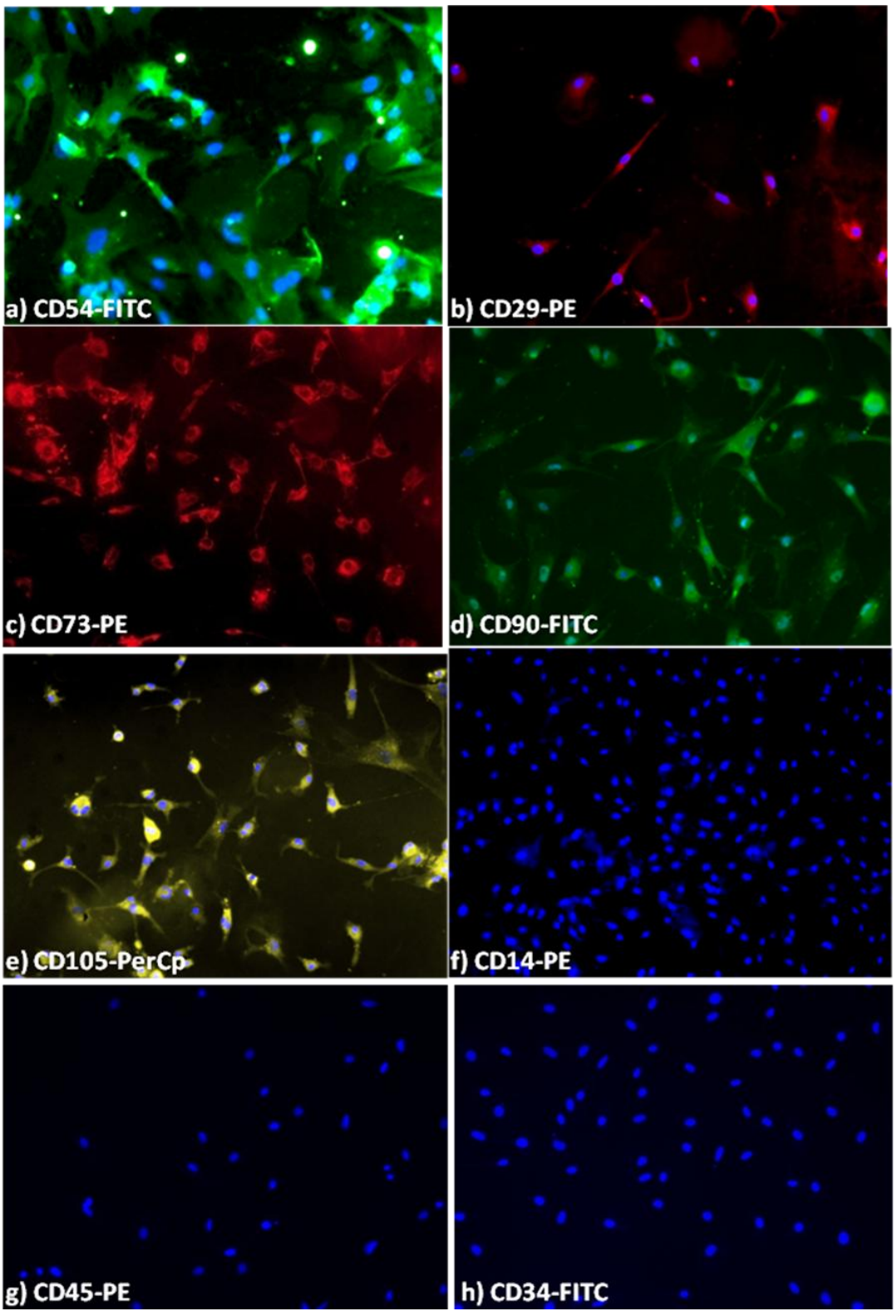

a) CD54-FITC
b) CD29-PE
c) CD73-PE
d) CD90-FITC
e) CD105-PerCp
f) CD14-PE
g) CD45-PE
h) CD34-FITC

Figure 6.2.7 Characterization of MSC by IHC shows positive for CD54 FITC, CD29 PE, CD73 PE, CD90 FITC, CD105 PerCp and negative for CD14 PE, CD45 PE, CD34 FITC.

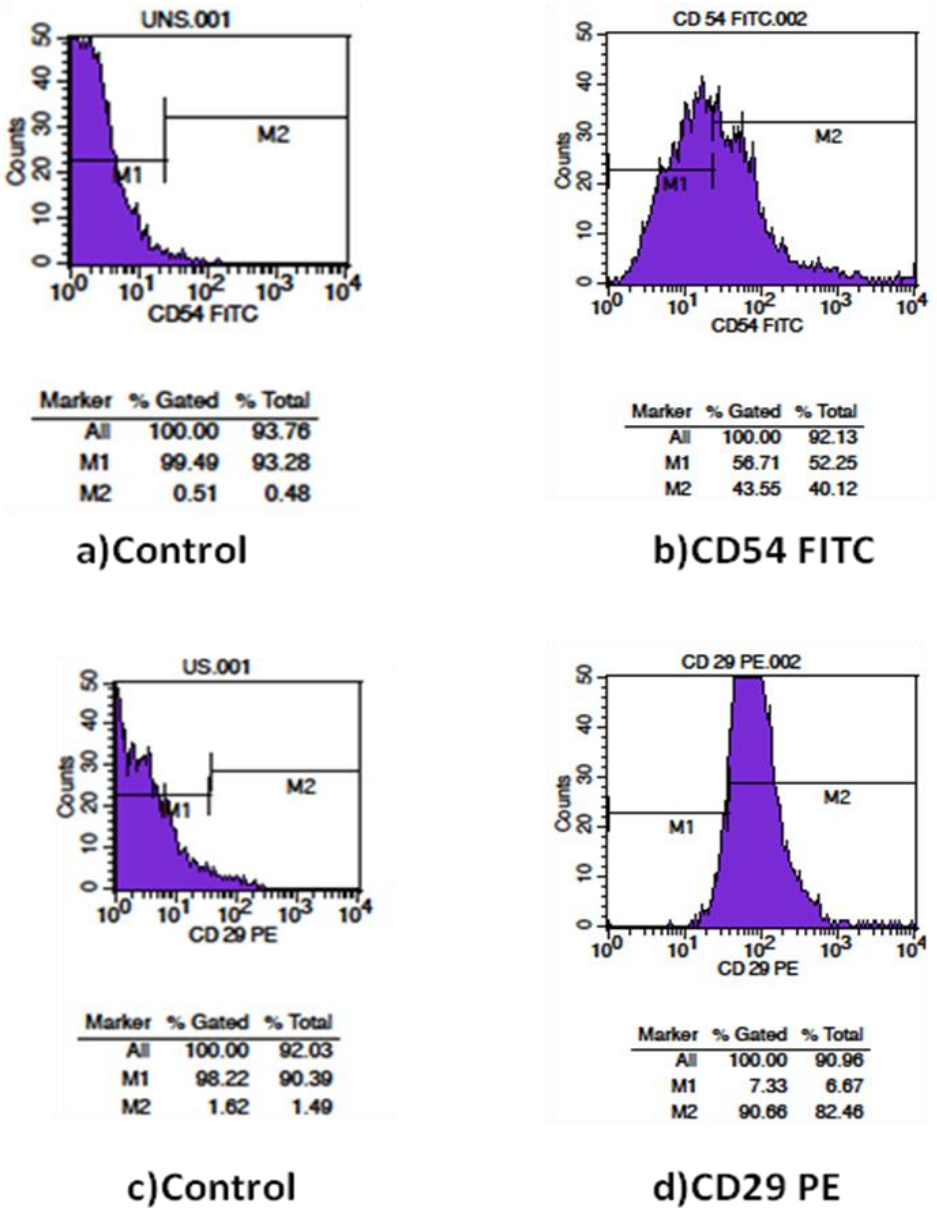
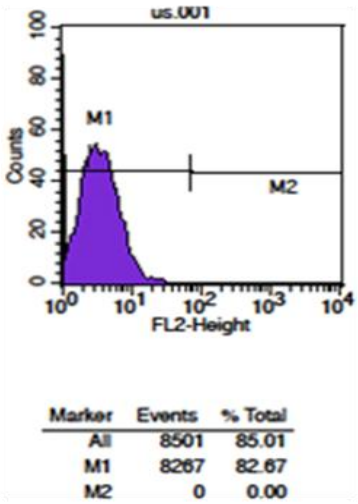
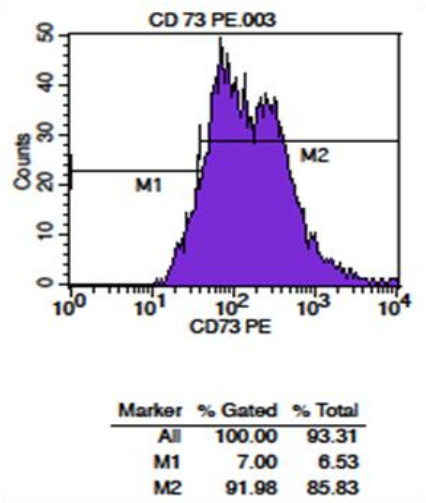


Figure 6.2.8 Flow cytometry analysis of MSC shows CD54-FITC positive of 40%, and CD29-PE positive of 82%.



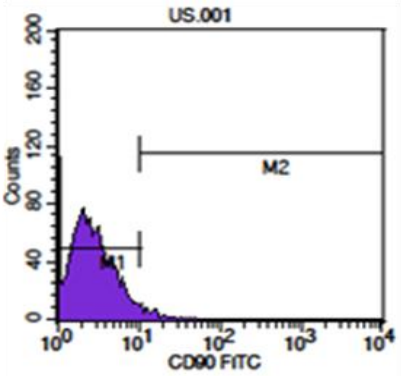
Marker	Events	% Total
All	8501	85.01
M1	8267	82.67
M2	0	0.00

e)Control



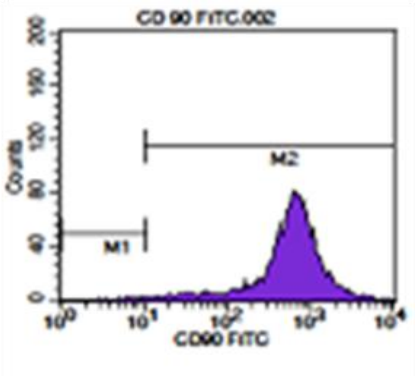
Marker	% Gated	% Total
All	100.00	93.31
M1	7.00	6.53
M2	91.98	85.83

f)CD73 PE



Marker	% Gated
All	100.00
M1	98.29
M2	1.76

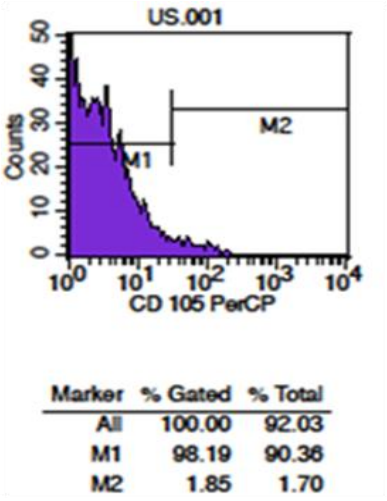
g)Control



Marker	% Gated
All	100.00
M1	0.26
M2	99.74

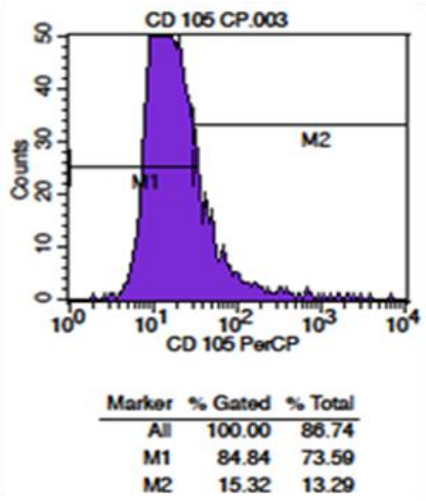
h)CD90 FITC

Figure 6.2.9 Flow cytometry analysis of MSC shows CD73-PE positive of 85%, and CD90-FITC positive of 99%.



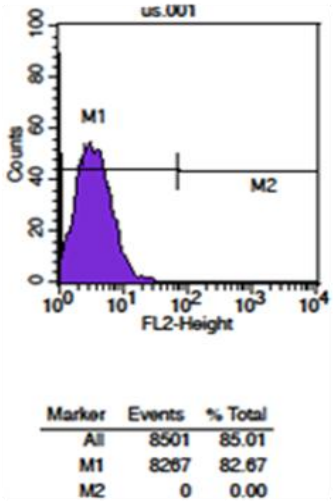
Marker	% Gated	% Total
All	100.00	92.03
M1	98.19	90.36
M2	1.85	1.70

i)Control



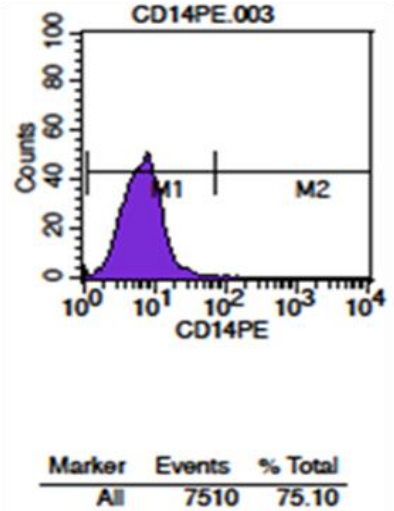
Marker	% Gated	% Total
All	100.00	86.74
M1	84.84	73.59
M2	15.32	13.29

j)CD105 PerCp



Marker	Events	% Total
All	8501	85.01
M1	8267	82.67
M2	0	0.00

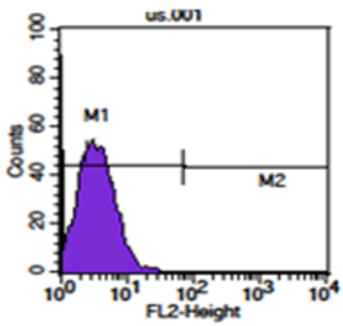
k)Control



Marker	Events	% Total
All	7510	75.10
M1	7449	74.49
M2	47	0.47

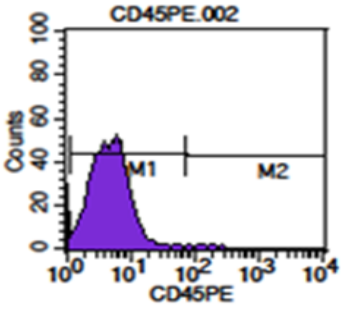
l)CD14 PE

Figure 6.3.0 Flow cytometry analysis of MSC shows CD105-PerCp positive of 14%, and CD14-PE positive of 0%.



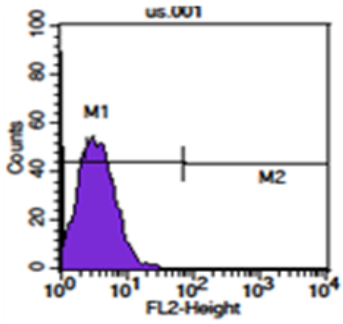
Marker	Events	% Total
All	8501	85.01
M1	8267	82.67
M2	0	0.00

m)Control



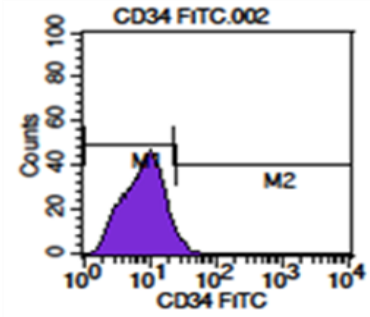
Marker	Events	% Total
All	8386	83.86
M1	8250	82.50
M2	63	0.63

n)CD45 PE



Marker	Events	% Total
All	8501	85.01
M1	8267	82.67
M2	0	0.00

o)Control



Marker	Events	% Total
All	7715	77.15
M1	7437	74.37
M2	223	2.23

p)CD34 FITC

Figure 6.3.1 Flow cytometry analysis of MSC shows CD45-PE positive of 0%, and CD34-FITC positive of 2%.

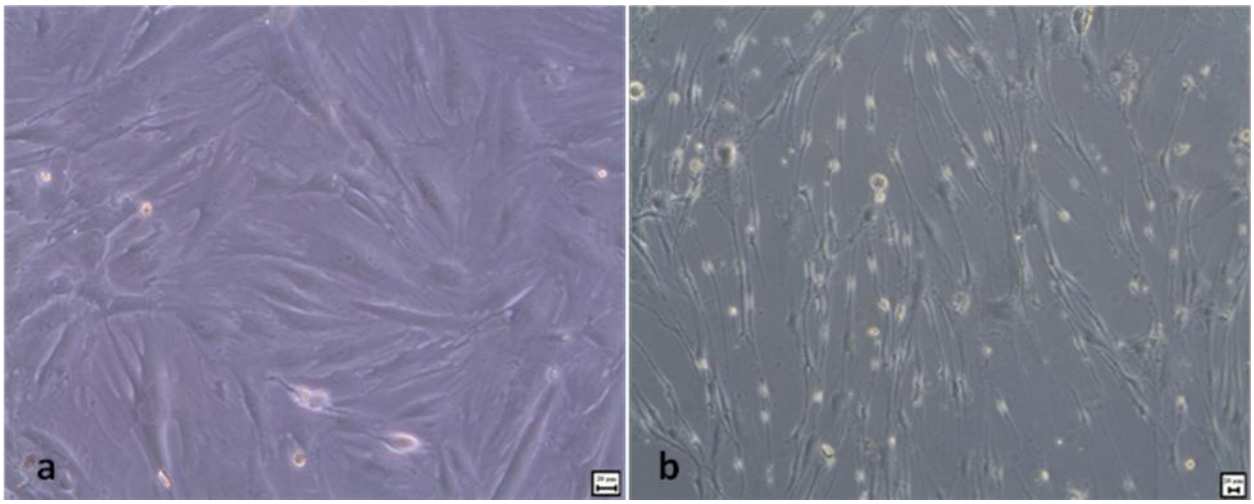


Figure 6.3.2 Before and after neuronal induction of MSC

(a) BMSC culture before neuronal induction.

(b) BMSC culture after neuronal incution, morphology changes from fibroblast to spindle shaped network.

Stem cells has the plasticity in morphological changes in invitro condition. On providing neuronal culture condition, the morphology changes from fibroblast-like to spindle shaped network. Morphology of spindle shaped cells taken on 12^{th} day post neuronal induction.

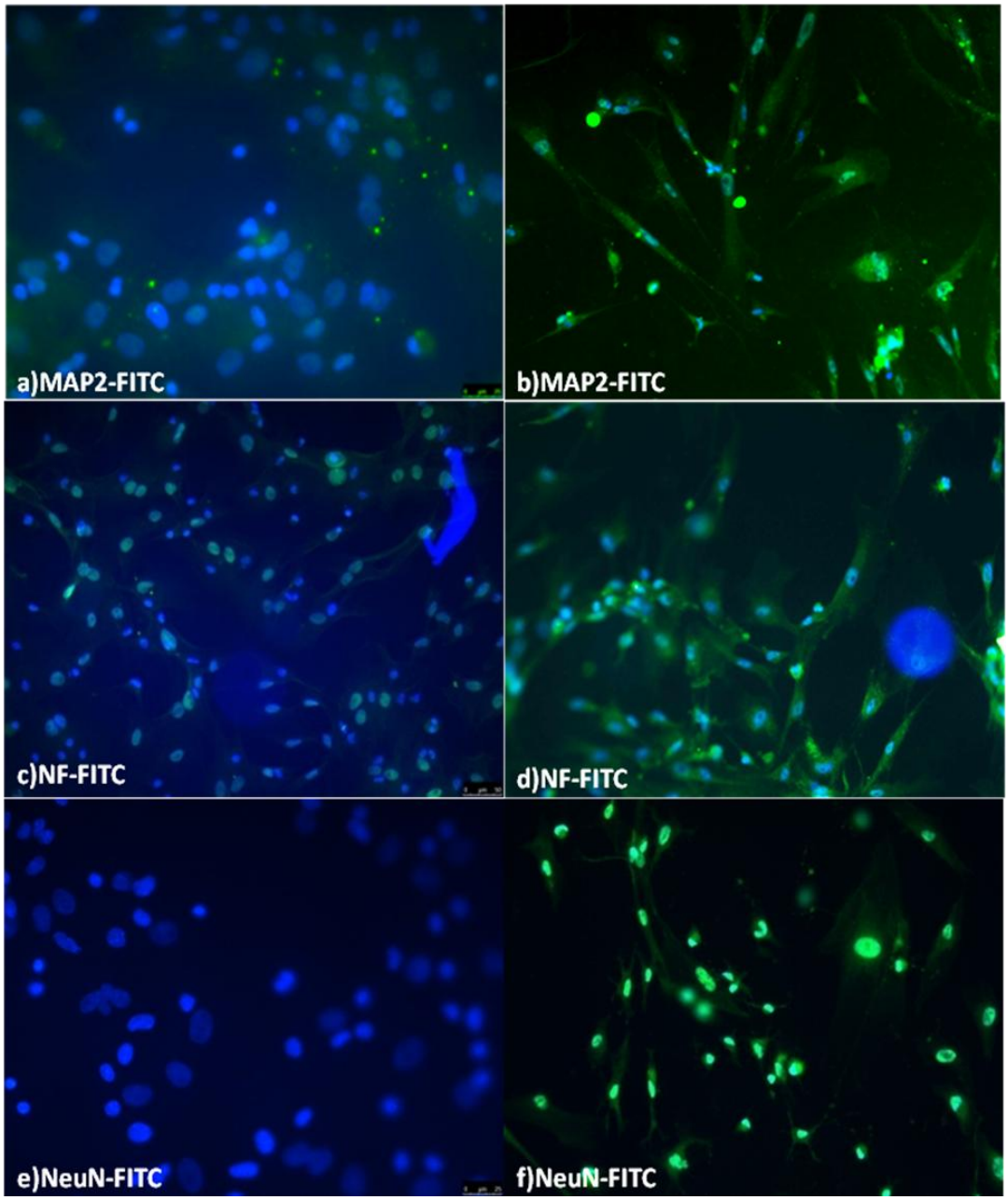


Figure 6.3.3 Characterization of before and after induction by IHC

MSC before neuronal induction shows negative for (a) MAP2-FITC, (c)NF-FITC, (e)NeuN-FITC. After neuronal induction shows positive for (b) MAP2-FITC, (d) NF-FITC, (F) NeuN-FITC.

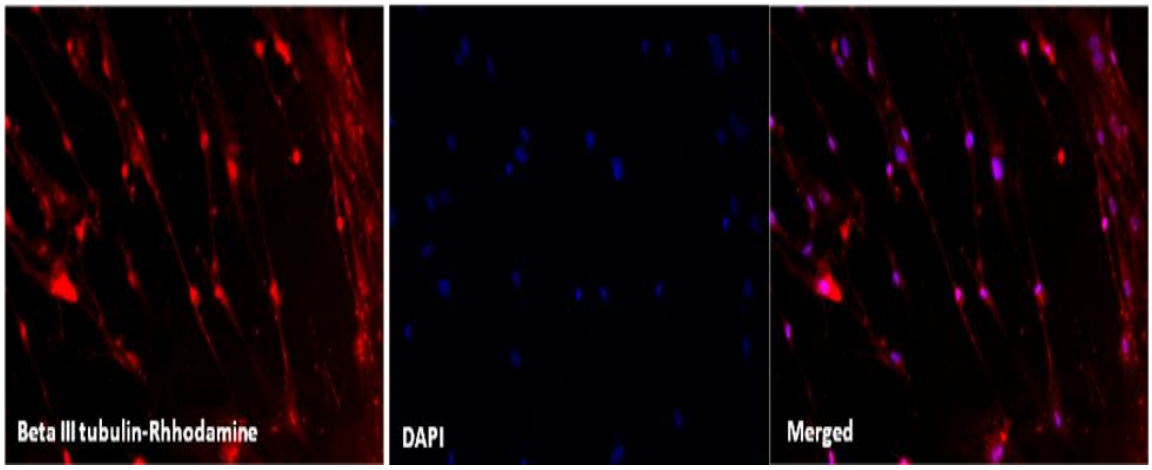


Figure 6.3.4 BMSC after neuronal induction shows positive for Beta III tubulin-Rhodamine.

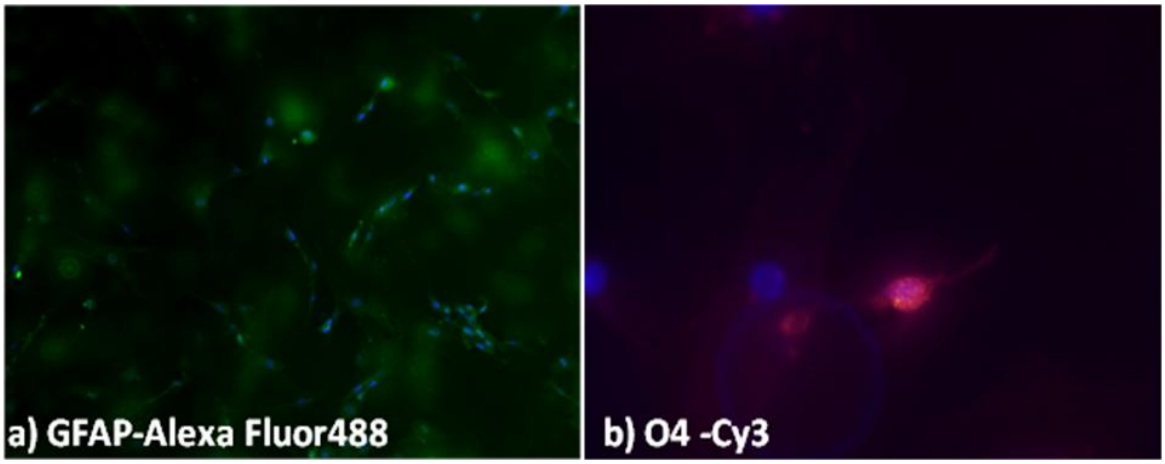


Figure 6.3.5 MSC after neuronal induction express glial marker,

GFAP(astrocyte marker) and O_4 (Oligodendrocyte marker)

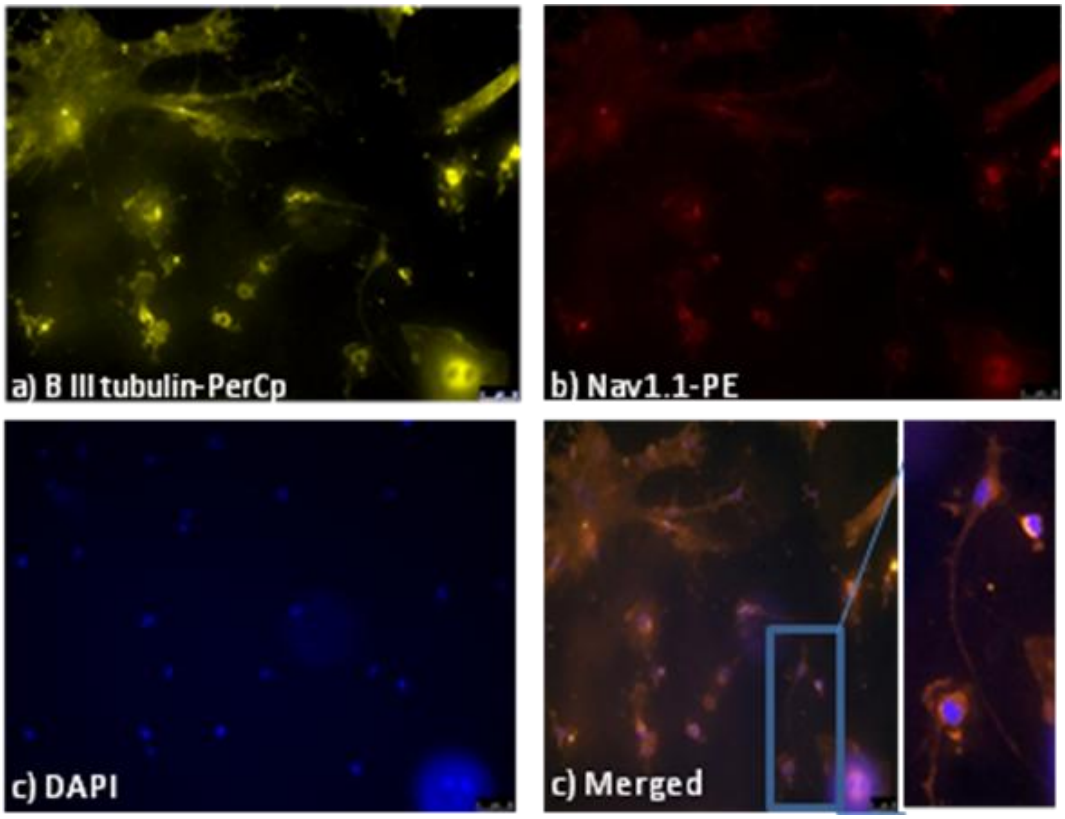


Figure 6.3.6 MSC after neuronal induction stained for β III tubulin-PerCp with Nav1.1-PE. Merged image shows β III tubulin-PerCp with Nav1.1-PE positive, which denotes neuron express voltage-gated sodium channel.

Patch-clamp studies

4 out of 4 mesenchymal stem cells in which it was possible to obtain giga seals did not exhibit voltage-gated sodium channels at the prepulse potential of -40mV. Small outward currents activating at about -40 mV were seen. The currents remained small in all 4 cells tested till about +40 mV. In two cells, using higher depolarizing test potentials (upto +180mV) the outward currents were recorded at reasonable magnitude. A representative tracing of this is shown in (figure 6.3.7)

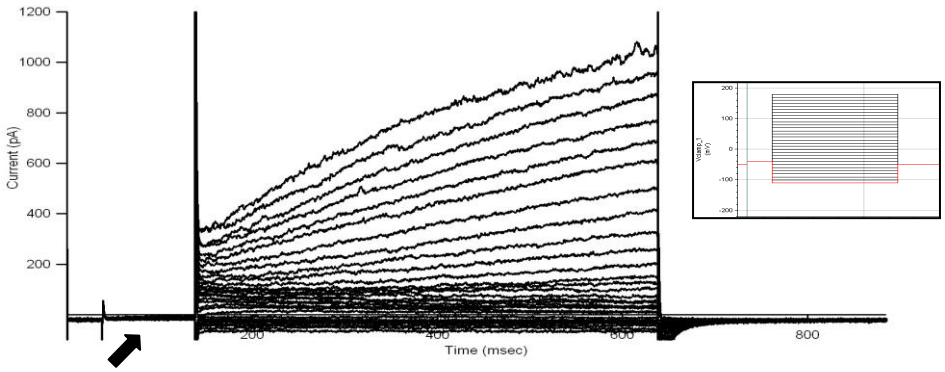


Figure 6.3.7 *Raw tracing from a mesechymal stem cell showing absence of inward currents at -40mV and presence of outward currents at higher depolarizing potentials. Note the absence of voltage-gated sodium currents at the prepulse of -40mV (shown by arrow) and the appearance of a family of depolarization-induced outward currents at depolarizing voltages. Voltage protocol shown in the inset:* V_{Hold} *= -50mV; Prepulse = -40mV; test pulses ranging from -110mV to +180mV, at 10mV increments. Offline filtering at 1kHz.*

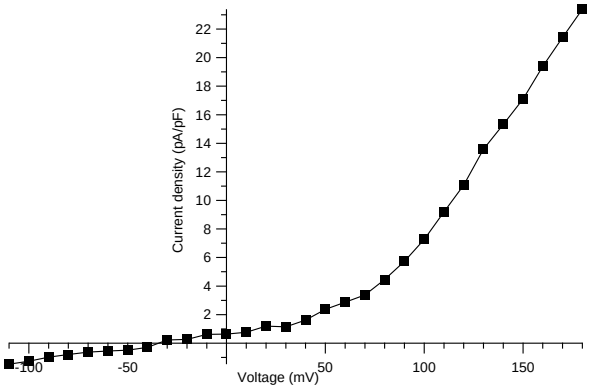


Figure 6.3.8 *The I-V curve generated using the recording shown in Figure 6.3.7. The currents were measured at end of the test pulse and presented here as current densities. The capacitance of the cell was 44.5pF.*

The profile of IV curves in both cells were higher depolarizing potentials were used, when normalized as current densities, showed very similar profiles.

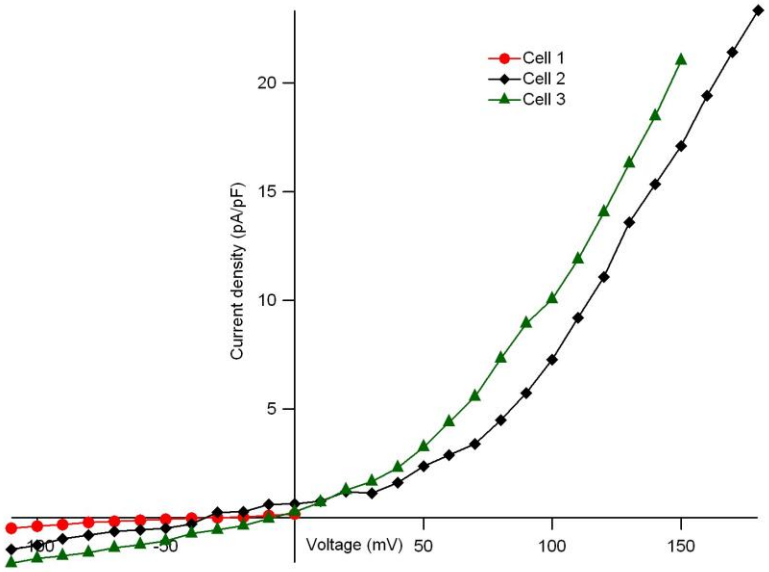


Figure 6.3.9 The I-V curves obtained from 3 different mesenchymal stem cells.

It has been reported that mesenchymal stem cells patched during second to fourth passage exhibit fast activating delayed rectifier currents in almost all cells (196). The profile of outward currents seen in our experiments is different from what is reported. While what is reported by Li et al (figure A), seems to be fast- activating delayed rectifier potassium channels, the currents that (B) recorded did not saturate and increased till the end of the pulse, though the duration of the pulse was longer than that of Li *et al.*

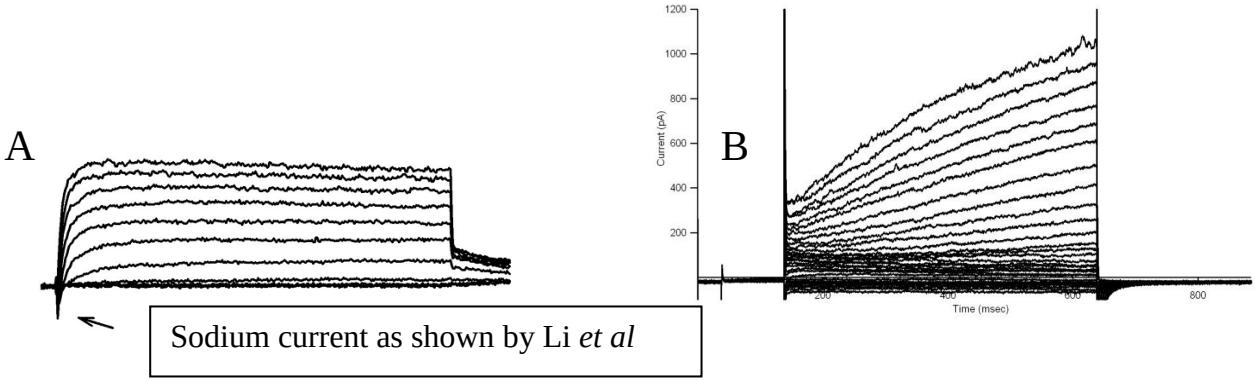


Figure : (A) Outward currents reported by Li et al (196). *The pulse duration was 300 msec in this case. (B) Outward currents obtained from the rat mesechymal stem cells in our lab (pulse duration 500 msec).*

In 4 out of 4 cells that were patched, inward sodium or calcium currents were not observed. However Li *et al* (196) have reported that 19% of rat MSCs had voltage-gated sodium currents and 8% had voltage-gated calcium currents. Mesenchymal stem cells from different days after the second passage express fast- activating delayed rectifier potassium channel and 12 day after neuronal induction, the profile of outward currents was observed, all patched cells did not express inward sodium or calcium currents. Voltage-gated sodium channel expression will be confirmatory for functional excitable cell neurons.

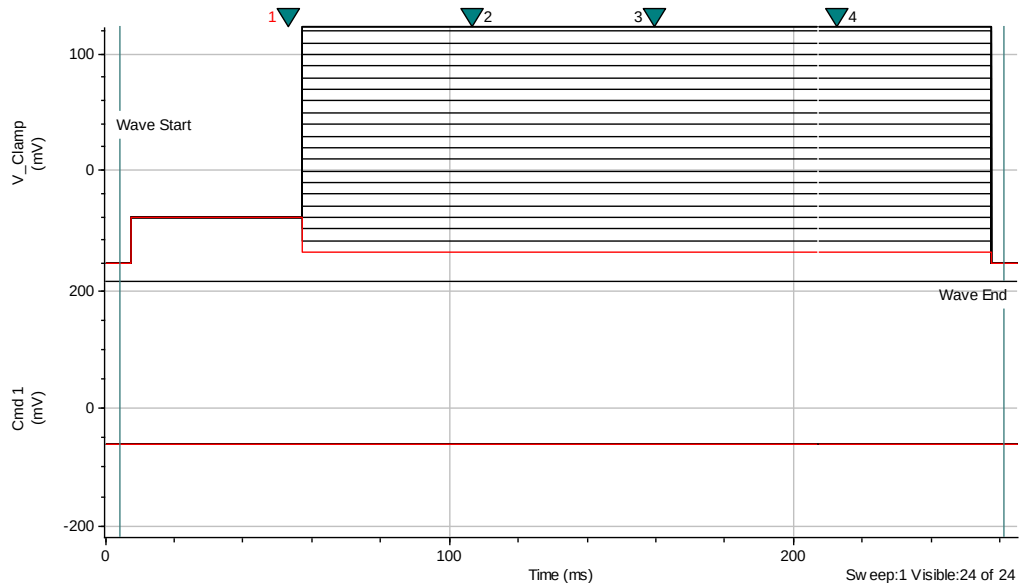


Figure 6.4.0 Family of raw current tracings. Voltage clamp protocol: V_{Hold} = -80mV; pre-step = -40mV; test pulses range from -70mV to +120mV in 10mV increments.

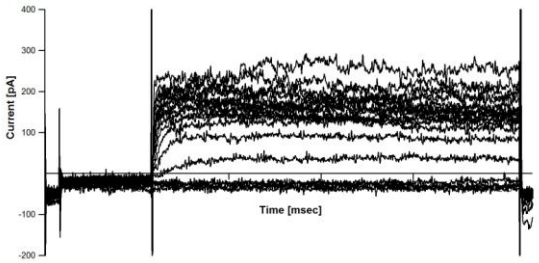


Figure 6.4.1 Outwardly rectifying k^+ current, absence of inward current at -40 and further depolarization. There is no Na^+ current seen in invitro neuronal induced mesenchymal stem cells.

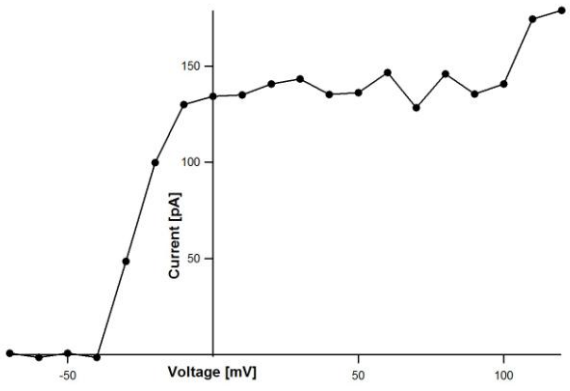


Figure 6.4.2 I-V relationship of the outwardly rectifying currents

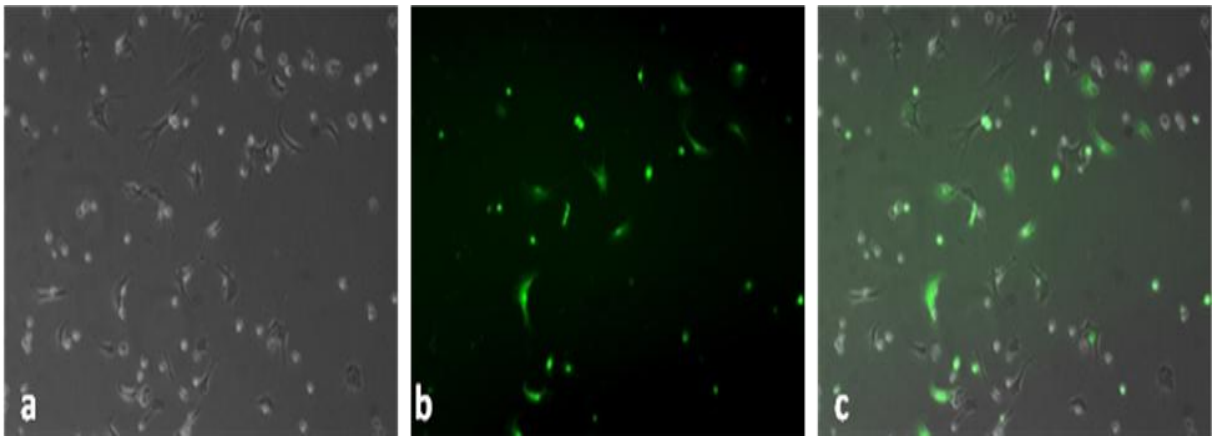


Figure 6.4.3 MSC transduced with lentiviral-GFP
(a) Culture, (b) GFP labelled cells, (c) Merged

To tract the transplanted cells surviving in the cord, the cells were labeled with lentiviral GFP and then transplanted on the 9^{th} day following spinal cord injury. The labeled cells express GFP.

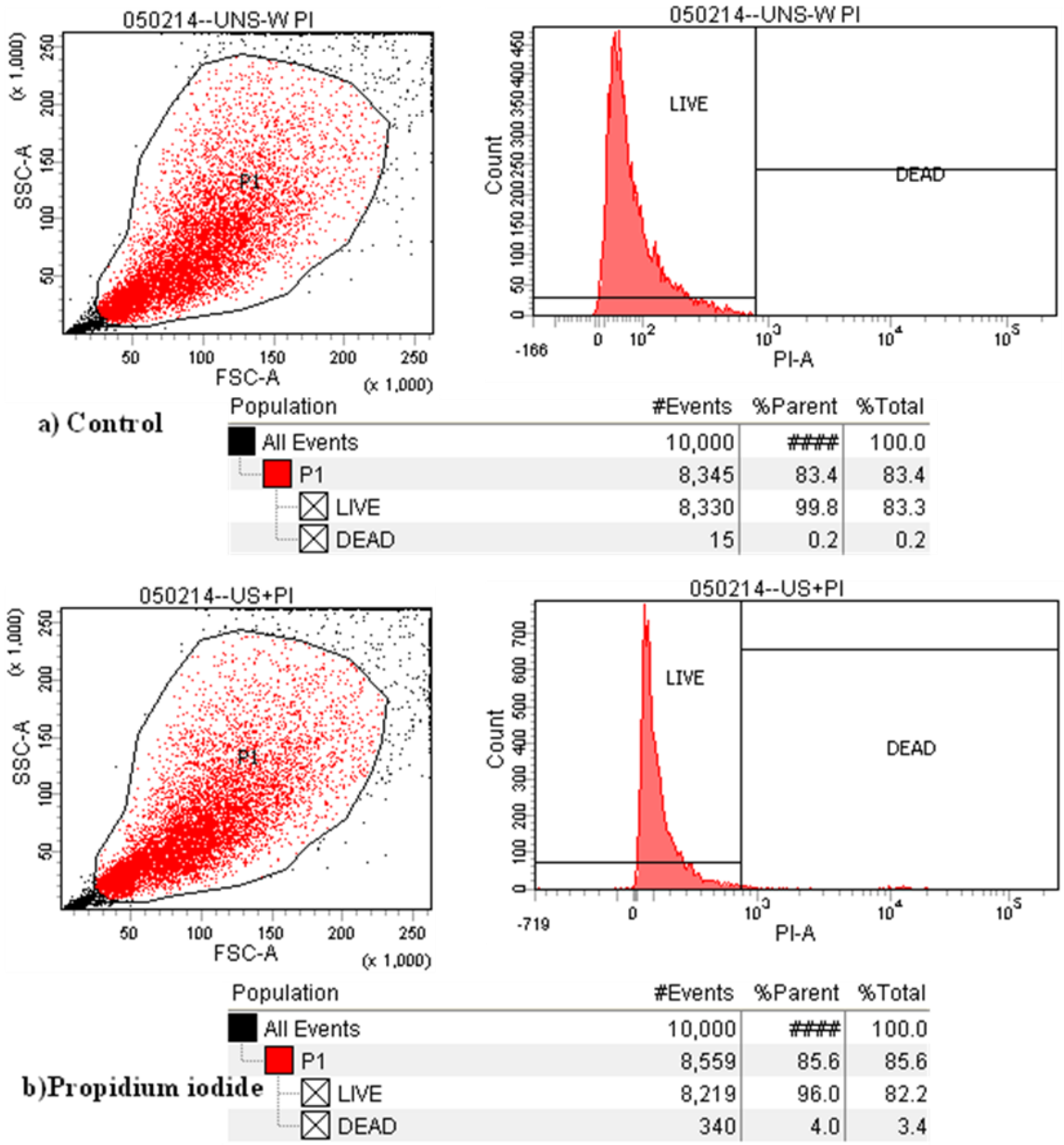


Figure 6.4.4 Viability of Bone marrow mesenchymal stem cell

a)Control, b) Propidium iodide stained cells of 3% (Dead cells).

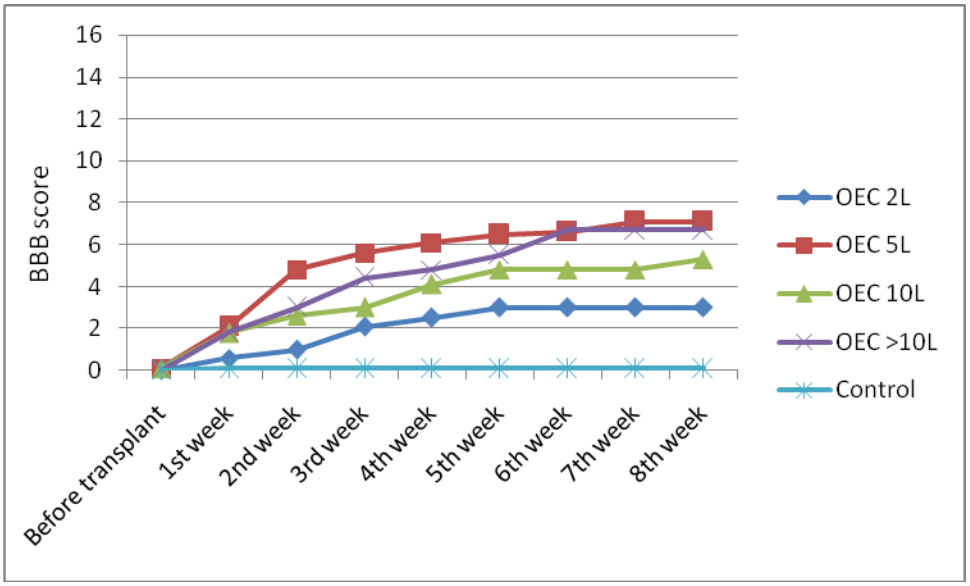
BBB score

Table 6.5.1 Mean BBB score after different dosage of OEC transplantation

Cell/ Dosage	Sample size	BBB before transplant (9th day after SCI)	BBB score after transplant in weeks							
			1st	2nd	3rd	4th	5th	6th	7th	8th
OEC 2L	n=6	0	0.6	1	2.1	2.5	3	3	3	3
OEC 5L	n=6	0.1	2.1	4.8	5.6	6.1	6.5	6.6	7.1	7.1
OEC 10L	n=6	0.1	1.8	2.6	3	4.1	4.8	4.8	4.8	5.3
OEC >10L	n=6	0	1.8	3	4.4	4.8	5.5	6.7	6.7	6.7
Control	n=11	0	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09

The results of different dosage of OEC transplants has been shown in (Table 6.5.1). 2 lakh of OEC transplanted rats (n=6) progressed to mean BBB score of 3.Whereas 10 lakh cells transplanted rats (n=6) scored 5.3. However 5 lakh treated rats (n=6) showed better motor recovery of 7.1. Although more than 10 lakh cells injected rats (n=6) improved to 6.7 in BBB score.

Graph 6.5.2 Mean BBB score after different dosage of OEC transplantation



(Graph 6.5.2) shows sequential recovery from 1st week post-transplant onwards and after 6th week attains plateau phase in motor score. As per dose, 2 lakh group (n=6) shows minimum recovery as compared to other group. OEC 10 lakh group (n=6) declines in motor recovery as compared to 5 lakh and more than 10 lakh group.

Table 6.5.3 Statistical analysis of BBB score after different dosage of OEC transplantation

OEC 2L (n=6)		OEC 5L (n=6)		OEC 10L (n=6)		OEC >10L (n=6)		Control (n=11)		P value
Mean	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	
3.00	2.190	7.16	2.562							0.021
3.00	2.190			5.33	2.503					0.375
3.00	2.190					7.16	3.371			0.021
3.00	2.190							0.090	0.301	0.096
		7.16	2.562	5.33	2.503					0.608
		7.16	2.562			7.16	3.371			1.000
		7.16	2.562					0.090	0.301	0.000
				5.33	2.503	7.16	3.371			0.608
				5.33	2.503			0.090	0.301	0.001
						7.16	3.371	0.090	0.301	0.000

In comparison of OEC 2 lakh (3.0±2.190) with OEC 5 lakh transplant group (7.1±2.562) shows significant (P=0.02) in motor recovery. Low dose of 2 lakh OEC (3.0±2.190) with high dose of more than 10 lakh of OEC (7.1±3.371) treated rats (n=6) has significant difference (P=0.02). Between 5 lakh (7.1±2.562) and 10 lakh (5.3±2.503) cell injected has no significant difference (P=0.608). There is no statistical difference between 5 lakh(7.1±2.562) and more than 10 lakh cells treated (7.1±3.371) group, where P=1.00. But in control (0.09±0.301) versus 5 lakh of OEC (7.1±2.562) shows highly significant (P=0.00). In higher dose group more than 10 lakh of OEC (7.1±3.371) and 10 lakh of OEC(5.3±2.503) shows no difference (P=0.608). However, on comparison of OEC 10 lakh (5.3±2.503) with control (0.09±0.301) shows much difference in hind limb motor recovery (P=0.001). Even on comparison control (0.09±0.301) with more than 10 lakhs proves remarkable difference (P=0.00).

Table 6.5.4 Statistical analysis of BBB score before and after different dosage of OEC transplantation

Cell/ Dosage	Sample size	Before transplant		After transplant		P value
		Mean	Std Dev	Mean	Std Dev	
OEC 2L	n=6	0.000	0.000	3.000	2.190	0.042
OEC 5L	n=6	0.1667	0.408	7.166	2.562	0.027
OEC 10L	n=6	0.1667	0.408	5.330	2.503	0.027
OEC >10L	n=6	0.000	0.000	7.166	3.371	0.027

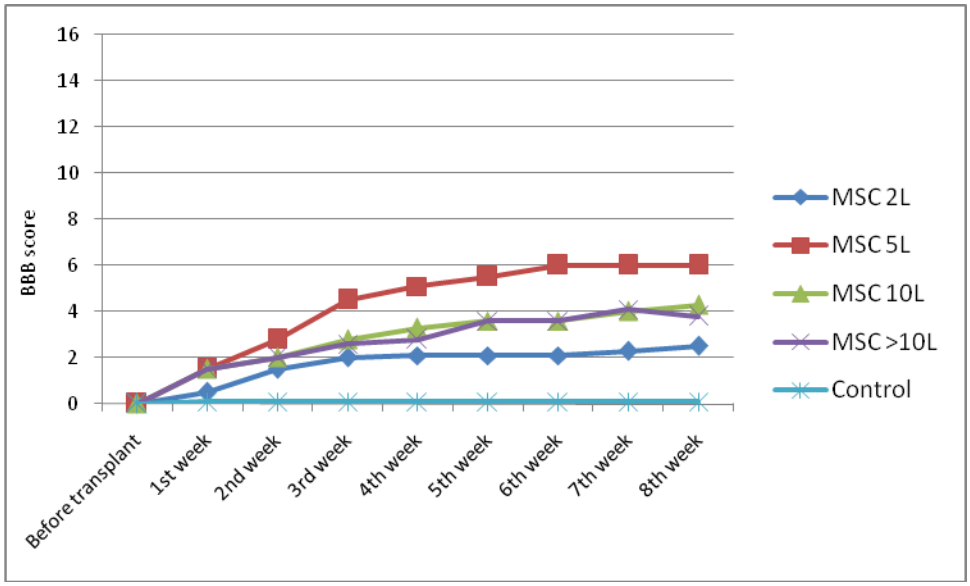
All the rats which received OEC transplantation after spinal cord injury with different dosages (n=6) in each group shows significant ($P < 0.05$) in motor recovery BBB score, when compared to before transplantation (after spinal cord injury) as shown in (Table 6.5.4).

Table 6.5.5 Mean BBB score after different dosage of MSC transplantation

Cell/ Dosage	Sample size	BBB before transplant (9^{th} day after SCI)	BBB score after transplant in weeks							
			1^{st}	2^{nd}	3^{rd}	4^{th}	5^{th}	6^{th}	7^{th}	8^{th}
MSC 2L	n=6	0	0.5	1.5	2	2.1	2.1	2.1	2.3	2.5
MSC 5L	n=6	0	1.5	2.8	4.5	5.1	5.5	6	6	6
MSC 10L	n=6	0	1.5	2	2.8	3.3	3.6	3.6	4	4.3
MSC >10L	n=6	0	1.5	2	2.6	2.8	3.6	3.6	4.1	3.8
Control	n=11	0	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09

The results of different dosage of mesenchymal stem cell as shown in (Table 6.5.5). In 5 lakhs treated rats (n=6) achieved the maximum mean BBB score of 6, in comparison to other groups. Low dosage 2 lakh and high dosage more than 10 lakh shows decline in recovery with that of 5 lakh and 10 lakh cells treated. However, in comparison to control group (n=11), all the treated group as beneficial therapeutic effects.

Graph 6.5.6 Mean BBB score after different dosage of MSC transplantation



Graph shows control group does not improve in motor score even on duration increases. But in treated group gradually progress and attains plateau around 6th to 7th weeks. As per dose response 2 lakh cell group (n=6) retains lowest recovery among all groups. Increasing in the dosage of MSC shows decline in the BBB as shown in (Graph 6.5.6).

Table 6.5.7 Statistical analysis of BBB score after different dosage of MSC transplantation

MSC 2L (n=6)		MSC 5L (n=6)		MSC 10L (n=6)		MSC >10L (n=6)		Control (n=11)		P value
Mean	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	
2.50	1.378	6.00	2.756							0.065
2.50	1.378			4.33	3.141					0.597
2.50	1.378					3.83	2.994			0.825
2.50	1.378							0.090	0.301	0.216
		6.00	2.756	4.33	3.141					0.679
		6.00	2.756			3.83	2.994			0.437
		6.00	2.756					0.090	0.301	0.000
				4.33	3.141	3.83	2.994			0.994
				4.33	3.141			0.090	0.301	0.005
						3.83	2.994	0.090	0.301	0.016

Although same cells with different dosage as different effect in motor recovery, but on statistical analysis there is no significant difference among the groups. 2 lakh of MSC (2.5±1.378) compared with MSC 5 lakh cells (6.0±2.756) shows no difference (P=0.065). Even with 10 lakh group (4.3±3.141) versus 2 lakh (2.5±1.378) there is no much difference (P=0.597). Low dose 2 lakh (2.5±1.378) compared with higher dose of more than 10 lakh (3.8±2.994) has no significant difference (P=0.825). 5 lakh treated rats (6.0±2.756) has remarkable difference (P=0.00) on comparison with control (0.09±0.301). 10 lakh transplant rats (4.3±3.141) proves significant (P=0.005) with that of control (0.09±0.301). Highest dose more than 10 lakh (3.8±2.994) supports motor recovery P=0.016, to that of untreated control rats (0.09±0.301).

Table 6.5.8 Statistical analysis of BBB score before and after different dosage of MSC transplantation

Cell/ Dosage	Sample size	Before transplant		After transplant		P value
		Mean	Std Dev	Mean	Std Dev	
MSC 2L	n=6	0.00	0.00	2.500	1.378	0.026
MSC 5L	n=6	0.00	0.00	6.00	2.756	0.027
MSC 10L	n=6	0.00	0.00	4.333	3.141	0.026
MSC >10L	n=6	0.00	0.00	3.833	2.994	0.042

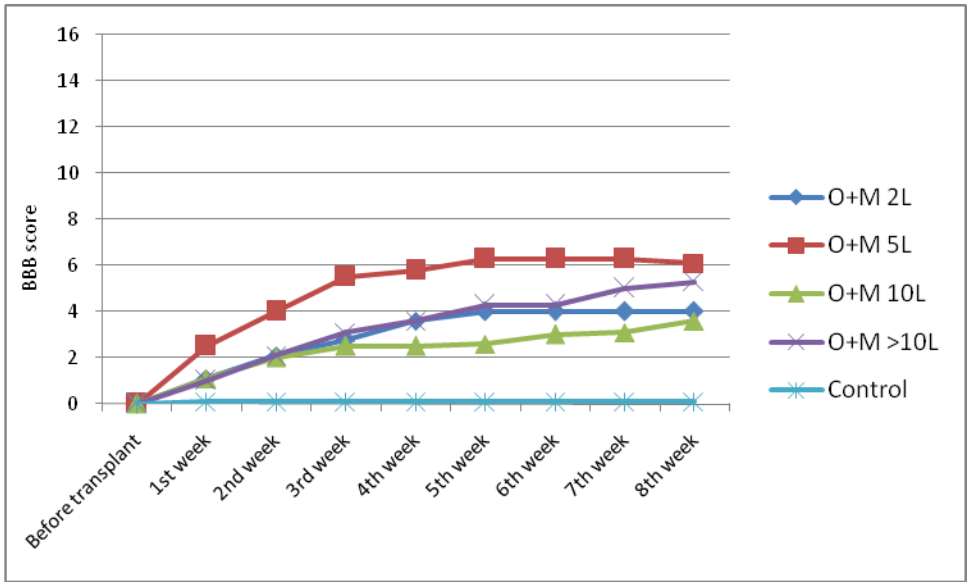
All the rats which received MSC transplantation after spinal cord injury with different dosages (n=6) in each group shows significant ($P< 0.05$) in motor recovery BBB score, when compared to before transplantation (after spinal cord injury) as shown in (Table 6.5.8).

Table 6.5.9 Mean BBB score after different dosage of OEC + MSC (1:1) transplantation

Cell/ Dosage	Sample size	BBB before transplant (9^{th} day after SCI)	BBB score after transplant in weeks							
			1^{st}	2^{nd}	3^{rd}	4^{th}	5^{th}	6^{th}	7^{th}	8^{th}
OEC+MSC(2L)	n=6	0	1.1	2.1	2.8	3.6	4	4	4	4
OEC+MSC(5L)	n=6	0	2.5	4	5.5	5.8	6.3	6.3	6.3	6.1
OEC+MSC(10L)	n=6	0	1.1	2	2.5	2.5	2.6	3	3.1	3.6
OEC+MSC(>10L)	n=6	0	1	2.1	3.1	3.6	4.3	4.3	5	5.3
Control	n=11	0	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09

The results of different dosage of OEC combined with MSC as shown in (Table 6.5.9). SCI rats (n=6) injected 2 lakh and 10 lakh cells recovered to 4 and 3.6 respectively in BBB score, whereas 5 lakh cells treated rats (n=6) improved to the maximum of 6.1. In comparison to control group, all other treated groups showed promising effects.

Graph 6.6.0 Mean BBB score after different dosage ofOEC+MSC(1:1) transplantation



(Graph 6.6.0) shows before transplant all the rats are paraplegic, with no hindlimb movement, but on 9th day transplanted after SCI, first week after transplant all rats slowly progressed and attains plateau at 7th to 8th week. Untreated rats, there is no spontaneous recovery still persist in baseline.

Table 6.6.1 Statistical analysis of BBB score after different dosage of OEC+MSC transplantation

O+M 2L (n=6)		O+M 5L (n=6)		O+M 10L (n=6)		O+M >10L (n=6)		Control (n=11)		P value
Mean	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	
4.00	2.828	6.16	4.622							0.681
4.00	2.828			3.66	3.559					1.000
4.00	2.828					5.33	2.503			0.925
4.00	2.828							0.090	0.301	0.076
		6.16	4.622	3.66	3.559					0.556
		6.16	4.622			5.33	2.503			0.986
		6.16	4.622					0.090	0.301	0.002
				3.66	3.559	5.33	2.503			0.846
				3.66	3.559			0.090	0.301	0.123
						5.33	2.503	0.090	0.301	0.008

Two different cells has different properties in regeneration, so combined the cells and looking for higher recovery. 2 lakh cells combination (OEC+MSC) group (4.0±2.828) has no recovery difference (P=1.00) with 10 lakh OEC+MSC group (3.6±3.559). No significant difference (P=0.925) between 2 lakh OEC+MSC (4.0±2.828) and more than 10 lakh OF OEC+MSC (5.3±2.503) groups. P=0.008 was achieved between more than 10 lakh of OEC +MSC (5.3±2.503) and control (0.09±0.301). Similar significant (P=0.002) found in 5 lakh of OEC+MSC (6.1±4.622) versus control (0.09±0.301). There is no statistical differences in motor recovery BBB score among doses, but there is a remarkable difference seen in treated versus control as shown in (Table 6.6.1).

Table 6.6.2 Statistical analysis of BBB score before and after different dosage of OEC+MSC transplantation

Cell/ Dosage	Sample size	Before transplant		After transplant		P value
		Mean	Std Dev	Mean	Std Dev	
O+M 2L	n=6	0.00	0.00	4.00	2.828	0.042
O+M 5L	n=6	0.00	0.00	6.166	4.622	0.028
O+M 10L	n=6	0.00	0.00	3.666	3.559	0.026
O+M >10L	n=6	0.00	0.00	5.333	2.503	0.026

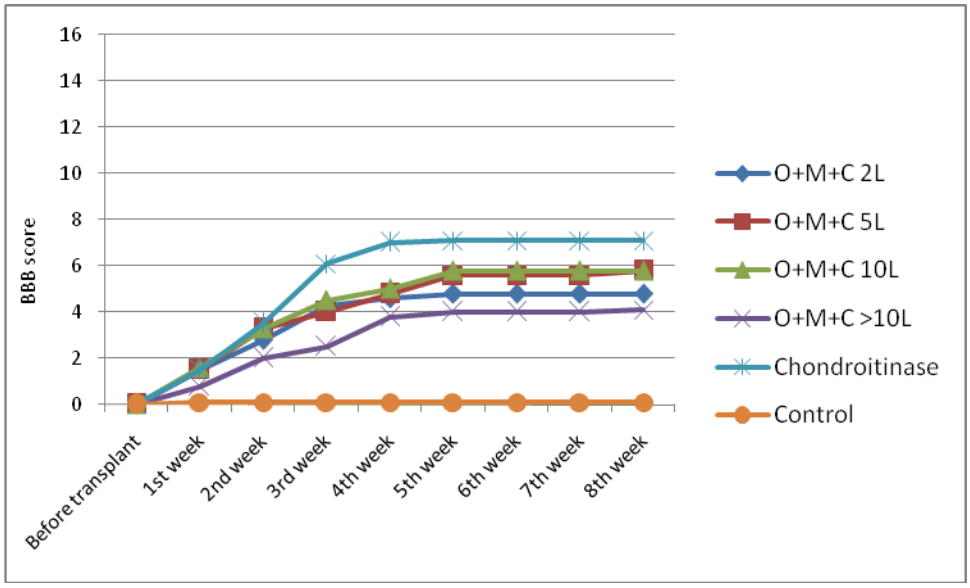
All the rats which received OEC+MSC(1:1) transplantation after spinal cord injury with different dosages (n=6) in each group shows significant ($P< 0.05$) in motor recovery BBB score, when compared to before transplantation (after spinal cord injury) as shown in (Table 6.6.2).

Table 6.6.3 Mean BBB score after different dosage of OEC+MSC+Chondroitinase (1:1+0.2U) transplantation

Cell/ Dosage/ enzymes	Sample size	BBB before transplant (9th day after SCI)	BBB score after transplant in weeks							
			1st	2nd	3rd	4th	5th	6th	7th	8th
OEC+MSC+ Chondroitinase (2L+0.2U)	n=6	0	1.5	2.8	4.3	4.6	4.8	4.8	4.8	4.8
OEC+MSC+ Chondroitinase (5L+0.2U)	n=6	0	1.5	3.3	4	4.8	5.6	5.6	5.6	5.8
OEC+MSC+ Chondroitinase (10L+0.2U)	n=6	0	1.6	3.3	4.5	5	5.8	5.8	5.8	5.8
OEC+MSC+ Chondroitinase (>10L+0.2U)	n=6	0	0.8	2	2.5	3.8	4	4	4	4.1
Chondroitinase (0.2U)	n=6	0	1.5	3.5	6.1	7	7.1	7.1	7.1	7.1
Control	n=11	0	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09

The results of different dosage of OEC combined with MSC and chondroitinase as shown in (Table 6.6.3). 5 lakh and 10 lakh cells with chondroitinase treated rats (n=6) progressed to the motor recovery score of 5.8. Both 2 lakh and more than 10 lakh cells combined with enzyme treated rats as mild recovery of 4.8 and 4.1 respectively. However, Chondroitinase alone group achieved maximum recovery of 7.1 in BBB score.

Graph 6.6.4 Mean BBB score after different dosage of OEC+MSC+Chondroitinase (1:1+0.2U) transplantation



(Graph 6.6.4) shows sequential hindlimb motor BBB score from 0 to 7. Upto 5th week post transplant rats progressed and later attain stable phase. Control rats stayed in the baseline without any BBB improvement. Both 5 lakh and 10 lakh group obtained same motor recovery, though the cell dose differs. Chondroitinase alone proves best than with cell combinations.

Table 6.6.5 Statistical analysis of BBB score after different dosage of OEC+MSC+Chondroitinase/ Chondroitinase transplantation

O+M+C 2L (n=6)		O+M+C 5L (n=6)		O+M+C 10L (n=6)		O+M+C >10L (n=6)		Chondroitinase (n=6)		Control (n=11)		P value
Mean	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	
4.83	2.316	5.83	3.816									0.994
4.83	2.316			5.83	4.215							0.994
4.83	2.316					4.16	5.231					0.999
4.83	2.316							7.16	2.483			0.801
4.83	2.316									0.09	0.301	0.061
		5.83	3.816	5.83	4.215							1.000
		5.83	3.816			4.16	5.231					0.943
		5.83	3.816					7.16	2.483			0.978
		5.83	3.816							0.09	0.301	0.013
				5.83	4.215	4.16	5.231					0.943
				5.83	4.215			7.16	2.483			0.978
				5.83	4.215					0.09	0.301	0.013
						4.16	5.231	7.16	2.483			0.587
						4.16	5.231			0.09	0.301	0.148
								7.16	2.483	0.09	0.301	0.001

Although all transplant groups shows clinically promising outcome with that of control group. However on statistical basis there is no significant difference among transplant groups. Control (0.09±0.301) compared with chondroitinase alone (7.1±2.483) shows both clinically and statistically significant (P=0.001). Another group of 5 lakh combination (5.8±3.816) versus control (0.09±0.301) supports $P<0.05$ that is P=0.013. Similarly like 5 lakh cells group, 10 lakh group (5.8±4.215) has significant difference (P=0.013) with that of control (0.09±0.301). As shown in(Table 6.6.5) rest other group does not show statistical significant.

Table 6.6.6 Statistical analysis of BBB score before and after different dosage of OEC+MSC+Chondroitinase/ Chondroitinase transplantation transplantation

Cell/ Dosage	Sample size	Before transplant		After transplant		P value
		Mean	Std Dev	Mean	Std Dev	
O+M+C 2L	n=6	0.00	0.00	4.833	2.316	0.027
O+M +C 5L	n=6	0.00	0.00	5.833	3.816	0.039
O+M +C 10L	n=6	0.00	0.00	5.833	4.2	0.027
O+M+C >10L	n=6	0.00	0.00	4.166	5.231	0.066
Chondroitinase	n=6	0.00	0.00	7.166	2.483	0.026

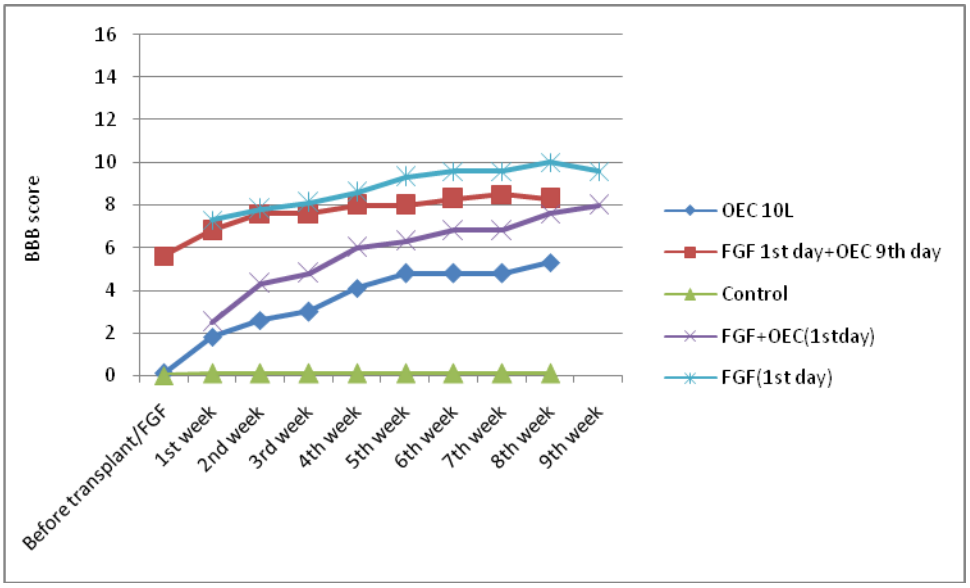
All the rats which received OEC+MSC+Chondroitinase (1:1+0.2U)/Chondroitinase (0.2U) transplantation after spinal cord injury with different dosages (n=6) in each group shows significant ($P< 0.05$) in motor recovery BBB score, when compared to before transplantation (after spinal cord injury) as shown in (Table 6.6.6).

Table 6.6.7 Mean BBB score after FGF/FGF+OEC/OEC transplantation

Cell/ Dosage / Growth factor	Sample size	BBB before transplant (9th day after SCI) /FGF	BBB score after transplant in weeks								
			1st	2nd	3rd	4th	5th	6th	7th	8th	9th
OEC 10L	n=6	0.1	1.8	2.6	3	4.1	4.8	4.8	4.8	5.3	
Control	n=11	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	
FGF(1st day) + OEC 10L (9th day)	n=6	5.6	6.8	7.6	7.6	8	8	8.3	8.5	8.3	
FGF(1st day)	n=6		7.3	7.8	8.1	8.6	9.3	9.6	9.6	10	9.6
FGF+OEC 10L (1st day)	n=6		2.5	4.3	4.8	6	6.3	6.8	6.8	7.6	8

The results of different groups of growth factor with and without OEC as shown in (Table 6.6.7). Fibroblast growth factor injected into the injured cord immediate after spinal cord injury (i.e) on the first day. FGF alone treated group (n=6) attains highest in motor recovery BBB score of 9.6. Both FGF combined with OEC 10 lakh cells injected on the first day shows BBB of 8 and FGF on first day treated with again OEC 10 lakh cells on 9th day group (n=6) improves to 8.3 in score. OEC 10 lakh cells on 9th day after SCI has progressed to 5.3.

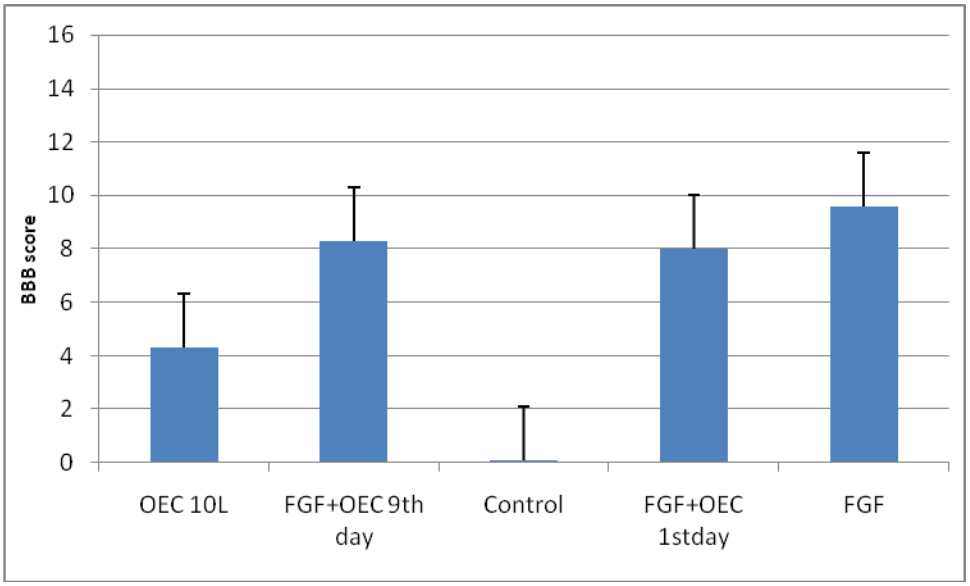
Graph 6.6.8 Mean BBB score after OEC/FGF/ FGF+OEC transplantation



(Graph 6.6.8) shows control group has not improved in BBB, but other group does. OEC alone group shows sequential recovery from 0 to 5.3, whereas FGF alone/FGF+OEC shows acute recovery because, FGF given immediate after spinal cord injury. The recovery is due to inhibition of secondary inflammatory cascade, which shows neuroprotective effect of acidic FGF.

Table 6.6.9 statistical analysis of BBB score after OEC/FGF/ FGF+OEC transplantation

OEC 10L (n=6)		FGF (n=6)		OEC+FGF (1) (n=6)		OEC(1)+FGF(9) (n=6)		Control (n=11)		P value
Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	
5.33	2.503	9.66	5.006							0.134
5.33	2.503			8.00	4.516					0.571
5.33	2.503					8.33	2.250			0.457
5.33	2.503							0.090	0.301	0.017
		9.66	5.006	8.00	4.516					0.880
		9.66	5.006			8.33	2.250			0.943
		9.66	5.006					0.090	0.301	0.000
				8.00	4.516	8.33	2.250			1.000
				8.00	4.516			0.090	0.301	0.000
						8.33	2.250	0.090	0.301	0.000



FGF alone (9.6±5.006) given immediate after SCI and OEC 10 lakh alone (5.3±2.503) administered on 9th day after SCI shows no significant difference (P=0.134). On adding up FGF with OEC on first day after SCI (immediate after injury) (8.0±4.516) compared

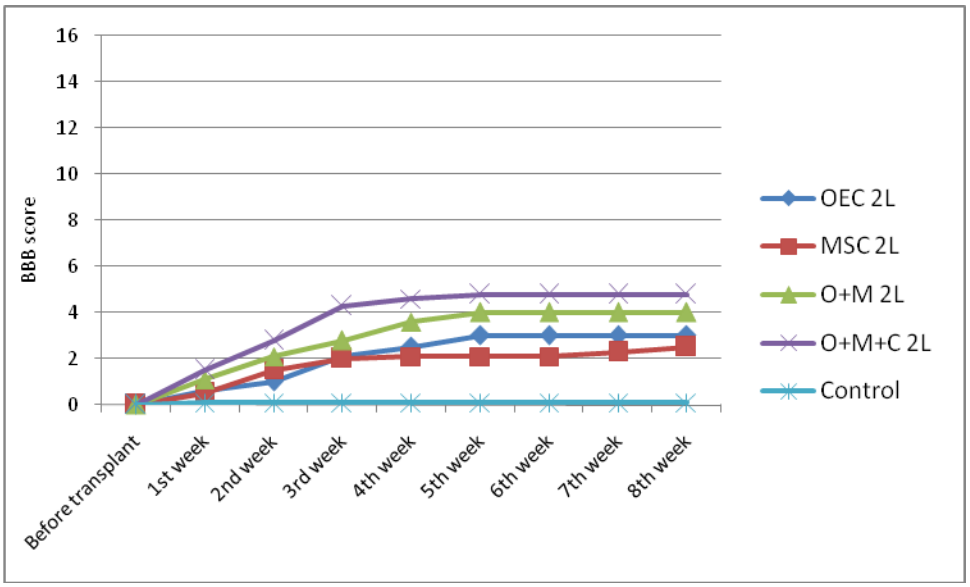
with FGF alone (9.6±5.006) proves no difference (P=0.880). Similarly OEC+FGF(1^{st} day) group (8.0±4.516) versus OEC alone (5.3±2.503) shows P=0.571. When compared to control (0.09±0.301) with FGF (9.6±5.006), OEC+FGF (1^{st} day) (8.0±4.516), OEC (1^{st} day)+FGF(9^{th} day) shows highly significant (P=0.000) in recovery.

Table 6.7.0 Efficacy of different groups in mean BBB score of 2 lakh cells transplantation.

Cell/ Enzyme	Sample size	BBB before transplant (9^{th} day after SCI)	BBB score after transplant in weeks							
			1^{st}	2^{nd}	3^{rd}	4^{th}	5^{th}	6^{th}	7^{th}	8^{th}
OEC 2L	n=6	0	0.6	1	2.1	2.5	3	3	3	3
MSC 2L	n=6	0	0.5	1.5	2	2.1	2.1	2.1	2.3	2.5
OEC+MSC 2L	n=6	0	1.1	2.1	2.8	3.6	4	4	4	4
OEC+MSC+ Chondroitinase 2L	n=6	0	1.5	2.8	4.3	4.6	4.8	4.8	4.8	4.8
Control	n=11	0	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09

The results of different cells/combination with same dosage as shown in (Table 6.7.0). Combinational therapy of OEC, MSC with Chondroitinase shows highest of 4.8 in BBB, when compare to other groups. But in comparison to control group, treated groups have significant recovery. MSC group and OEC group exhibit approximately similar outcomes in BBB of 2.5 and 3 respectively. OEC combined MSC group (n=6) shows better than individual cell treated group.

Graph 6.7.1 Efficacy of different groups in mean BBB score of 2 Lakh cells transplantation



(Graph 6.7.1) shows mild recovery of BBB in all groups except control. Individual cells OEC or MSC with same dose has approximately similar outcome, whereas combination of OEC+MSC and OEC+MSC+Chondroitinase shows increasing outcome of 4 and 4.8 respectively as expected.

Table 6.7.2 statistical analysis of BBB score of 2 lakh cells with different groups after transplantation

OEC 2L (n=6)		MSC 2L (n=6)		O+M 2L (n=6)		O+M+C 2L (n=6)		Control (n=11)		P value
Mean	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	
3.00	2.190	2.50	1.378							0.989
3.00	2.190			4.00	2.828					0.878
3.00	2.190					4.83	2.316			0.433
3.00	2.190							0.090	0.301	0.030
		2.50	1.378	4.00	2.828					0.624
		2.50	1.378			4.83	2.316			0.207
		2.50	1.378					0.090	0.301	0.099
				4.00	2.828	4.83	2.316			0.933
				4.00	2.828			0.090	0.301	0.002
						4.83	2.316	0.090	0.301	0.000

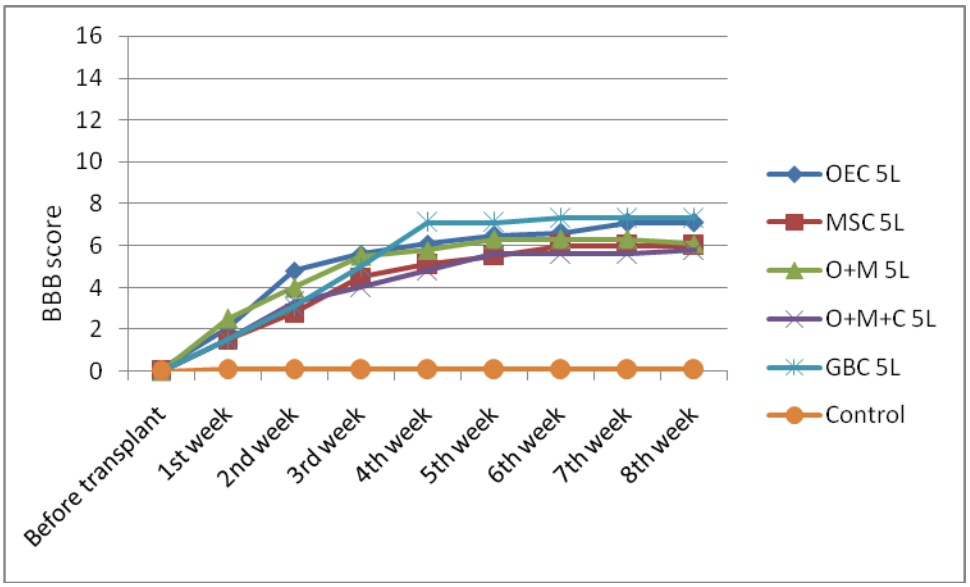
2 lakh combination of OEC with 2 lakh of MSC (4.0±2.828), 2 lakh of OEC+MSC+ Chondroitinase (4.8±2.316) on comparison with control (0.09±0.301) shows highly significant (P=0.00), whereas control (0.09±0.301) with MSC 2 lakh alone (2.5±1.378) does not support P<0.05 (P=0.099), this shows 2 lakh MSC dose was not sufficient for recovery. But OEC 2 lakh (3.0±2.190) versus control (0.09±0.301) shows significant (P=0.030). However, there is no statistical difference among the treated groups as shown in (Table 6.7.2), even though the dose is same but combination differs.

Table 6.7.3 Efficacy of different groups in mean BBB score of 5 lakh cells transplantation

Cell/ Enzyme	Sample size	BBB before transplant (9^{th} day after SCI)	BBB score after transplant in weeks							
			1^{st}	2^{nd}	3^{rd}	4^{th}	5^{th}	6^{th}	7^{th}	8^{th}
OEC 5L	n=6	0.1	2.1	4.8	5.6	6.1	6.5	6.6	7.1	7.1
MSC 5L	n=6	0	1.5	2.8	4.5	5.1	5.5	6	6	6
OEC+MSC 5L	n=6	0	2.5	4	5.5	5.8	6.3	6.3	6.3	6.1
OEC+MSC+ Chondroitinase 5L+0.2U	n=6	0	1.5	3.3	4	4.8	5.6	5.6	5.6	5.8
Control	n=11	0	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
GBC 5L	n=6	0	1.5	3.1	5	7.1	7.1	7.3	7.3	7.3

The results of different cells/combination with same dosage as shown in (Table 6.7.3). 5 lakh cells of MSC, OECwith MSC and OEC,MSC with chondroitinasse transplanted rats almost approximately scored equal in BBB score. But OEC alone treated rats improves to 7.1. Globose basal stem cell (GBC) proves highest in recovery of 7.3 in BBB.

Graph 6.7.4 Efficacy of different groups in mean BBB score of 5 Lakh cells transplantation



(Graph 6.7.4) shows all the groups progressed from 0 and attains approximately 6 to 7 in BBB after transplantation. All 5 lakh groups recovered similar in motor recovery though the cells and combination differs, except control group (n=11).

Table 6.7.5 Statistical analysis of BBB score of 5 Lakh cells with different groups after transplantation

OEC 5L (n=6)		MSC 5L (n=6)		O+M 5L (n=6)		O+M+C 5L (n=6)		GBC 5L (n=6)		Control (n=11)		P value
Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	
7.16	2.56	6.00	2.75									0.975
7.16	2.56			6.16	4.622							0.988
7.16	2.56					5.83	3.816					0.956
7.16	2.56							7.33	1.366			1.000
7.16	2.56									0.09	0.301	0.000
		6.00	2.75	6.16	4.622							1.000
		6.00	2.75			5.83	3.816					1.000
		6.00	2.75					7.33	1.366			0.956
		6.00	2.75							0.09	0.301	0.002
				6.16	4.622	5.83	3.816					1.000
				6.16	4.622			7.33	1.366			0.975
				6.16	4.622					0.09	0.301	0.001
						5.83	3.816	7.33	1.366			0.930
						5.83	3.816			0.09	0.301	0.003
								7.33	1.366	0.09	0.301	0.000

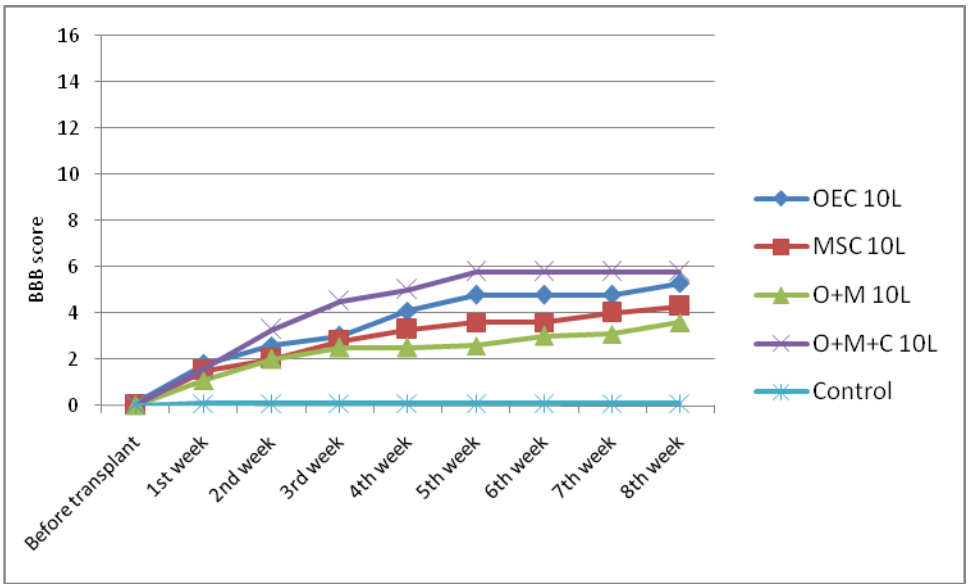
Olfactory ensheathing glial cells of 5 lakh dose (7.1±2.56) compared with MSC of same dose (6.0±2.75) shows no difference (P=0.975). Two different stem cells, olfactory globose basal cell (7.3±1.366) with BMSC (6.0±2.75) as no significance (P=0.956) although the dose (5 lakh) is same. Combination therapy, OEC+MSC of 5 lakh (6.1±4.622) group analysed with OEC+MSC+Chondroitinase of 5 lakh (5.8±3.816) group shows no difference (P=1.000). However on comparison with control (0.09±0.301) versus GBC of 5 lakh (7.3±1.366), OEC+MSC+Chondroitinase of 5 lakh (5.8±3.816),OEC+MSC of 5 lakh (6.1±4.622), MSC of 5 lakh (6.0±2.75), OEC of 5 lakh (7.1±2.56) indicates remarkable statistical difference of P=0.003.

Table 6.7.6 Efficacy of different groups in mean BBB score of 10 lakh cells transplantation

Cell/ Enzyme	Sample size	BBB before transplant (9^{th} day after SCI)	BBB score after transplant in weeks							
			1^{st}	2^{nd}	3^{rd}	4^{th}	5^{th}	6^{th}	7^{th}	8^{th}
OEC 10L	n=6	0.1	1.8	2.6	3	4.1	4.8	4.8	4.8	5.3
MSC 10L	n=6	0	1.5	2	2.8	3.3	3.6	4	4	4.3
OEC+MSC 10L	n=6	0	1.1	2	2.5	2.5	2.6	3	3.1	3.6
OEC+MSC+ Chondroitinase 10L+0.2U	n=6	0	1.6	3.3	4.5	5	5.8	5.8	5.8	5.8
Control	n=11	0	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09

The results of different cells/combination with same dosage as shown in (Table 6.7.6) OEC, MSC with Chondroitinase group (n=6) attains highest 5.8 in BBB. When compared to control group all other groups as significant recovery.

Graph 6.7.7 Efficacy of different groups in mean BBB score of 10 Lakh cells transplantation



(Graph 6.7.7) shows the sequential recovery after transplantation of SCI rats. OEC alone, MSC alone group followed upto 8th week, which scored 5.3 and 4.3 respectively. Combination OEC+MSC declines in recovery as compared to OEC or MSC.

Table 6.7.8 Statistical analysis of BBB score of 10 Lakh cells with different groups after transplantation

OEC 10L (n=6)		MSC 10L (n=6)		O+M 10L (n=6)		O+M+C 10L (n=6)		Control (n=11)		P value
Mean	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	
5.33	2.503	4.33	3.141							0.971
5.33	2.503			3.66	3.559					0.838
5.33	2.503					5.83	4.215			0.998
5.33	2.503							0.090	0.301	0.007
		4.33	3.141	3.66	3.559					0.994
		4.33	3.141			5.83	4.215			0.883
		4.33	3.141					0.090	0.301	0.040
				3.66	3.559	5.83	4.215			0.666
				3.66	3.559			0.090	0.301	0.112
						5.83	4.215	0.090	0.301	0.003

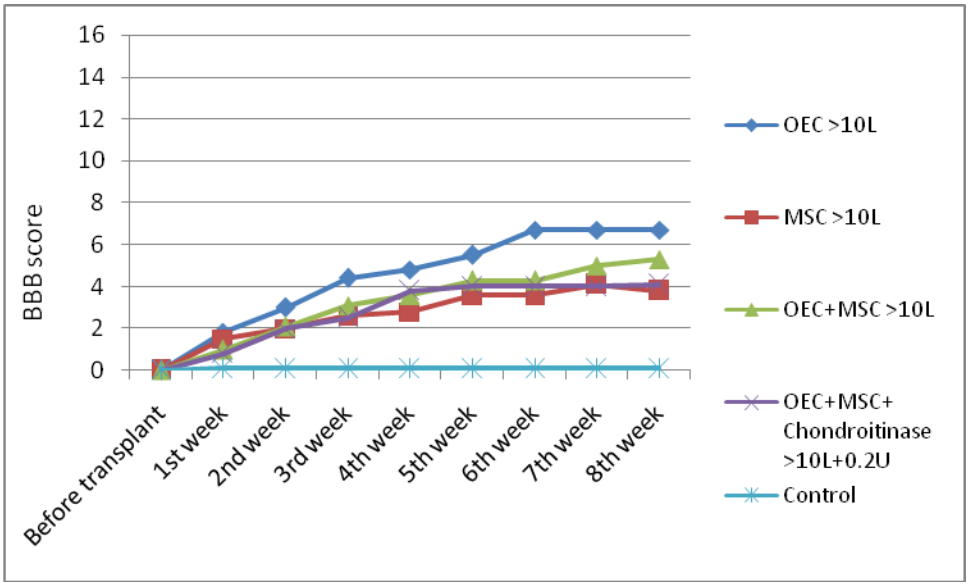
10 lakh of OEC (5.3±2.503) analysed with 10 lakh of MSC (4.3±3.141) as shows no difference (P=0.971). OEC+MSC combination of 10 lakh (3.6±3.559) compared with 10 lakh of MSC (4.3±3.141) treated rats proves no statistical difference (P=0.994). 10 lakh of OEC+MSC (3.6±3.559) groups evaluated with 10 lakh of OEC+MSC+Chondroitinase (5.8±4.215) but there is no statistical difference (P=0.666). Among the 10 lakh treated groups there is no significances was attained; but in comparison with control (0.09±0.301) all group establish statistical significant as shown in (Table 6.7.8).

Table 6.7.9 Efficacy of different groups in mean BBB score of >10 lakh cells transplantation

Cell/ Enzyme	Sample size	BBB before transplant (9^{th} day after SCI)	BBB score after transplant in weeks							
			1^{st}	2^{nd}	3^{rd}	4^{th}	5^{th}	6^{th}	7^{th}	8^{th}
OEC >10L	n=6	0	1.8	3	4.4	4.8	5.5	6.7	6.7	6.7
MSC >10L	n=6	0	1.5	2	2.6	2.8	3.6	3.6	4.1	3.8
OEC+MSC >10L	n=6	0	1	2.1	3.1	3.6	4.3	4.3	5	5.3
OEC+MSC+ Chondroitinase >10L+0.2U	n=6	0	0.8	2	2.5	3.8	4	4	4	4.1
Control	n=11	0	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09

The results of different cells/combination with same dosage as shown in (Table 6.7.9). OEC group (n=6) shows highest improvement with that of other groups. But in comparison to control group all treated groups has beneficial effects.

Graph 6.8.0 Efficacy of different groups in mean BBB score of more than 10 Lakh cells transplantation



(Graph 6.8.0) shows the recovery status of each group after transplantation, but control stay stable without improvement in motor recovery scale. OEC group (n=6) shows elicit better recovery than other groups. OEC+MSC obtained better BBB score than OEC+MSC+Chondroitinase group.

Table 6.8.1 Statistical analysis of BBB score of more than 10 Lakh cells with different groups after transplantation

OEC >10L (n=6)		MSC>10L (n=6)		O+M>10L (n=6)		O+M+C>10L (n=6)		Control (n=11)		P value
Mean	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	
7.16	3.371	3.83	2.994							0.328
7.16	3.371			5.33	2.503					0.827
7.16	3.371					4.16	5.231			0.432
7.16	3.371							0.090	0.301	0.001
		3.83	2.994	5.33	2.503					0.907
		3.83	2.994			4.16	5.231			1.000
		3.83	2.994					0.090	0.301	0.129
				5.33	2.503	4.16	5.231			0.961
				5.33	2.503			0.090	0.301	0.014
						4.16	5.231	0.090	0.301	0.082

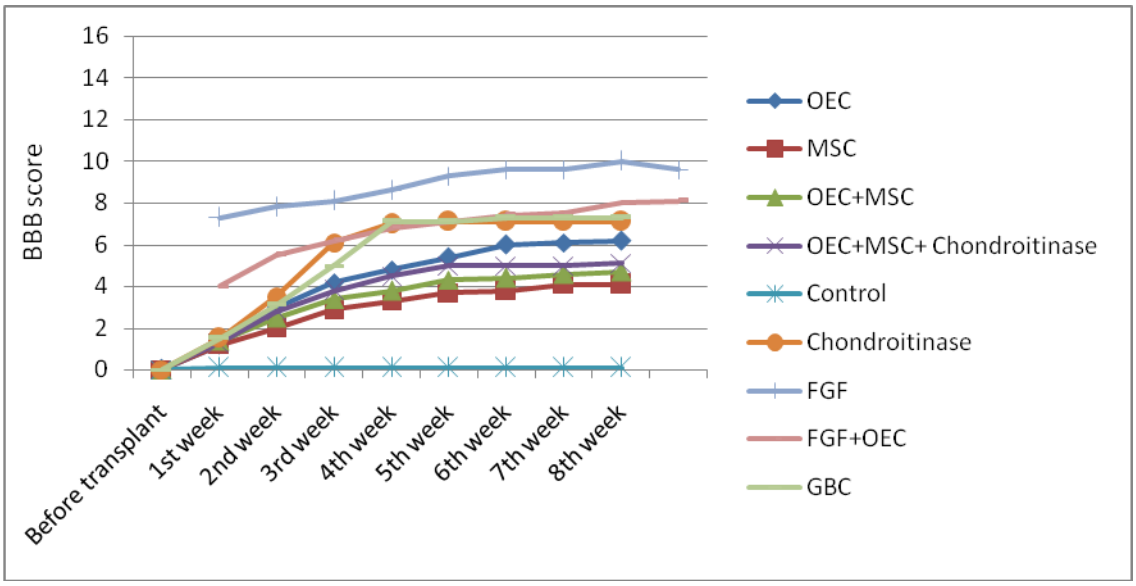
More than 10 lakh of OEC+MSC group (5.3±2.503) analysed with control (0.09±0.301) shows significant (P=0.014) in BBB. Similarily, more than 10 lakh of OEC group (7.1±3.371) with control (0.09±0.301) obtained remarkable difference (P=0.001). No significant was seen between more than 10 lakh of MSC (3.8±2.994) versus control (0.09±0.301) and more than 10 lakh of OEC+MSC+Chondroitinase (4.1±5.231) versus control (0.09±0.301), where P=0.129 and P=0.082 respectively. Among transplanted groups there is no statistical significances as shown in (Table 6.8.1).

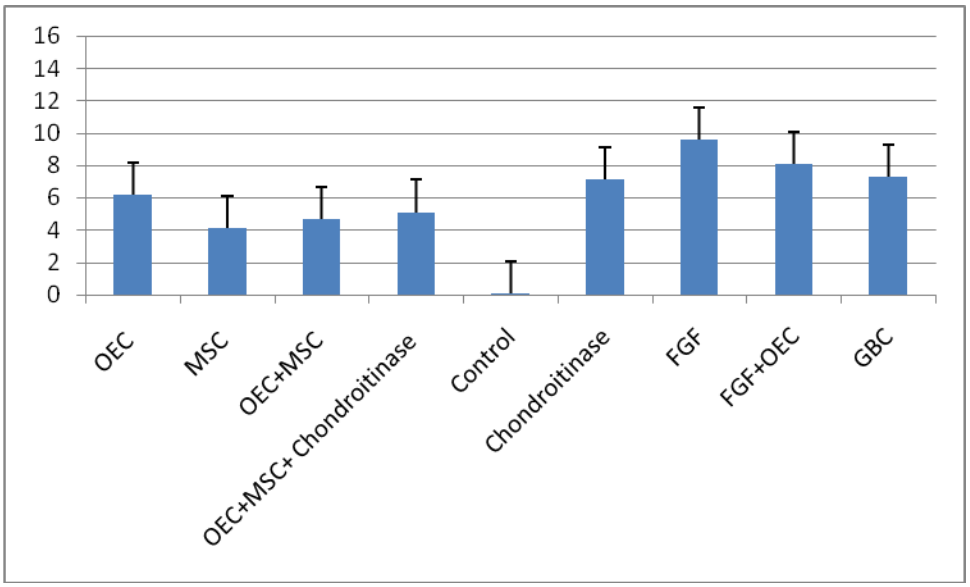
Table 6.8.2 Efficacy of different groups after transplantation

Cell/ Enzyme/ Growth factor	Sample size	BBB before transplant (9th day after SCI)	BBB score after transplant in weeks								
			1st	2nd	3rd	4th	5th	6th	7th	8th	9th
OEC	n=24	0.025	1.2	3.0	4.2	4.8	5.4	6.0	6.1	6.2	
MSC	n=24	0	1.2	2	2.9	3.3	3.7	3.8	4.1	4.1	
OEC+MSC	n=24	0	1.4	2.5	3.4	3.8	4.3	4.4	4.6	4.7	
OEC+MSC+ Chondroitinase	n=24	0	1.3	2.8	3.8	4.5	5	5	5	5.1	
Control	n=11	0	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	
Chondroitinase	n=6	0	1.5	3.5	6.1	7	7.1	7.1	7.1	7.1	
FGF	n=6		7.3	7.8	8.1	8.6	9.3	9.6	9.6	10	9.6
FGF+OEC	n=12		4	5.5	6.2	6.8	7.1	7.4	7.5	8	8.1
GBC	n=6	0	1.5	3.1	5	7.1	7.1	7.3	7.3	7.3	

Even though the number of rats, cell dosage, cell combination, enzyme/ growth factor combination varies in transplantation but all have better motor recovery except control group. Overall outcome differs from one group to another group as shown in (Table 6.8.2). MSC group (n=24) as least outcome of 4.1 in BBB as compared to other group. OEC+ MSC combination group (n=24) and OEC+MSC+Chondroitinase group (n=24) exhibit similar in outcome of 4.7 and 5.1 respectively. Chondroitinase alone and FGF combination proves better as compared to cell groups. GBC is better than OEC and MSC, in terms of BBB score (Table 6.8.2).

Graph 6.8.3 Efficacy of different groups after transplantation



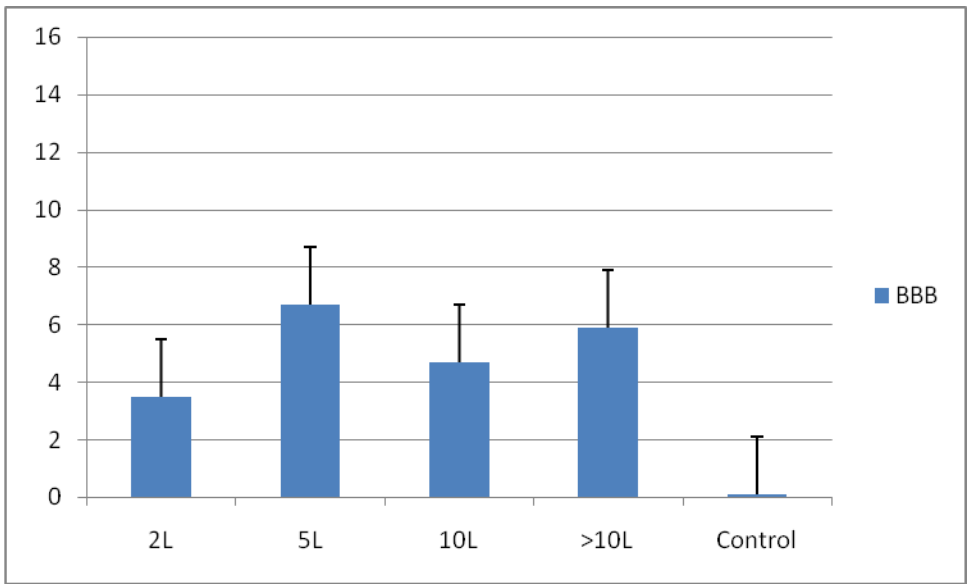


Overall, FGF group (n=6) and FGF with OEC group (n=12) proving better in hind limb motor recovery after SCI. Chondroitinase alone shows the improvement of 7.1 in BBB score. But in comparison to control group all the groups shows significant improvement in motor recovery.

Dose response relationship

Table 6.8.4 Efficacy of mean BBB score in dose response relationship

Dosage	Sample size	Mean BBB score
2 Lakh cells	**n=24**	**3.5**
5Lakh cells	**n=30**	**6.7**
10 Lakh cells	**n=24**	**4.7**
>10 Lakh cells	**n=24**	**5.9**
Control	**n=11**	**0.09**



Graph 6.8.5 Efficacy of mean BBB score in dose response relationship

5 lakh cells (n=30) shows promising outcome with that of other dosage. Although different cells/combination has different effects, transplantation of 5 lakh cells showed highest recovery (6.7 mean BBB score). 10 lakh cells group (n=24) and more than 10

lakh cells group (n=24) shows motor recovery of 4.7 and 5.9 respectively, though the combination of cell differs. As per low dose of 2 lakh cell combination group (n=24) attains lowest recovery of 3.5 in BBB score as compared to other higher dosage. However, control (n=11) group (spinal cord injured without any treatment) shows no motor recovery in terms of BBB score (0.09), this shows there is no spontaneous recovery after SCI (Table 6.8.4).

In comparison of OEC 2 lakh (3.0±2.190) with OEC 5 lakh transplant group (7.1±2.562) shows significant (P=0.02) in motor recovery. Low dose of 2 lakh OEC (3.0±2.190) with high dose of more than 10 lakh of OEC (7.1±3.371) treated rats (n=6) has significant difference (P=0.02). Between 5 lakh (7.1±2.562) and 10 lakh (5.3±2.503) cell injected has no significant difference (P=0.608). There is no statistical difference between 5 lakh OEC (7.1±2.562) and more than 10 lakh OEC treated (7.1±3.371) group, where P=1.00. But in control (0.09±0.301) versus 5 lakh of OEC (7.1±2.562) shows highly significant (P=0.00). In higher dose group more than 10 lakh of OEC (7.1±3.371) and 10 lakh of OEC(5.3±2.503) shows no difference (P=0.608). However, on comparison of OEC 10 lakh (5.3±2.503) with control (0.09±0.301) shows much difference in hind limb motor recovery (P=0.001). Even on comparison control (0.09±0.301) with more than 10 lakh OEC proves remarkable difference (P=0.00)(Table 6.5.3). However, BBB score of before and after transplantation of OEC group shows significant (Table 6.5.4).

5 lakh of MSC treated rats (6.0±2.756) has remarkable difference (P=0.00) on comparison with control (0.09±0.301).Similary, 10 lakh of MSC transplant rats (4.3±3.141) proves significant (P=0.005) with that of control (0.09±0.301). Highest dose more than 10 lakh of MSC (3.8±2.994) supports motor recovery P=0.016, to that of untreated control rats (0.09±0.301). However, there is no statistical difference in BBB score of different dosages of MSC transplanted rats (Table 6.5.7). BBB score of before and after transplantation of MSC group shows significant (Table 6.5.8).

The results of different dosage of OEC combined with MSC as shown in table (6.5.9). SCI rats (n=6) injected 2 lakh OEC+MSC and 10 lakh cells of OEC+MSC recovered to 4 and 3.6 respectively in BBB score, whereas 5 lakh cells of OEC+MSC treated rats (n=6) improved to the maximum of 6.1. In comparison to control group, all other treated groups showed promising effects.

2 lakh cells combination (OEC+MSC) group (4.0±2.828) has no recovery difference (P=1.00) with 10 lakh OEC+MSC group (3.6±3.559). No significant difference (P=0.925) between 2 lakh OEC+MSC (4.0±2.828) and more than 10 lakh OF OEC+MSC (5.3±2.503) groups. P=0.008 was achieved between more than 10 lakh of OEC +MSC (5.3±2.503) and control (0.09±0.301). Similar significant (P=0.002) found in 5 lakh of OEC+MSC (6.1±4.622) versus control (0.09±0.301). There is no statistical differences in motor recovery BBB score among doses, but there is a remarkable difference seen in treated versus control (Table 6.6.1). However, on comparing before and after transplantation of OEC+MSC shows remarkable significant differences (Table 6.6.2).

Although all transplant groups shows clinically promising outcome with that of control group. However on statistical basis there is no significant difference among OEC+MSC+Chondroitinase transplant groups. Control (0.09±0.301) compared with chondroitinase alone (7.1±2.483) shows both clinically and statistically significant (P=0.001). Another group of 5 lakh of OEC+MSC+chondritinase combination (5.8±3.816) versus control (0.09±0.301) supports $P<0.05$ that is P=0.013. Similarly like 5 lakh of OEC+MSC+chondritinase group, 10 lakh of OEC+MSC+chondritinase group

(5.8±4.215) has significant difference (P=0.013) with that of control (0.09±0.301) (Table 6.6.5). However, on comparing before and after transplantation of OEC+MSC+ Chondroitinase/ Chondroitinase shows remarkable significant differences (Table 6.6.6).

Fibroblast growth factor injected into the injured cord immediate after spinal cord injury (i.e) on the first day. FGF alone treated group (n=6) attains highest in motor recovery BBB score of 9.6. Both FGF combined with OEC 10 lakh cells injected on the first day shows BBB of 8 and FGF on first day treated with again OEC 10 lakh cells on 9^{th} day group (n=6) improves to 8.3 in score. OEC 10 lakh cells on 9^{th} day after SCI has progressed to 5.3 (Table 6.6.7). FGF alone (9.6±5.006) given immediate after SCI and OEC 10 lakh alone (5.3±2.503) administered on 9^{th} day after SCI shows no significant difference (P=0.134). On adding up FGF with OEC on first day after SCI (immediate after injury) (8.0±4.516) compared with FGF alone (9.6±5.006) proves no difference (P=0.880). Similarly OEC+FGF (1^{st} day) group (8.0±4.516) versus OEC alone (5.3±2.503) shows P=0.571. When compared to control (0.09±0.301) with FGF (9.6±5.006), OEC+FGF (1^{st} day) (8.0±4.516), OEC (1^{st} day)+FGF(9^{th} day) shows highly significant (P=0.000) in recovery (Table 6.6.9).

The results of different cells/combination with same dosage of 2 lakhs shows combinational therapy of OEC, MSC with Chondroitinase shows highest of 4.8 in BBB, when compare to other groups. But in comparison to control group, treated groups have significant recovery. MSC group and OEC group exhibit approximately similar outcomes

in BBB of 2.5 and 3 respectively. OEC combined MSC group (n=6) shows better than individual cell treated group (Table 6.7.0).

2 lakh combination of OEC with 2 lakh of MSC (4.0±2.828), 2 lakh of OEC+MSC+ Chondroitinase (4.8±2.316) on comparison with control (0.09±0.301) shows highly significant (P=0.00), whereas control (0.09±0.301) with MSC 2 lakh alone (2.5±1.378) does not support P<0.05 (P=0.099), this shows 2 lakh MSC dose was not sufficient for recovery. But OEC 2 lakh (3.0±2.190) versus control (0.09±0.301) shows significant (P=0.030). However, there is no statistical difference among the 2 lakhs treated groups, even though the dose is same but combination differs (Table 6.7.2).

The results of different cells/combination with same dosage of 5 lakh cells of MSC, OEC with MSC and OEC, MSC with chondroitinasse transplanted rats almost approximately scored equal in BBB score. But OEC alone treated rats improves to 7.1. Globose basal stem cell (GBC) proves highest in recovery of 7.3 in BBB (Table 6.7.3).

Olfactory ensheathing glial cells of 5 lakh dose (7.1±2.56) compared with MSC of same dose (6.0±2.75) shows no difference (P=0.975). Two different stem cells, olfactory globose basal cell (7.3±1.366) with BMSC (6.0±2.75) as no significance (P=0.956) although the dose (5 lakh) is same. Combination therapy, OEC+MSC of 5 lakh (6.1±4.622) group analysed with OEC+MSC+Chondroitinase of 5 lakh (5.8±3.816) group shows no difference (P=1.000). However on comparison with control (0.09±0.301) versus GBC of 5 lakh (7.3±1.366), OEC+MSC+Chondroitinase of 5 lakh (5.8±3.816),

OEC+MSC of 5 lakh (6.1±4.622), MSC of 5 lakh (6.0±2.75), OEC of 5 lakh (7.1±2.56) indicates remarkable statistical difference of P=0.003(Table 6.7.5).

10 lakh of OEC (5.3±2.503) analysed with 10 lakh of MSC (4.3±3.141) as shows no difference (P=0.971). OEC+MSC combination of 10 lakh (3.6±3.559) compared with 10 lakh of MSC (4.3±3.141) treated rats proves no statistical difference (P=0.994). 10 lakh of OEC+MSC (3.6±3.559) groups evaluated with 10 lakh of OEC+MSC+Chondroitinase (5.8±4.215) but there is no statistical difference (P=0.666). Among the 10 lakh treated groups there is no significances was attained; but in comparison with control (0.09±0.301) all group establish statistical significant (Table 6.7.8).

More than 10 lakh of OEC group (n=6) shows highest improvement with that of other groups, even though different cells/combination with same dosage of more than 10 lakh. But in comparison to control group all treated more than 10 lakh groups has beneficial effects (Table 6.7.9)

More than 10 lakh of OEC+MSC group (5.3±2.503) analysed with control (0.09±0.301) shows significant (P=0.014) in BBB. Similarily, more than 10 lakh of OEC group (7.1±3.371) with control (0.09±0.301) obtained remarkable difference (P=0.001). No significant was seen between more than 10 lakh of MSC (3.8±2.994) versus control (0.09±0.301) and more than 10 lakh of OEC+MSC+Chondroitinase (4.1±5.231) versus control (0.09±0.301), where P=0.129 and P=0.082 respectively. Among transplanted groups there is no statistical significances observed (Table 6.8.1).

Even though the number of rats, cell dosage, cell combination, enzyme/ growth factor combination varies in transplantation but all have better motor recovery except control group. Overall outcome differs from one group to another group as shown in (Table 6.8.2). MSC group (n=24) as least outcome of 4.1 in BBB as compared to other group. OEC+ MSC combination group (n=24) and OEC+MSC+Chondroitinase group (n=24) exhibit similar in outcome of 4.7 and 5.1 respectively. Chondroitinase alone and FGF combination proves better as compared to cell groups. GBC is better than OEC and MSC, in terms of BBB score (Table 6.8.2).

5 lakh cells (n=30) shows promising outcome with that of other dosage. Although different cells/combination has different effects, transplantation of 5 lakh cells showed highest recovery (6.7 mean BBB score). 10 lakh cells group (n=24) and more than 10 lakh cells group (n=24) shows motor recovery of 4.7 and 5.9 respectively, though the combination of cell differs. As per low dose of 2 lakh cell combination group (n=24) attains lowest recovery of 3.5 in BBB score as compared to other higher dosage. However, control (n=11) group (spinal cord injured without any treatment) shows no motor recovery in terms of BBB score (0.09), this shows there is no spontaneous recovery after SCI (Table 6.8.4).

Motor evoked potential studies

2 lakh of OEC group (0.6967±0.3342) with 5 lakh of OEC group (1.207±0.4167) has no much difference in amplitude (P=0.061), but with 10 lakh of OEC (1.21±0.4876) shows significant (P=0.05). There is no variation in amplitude of more than 10 lakh of OEC

group (0.56±0.0494) versus 2 lakh of OEC group (0.69±0.3342), where P=0.955. Amplitude of 5 lakh of OEC group (1.20±0.4167) compared with more than 10 lakh of OEC group shows significant (P=0.012) and also 5 lakh of OEC group with control (0.25±0.1845) has highly significant (P=0.000). Ten lakh of OEC group (1.21±0.4876) analysed with control group (0.25±0.1845) proves highly significant (P=0.000) in amplitude. But more than 10 lakh of OEC group (0.56±0.0494) and control group (0.25±0.1845) has almost similar amplitude (P=0.307) (Table 6.8.7)

Though transplanted cells are MSC but differ in dosage, these doses have impact on recovery analysed by the motor evoked potential in amplitude. Amplitude of 2 lakh of MSC (0.41±0.1662) differs with 5 lakh of MSC (1.55±1.0625) and thus P=0.038. 2 lakh of MSC (0.41±0.1662) versus 10 lakh of MSC (1.23±1.1002) amplitude as no difference (P=0.216). Control group (0.25±0.1845) compared with 2 lakh of MSC (0.41±0.1662), more than 10 lakh of MSC (0.67±0.3349) shows no statistical significant (P=0.988), (P=0.715) respectively. Significant difference (P=0.043), (P=0.004) seen in 10 lakh of MSC (1.23±1.1002), 5 lakh of MSC (1.55±1.0625) respectively when compared with control group (0.25±0.1845). 10 lakh of MSC group (1.23±1.1002) with more than 10 lakh of MSC group (0.67±0.3349) exhibit P=0.573, which shows no variation in amplitude (Table 6.8.9).

Combination treatment shows effective regeneration was evaluated by amplitude response in different dosage. Control group (0.25±0.1845) compared with treated group 5 lakh of OEC+MSC (1.64±0.8734), more than 10 lakh of OEC+MSC (1.80±0.6268) proves highly significant (P=0.000). 10 lakh of OEC+MSC (1.23±0.5404) versus more

than 10 lakh of OEC+MSC (1.80±0.6268) as no much difference in amplitude (P=0.398). Two lakh of OEC+MSC (0.83±0.5467) compared with more than 10 lakh of OEC+MSC (1.80±0.6268) denotes P=0.035. Control group (0.25±0.1845) with 2 lakh of OEC+MSC group (0.83±0.5467) as no significance (P=0.262) may be due low dosage (Table 6.9.1).

Chondroitinase with different dosage of cell transplant yield different outcome in amplitude of motor evoked potential studies. Chondroitinase alone treated group (1.67±0.596) has remarkable difference (P=0.000) when compared with control (0.25±0.184). 10 lakh of OEC+MSC+Chondroitinase (1.45±1.192) increased in amplitude (P=0.001) with that of control group (0.25±0.184). 2 lakh of OEC+MSC+Chondroitinase (0.9±0.3065) compared with 5 lakh of OEC+MSC+Chondroitinase (0.87±0.1745), 10 lakh of OEC+MSC+Chondroitinase (1.4±1.192), more than 10 lakh of OEC+MSC+Chondroitinase (0.9±0.2574) shows statistically non-significant P=0.99, P=0.674, P=1.00 respectively. Overall there is no significant difference in amplitude among the OEC+MSC+Chondroitinase/ Chondroitinase treated groups (Table 6.9.3)

Amplitude of 10 lakh of OEC group (1.211±0.4876), FGF alone group (1.088±0.2245), OEC+FGF (1^{st} day) treated group (1.769±0.8129) and OEC (1^{st} day)+FGF(9^{th} day) treated group (1.184±0.4694) as considerable improvement in motor recovery analysed by MEP, P=0.002, P=0.009, P=0.000, P=0.003 respectively, when compared to control group amplitude (0.255±0.1845). Among the treated groups shows no significant difference in amplitude (Table 6.9.5)

2 lakh of OEC (0.69±0.3342) compared with MSC 2 lakh group (0.41±0.1662), 2 lakh of OEC+MSC group (0.83±0.5467) and 2 lakh of OEC+MSC+Chondroitinase group (0.99±0.3065) shows no statistical difference in amplitude P=0.552, P=0.946, and P=0.485 respectively. Control group (0.25±0.1845) analysed with 2 lakh of OEC+MSC group (0.83±0.5467), 2 lakh of OEC+MSC+Chondroitinase group (0.99±0.3065) proves significant in amplitude, where P=0.01, P=0.001 respectively. 2 lakh of MSC group (0.41±0.1662) versus 2 lakh of OEC+MSC+Chondroitinase group (0.99±0.3065) exhibit significant (P=0.027) in MEP amplitude (Table 6.9.6).

Amplitude of control group (0.25±0.1845) compared with 5 lakh of GBC group (1.22±0.5596), 5 lakh of OEC+MSC+Chondroitinase group (0.99±0.3065) shows P=0.000, P=0.005 respectively in significant. In treated group, 5 lakh of OEC group (1.20±0.4167) versus 5 lakh of MSC group (0.41±0.1662) as difference in amplitude (P=0.010). When 5 lakh of MSC group (0.41±0.1662) analysed with 5 lakh of OEC+MSC group (0.83±0.5467), 5 lakh of OEC+MSC+Chondroitinase group (0.99±0.3065) and control group (0.25±0.1845) gives P=0.406, P=0.104, P=0.955 respectively, Which denotes there is no statistical difference in amplitude. When 5 lakh of GBC group (1.22±0.5596) compared with 5 lakh of MSC group (0.41±0.1662) shows significant (P=0.008) in peak amplitude. 5 lakh of OEC+MSC group (0.83±0.5467) shows significant in amplitude (P=0.04) with that of control group (0.25±0.1845) (Table 6.9.7)

There is a significant in amplitude (P=0.023) between control group (0.25±0.1845) and 10 lakh of OEC+MSC+Chondroitinase group (1.45±1.1929). 10 lakh of OEC group

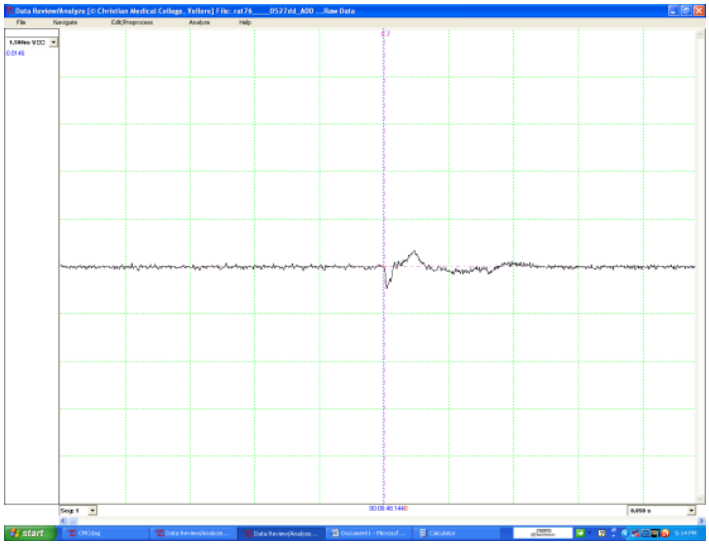
(1.21±0.4876), 10 lakh of MSC group (1.23±1.1002), 10 lakh of OEC+MSC group (1.23±0.5404) compared with control group (0.25±0.1845) yields P=0.103, P=0.090, P=0.091, which shows no differences in amplitude (Table 6.9.8).

Amplitude of combinational treatment of more than 10 lakh of OEC+MSC+ Chondroitinase group (0.958±0.2574) shows remarkable increases in action potential (P=0.002) on comparison with control group (0.255±0.1845). More than 10 lakh of OEC with MSC group (1.801±0.6268) proves significant (P=0.000), when analysed with control group (0.255±0.1845). Individual cells, more than 10 lakh of OEC group (0.569±0.049), more than 10 lakh of MSC group (0.673±0.3349) shows no significant (P=0.344) and (P=0.114) respectively, when compared with control group (0.255±0.184). More than 10 lakh of OEC+MSC group (1.801±0.6268) statistical significant was seen (P=0.000), on comparison with more than 10 lakh of OEC (0.569±0.0494), more than 10 lakh of MSC (0.673±0.3349), more than 10 lakh of OEC+MSC+Chondroitinase (0.958±0.2574). However, combination therapy proves effective on evaluation by motor evoked potential studies than individual cells (Table 6.9.9).

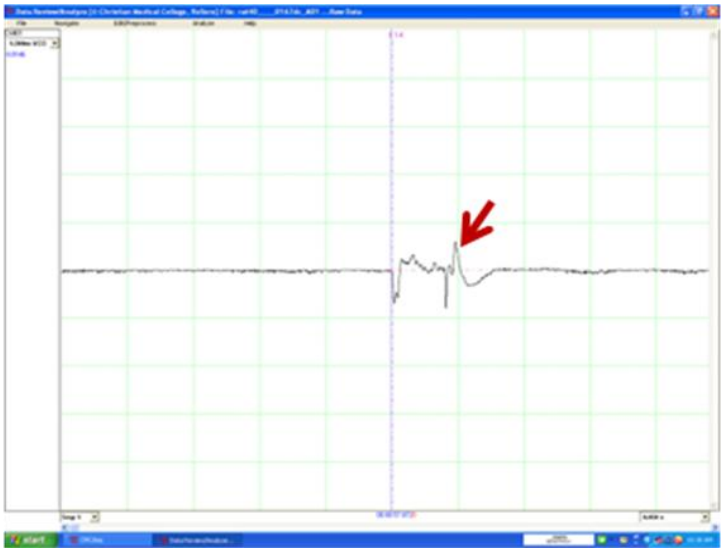
Mean motor evoked potential studies of different groups

A sample of motor response following transcortical stimulation is shown below.

Control :



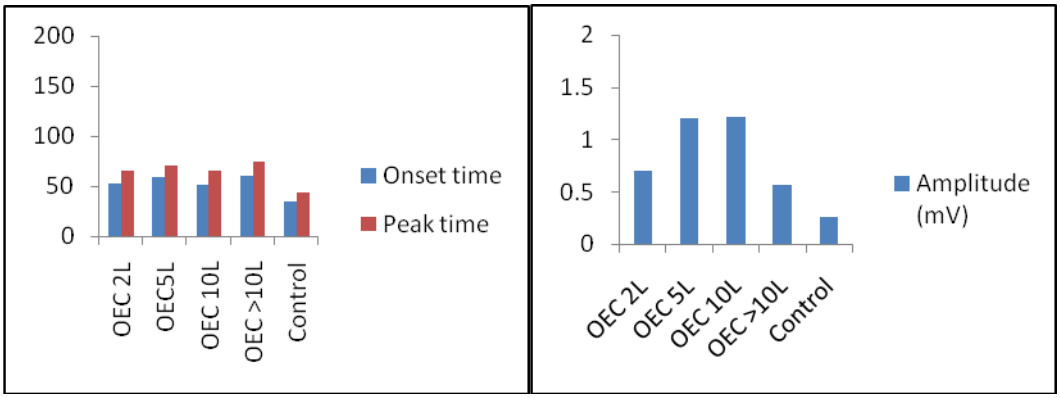
Transplanted rat:



Motor evoked potential studies shows increased peak amplitude (red arrow) in treated rats than control group. This increase in amplitude shows transplant mediated repair of injured spinal cord.

Table 6.8.6 Motor evoked potential of different dosage of OEC transplant groups mean onset time, mean peak time and mean amplitude.

Cells/Dosage	Sample size	Onset time (ms)	Peak time (ms)	Amplitude (mV)
OEC 2L	n=6	53	66	0.6967
OEC 5L	n=6	59	71	1.2072
OEC 10L	n=6	51	66	1.2117
OEC >10L	n=6	61	75	0.5695
Control	n=11	35	44	0.2557



In 2 lakh of OEC treated group the onset of action potential is 53 msec and the peak attains maximum at 66 msec. Based on the magnitude of regeneration the amplitude has exhibited. The delayed onset of action potential is around 50-60 msec is due to the distance from the motor cortex to hind limb muscle. Onset of action potential is followed by peak time, where the peak attains maximum. Both 5 lakh and 10 lakh of OEC transplanted rat's shows higher in amplitude as shown in (Figure 6.8.6). Graph shows similar of onset time and peak time in all groups, but differs in amplitude.

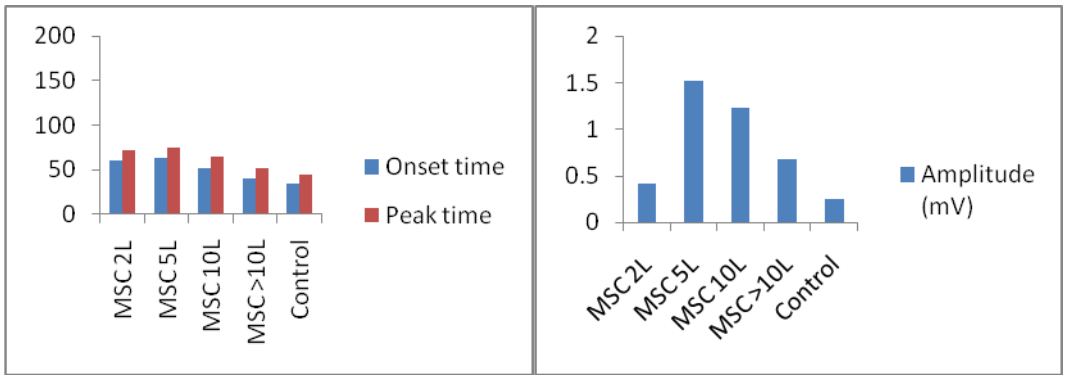
Table 6.8.7 statistical analysis of motor evoked potential -amplitude after different dosage of OEC transplantation

OEC 2L		OEC 5L		OEC 10L		OEC >10L		Control (n=11)		P value
Mean	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	
0.6967	0.3342	1.207	0.4167							0.061
0.6967	0.3342			1.2117	0.4876					0.058
0.6967	0.3342					0.5695	0.0494			0.955
0.6967	0.3342							0.2557	0.1845	0.068
		1.207	0.4167	1.2117	0.4876					1.000
		1.207	0.4167			0.5695	0.0494			0.012
		1.207	0.4167					0.2557	0.1845	0.000
				1.2117	0.4876	0.5695	0.0494			0.011
				1.2117	0.4876			0.2557	0.1845	0.000
						0.5695	0.0494	0.2557	0.1845	0.307

Transcranial stimulation done and the response were collected for analysis between different groups of OEC transplantation (Table 6.8.7). 2 lakh of OEC group (0.6967±0.3342) with 5 lakh of OEC group (1.207±0.4167) has no much difference in amplitude (P=0.061), but with 10 lakh of OEC (1.21±0.4876) shows significant (P=0.05). There is no variation in amplitude of more than 10 lakh of OEC group (0.56±0.0494) versus 2 lakh of OEC group (0.69±0.3342), where P=0.955. Amplitude of 5 lakh of OEC group (1.20±0.4167) compared with more than 10 lakh of OEC group shows significant (P=0.012) and also 5 lakh of OEC group with control (0.25±0.1845) has highly significant (P=0.000). Ten lakh of OEC group (1.21±0.4876) analysed with control group (0.25±0.1845) proves highly significant (P=0.000) in amplitude. But more than 10 lakh of OEC group (0.56±0.0494) and control group (0.25±0.1845) has almost similar amplitude (P=0.307).

Table 6.8.8 Motor evoked potential of different dosage of MSC transplant groups mean onset time, mean peak time and mean amplitude.

Cells/Dosage	Sample size	Onset time (ms)	Peak time (ms)	Amplitude (mV)
MSC 2L	n=6	60	72	0.4172
MSC 5L	n=6	63	75	1.5553
MSC 10L	n=6	51	64	1.2361
MSC >10L	n=6	40	51	0.6733
Control	n=11	35	44	0.2557



Onset of action potential is around 40-60 msec and it is followed by peak time as shown in (Table 6.8.8).There is no much difference in onset and peak time of MEP, but difference in amplitude based on degree of axonal conduction and regeneration.

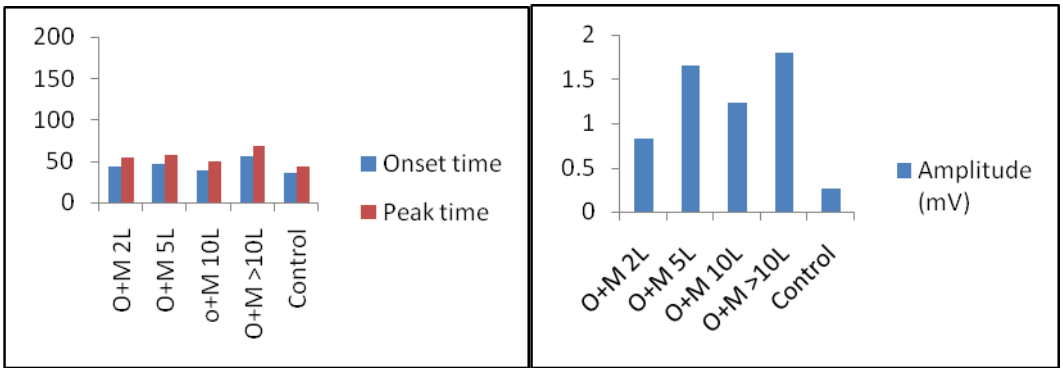
Table 6.8.9 statistical analysis of motor evoked potential -amplitude after different dosage of MSC transplantation

MSC 2L (n=6)		MSC 5L(n=6)		MSC 10L (n=6)		MSC >10L(n=6)		Control (n=11)		P value
Mean	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	
0.4172	0.1662	1.555	1.0625							0.038
0.4172	0.1662			1.2361	1.1002					0.216
0.4172	0.1662					0.6733	0.3349			0.959
0.4172	0.1662							0.2557	0.1845	0.988
		1.555	1.0625	1.2361	1.1002					0.913
		1.555	1.0625			0.6733	0.3349			0.159
		1.555	1.0625					0.2557	0.1845	0.004
				1.2361	1.1002	0.6733	0.3349			0.573
				1.2361	1.1002			0.2557	0.1845	0.043
						0.6733	0.3349	0.2557	0.1845	0.715

Though transplanted cells are same by dose's are different, this doses have impact on recovery analysed by the motor evoked potential in amplitude (Table 6.8.9). Amplitude of 2 lakh of MSC (0.41±0.1662) differs with 5 lakh of MSC (1.55±1.0625) and thus P=0.038. 2 lakh of MSC (0.41±0.1662) versus 10 lakh of MSC (1.23±1.1002) amplitude as no difference (P=0.216). Control group (0.25±0.1845) compared with 2 lakh of MSC (0.41±0.1662), more than 10 lakh of MSC (0.67±0.3349) shows no statistical significant (P=0.988), (P=0.715) respectively. Significant difference (P=0.043), (P=0.004) seen in 10 lakh of MSC (1.23±1.1002), 5 lakh of MSC (1.55±1.0625) respectively when compared with control group (0.25±0.1845). 10 lakh of MSC group (1.23±1.1002) with more than 10 lakh of MSC group (0.67±0.3349) exhibit P=0.573, which shows no variation in amplitude.

Table 6.9.0 Motor evoked potential of different dosage of OEC+MSC transplant groups mean onset time, mean peak time and mean amplitude.

Cells/Dosage	Sample size	Onset time (ms)	Peak time (ms)	Amplitude (mV)
O+M 2L	n=6	44	55	0.8316
O+M 5L	n=6	46	58	1.6485
O+M 10L	n=6	39	49	1.2328
O+M >10L	n=6	56	68	1.801
Control	n=11	35	44	0.2557



Though the cell combinations are same, but doses are different. This dosage of cell transplant has varying in regeneration, which reflects in amplitude. Onset time and peak time exhibit almost similar as shown in (Table 6.9.0).

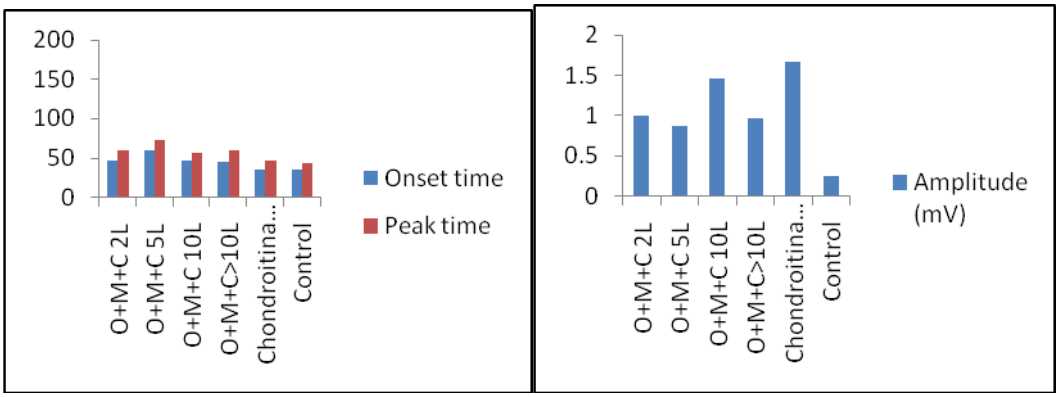
Table 6.9.1 statistical analysis of motor evoked potential -amplitude after different dosage of OEC+MSC transplantation

O+M 2L (n=6)		O+M 5L(n=6)		O+M 10L(n=6)		O+M >10L(n=6)		Control (n=11)		P value
Mean	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	
0.8316	0.5467	1.6485	0.8734							0.101
0.8316	0.5467			1.2328	0.5404					0.715
0.8316	0.5467					1.8010	0.6268			0.035
0.8316	0.5467							0.2557	0.1845	0.262
		1.6485	0.8734	1.2328	0.5404					0.688
		1.6485	0.8734			1.8010	0.6268			0.989
		1.6485	0.8734					0.2557	0.1845	0.000
				1.2328	0.5404	1.8010	0.6268			0.398
				1.2328	0.5404			0.2557	0.1845	0.012
						1.8010	0.6268	0.2557	0.1845	0.000

Combination treatment shows effective regeneration was evaluated by amplitude response in different dosage (Table 6.9.1). Control group (0.25±0.1845) compared with treated group 5 lakh of OEC+MSC (1.64±0.8734), more than 10 lakh of OEC+MSC (1.80±0.6268) proves highly significant (P=0.000). 10 lakh of OEC+MSC (1.23±0.5404) versus more than 10 lakh of OEC+MSC (1.80±0.6268) as no much difference in amplitude (P=0.398). Two lakh of OEC+MSC (0.83±0.5467) compared with more than 10 lakh of OEC+MSC (1.80±0.6268) denotes P=0.035. Control group (0.25±0.1845) with 2 lakh of OEC+MSC group (0.83±0.5467) as no significance (P=0.262) may be due low dosage.

Table 6.9.2 Motor evoked potential of different dosage of OEC+MSC+ Chondroitinase/Chondroitinase transplant mean onset time, mean peak time and mean amplitude.

Cells/Dosage/Enzymes	Sample size	Onset time (ms)	Peak time (ms)	Amplitude (mV)
O+M+C 2L	n=6	47	59	0.9959
O+M+C 5L	n=6	59	72	0.8711
O+M+C 10L	n=6	46	57	1.4593
O+M+C >10L	n=6	45	59	0.9587
Chondroitinase	n=6	36	46	1.6745
Control	n=11	35	44	0.2557



Onset of action potential is around 35-59 msec and it is followed by peak amplitude. Chondroitinase alone treated exhibit highest in amplitude (1.6745), as compared to other treated groups. Control shows least in amplitude, which shows axonal regeneration is less and action potential also lesser (Table 6.9.2).

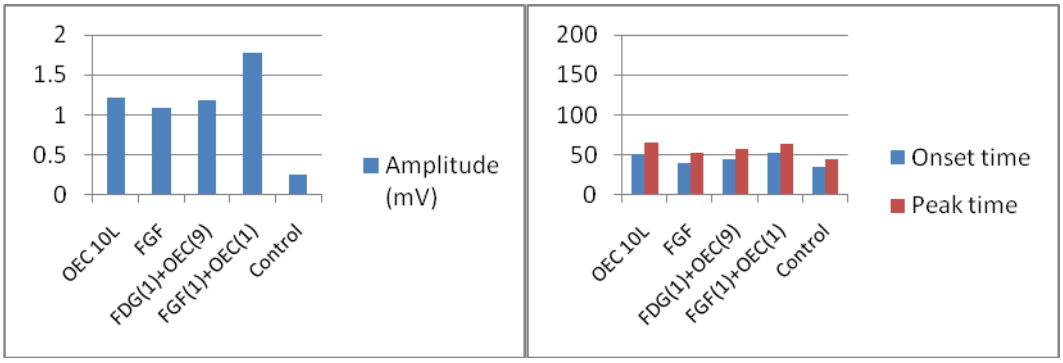
Table 6.9.3 statistical analysis of motor evoked potential -amplitude after different dosage of OEC+MSC+ Chondroitinase/Chondroitinase transplantation

O+M+C 2L (n=6)		O+M+C 5L (n=6)		O+M+C 10L (n=6)		O+M+C >10L (n=6)		Chondroitinase (n=6)		Control (n=11)		P value
Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	
0.995	0.3065	0.871	0.1745									0.999
0.995	0.3.65			1.459	1.192							0.674
0.995	0.3065					0.958	0.2574					1.00
0.995	0.3.65							1.674	0.596			0.273
0.995	0.3065									0.255	0.184	0.100
		0.871	0.1745	1.459	1.192							0.426
		0.871	0.1745			0.958	0.2574					1.000
		0.871	0.1745					1.674	0.596			0.129
		0.871	0.1745							0.255	0.184	0.243
				1.459	1.192	0.958	0.2574					0.600
				1.459	1.192			1.674	0.596			0.982
				1.459	1.192					0.255	0.184	0.001
						0.958	0.2574	1.674	0.596			0.222
						0.958	0.2574			0.255	0.184	0.133
								1.674	0.596	0.255	0.184	0.000

Chondroitinase with different dosage of cell transplant yield different outcome in amplitude of motor evoked potential studies (Table 6.9.3). Chondroitinase alone treated group (1.67±0.596) has remarkable difference (P=0.000) when compared with control (0.25±0.184). 10 lakh of OEC+MSC+Chondroitinase (1.45±1.192) increased in amplitude (P=0.001) with that of control group (0.25±0.184). 2 lakh of OEC+MSC+Chondroitinase (0.9±0.3065) compared with 5 lakh of OEC+MSC+Chondroitinase (0.87±0.1745), 10 lakh of OEC+MSC+Chondroitinase (1.4±1.192), more than 10 lakh of OEC+MSC+Chondroitinase (0.9±0.2574) shows statistically non-significant P=0.99, P=0.674, P=1.00 respectively. Overall there is no significant difference in amplitude among the OEC+MSC+Chondroitinase/ Chondroitinase treated groups.

Table 6.9.4 Motor evoked potential of FGF/FGF+OEC/OEC treated groups mean onset time, mean peak time and mean amplitude

Cells/Dosage/Enzymes/Growth factor	Sample size	Onset time (ms)	Peak time (ms)	Amplitude (mV)
OEC 10L	n=6	51	66	1.2117
FGF	n=6	40	52	1.0889
FGF(1)+OEC(9)	n=6	44	58	1.1847
FGF(1)+OEC(1)	n=6	52	64	1.7691
Control	n=11	35	44	0.2557



Onset of action potential is around 40-50 msec and the peak attains maximum at 50-60 msec as shown in (Table 6.9.4). There is no much difference in onset and peak time, but huge difference seen between control and treated rats in amplitude.

Table 6.9.5 Statistical analysis of motor evoked potential- amplitude after treatment of FGF/FGF+OEC/OEC

OEC 10L (n=6)		FGF (n=6)		OEC+FGF (1) (n=6)		OEC(1)+FGF(9) (n=6)		Control (n=11)		P value
Mean	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	
1.211	0.4876	1.0889	0.2245							0.990
1.211	0.4876			1.7691	0.8129					0.236
1.211	0.4876					1.1847	0.4694			1.000
1.211	0.4876							0.2557	0.1845	0.002
		1.0889	0.2245	1.7691	0.8129					0.097
		1.0889	0.2245			1.1847	0.4694			0.996
		1.0889	0.2245					0.2557	0.1845	0.009
				1.7691	0.8129	1.1847	0.4694			0.197
				1.7691	0.8129			0.2557	0.1845	0.000
						1.1847	0.4694	0.2557	0.1845	0.003

Amplitude of 10 lakh of OEC group (1.211±0.4876), FGF alone group (1.088±0.2245), OEC+FGF(1^{st} day) treated group (1.769±0.8129) and OEC(1^{st} day)+FGF(9^{th} day) treated group (1.184±0.4694) as considerable improvement in motor recovery analysed by MEP, P=0.002, P=0.009, P=0.000, P=0.003 respectively, when compared to control group amplitude (0.255±0.1845). Among the treated groups there is no significant seen as shown in (Table 6.9.5).

Table 6.9.6 statistical analysis of motor evoked potential -amplitude of different groups of 2 Lakh cells after transplantation

OEC 2L (n=6)		MSC 2L (n=6)		O+M 2L (n=6)		O+M+C 2L (n=6)		Control (n=11)		P value
Mean	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	
0.6967	0.3342	0.4172	0.1662							0.552
0.6967	0.3342			0.8316	0.5467					0.946
0.6967	0.3342					0.9959	0.3065			0.485
0.6967	0.3342							0.2557	0.1845	0.070
		0.4172	0.1662	0.8316	0.5467					0.183
		0.4172	0.1662			0.9959	0.3065			0.027
		0.4172	0.1662					0.2557	0.1845	0.850
				0.8316	0.5467	0.9959	0.3065			0.895
				0.8316	0.5467			0.2557	0.1845	0.010
						0.9959	0.3065	0.2557	0.1845	0.001

2 lakh of OEC (0.69±0.3342) compared with MSC 2 lakh group (0.41±0.1662), 2 lakh of OEC+MSC group (0.83±0.5467) and 2 lakh of OEC+MSC+Chondroitinase group (0.99±0.3065) shows no statistical difference in amplitude P=0.552, P=0.946, and P=0.485 respectively. Control group (0.25±0.1845) analysed with 2 lakh of OEC+MSC group (0.83±0.5467), 2 lakh of OEC+MSC+Chondroitinase group (0.99±0.3065) proves significant in amplitude, where P=0.01, P=0.001 respectively. 2 lakh of MSC group (0.41±0.1662) versus 2 lakh of OEC+MSC+Chondroitinase group (0.99±0.3065) exhibit significant (P=0.027) in MEP amplitude. Among all other treated groups shows similar in amplitude (Table 6.9.6).

Table 6.9.7 statistical analysis of motor evoked potential -amplitude of different groups of 5 Lakh cells after transplantation

OEC 5L (n=6)		MSC 5L (n=6)		O+M 5L (n=6)		O+M+C 5L (n=6)		GBC 5L (n=6)		Control (n=11)		P value
Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	
1.2072	0.4167	0.4172	0.1662									0.010
1.2072	0.4167			0.8316	0.5467							0.514
1.2072	0.4167					0.9959	0.3065					0.921
1.2072	0.4167							1.2231	0.5596			1.000
1.2072	0.4167									0.2557	0.1845	0.000
		0.4172	0.1662	0.8316	0.5467							0.406
		0.4172	0.1662			0.9959	0.3065					0.104
		0.4172	0.1662					1.2231	0.5596			0.008
		0.4172	0.1662							0.2557	0.1845	0.955
				0.8316	0.5467	0.9959	0.3065					0.972
				0.8316	0.5467			1.2231	0.5596			0.469
				0.8316	0.5467					0.2557	0.1845	0.047
						0.9959	0.3065	1.2231	0.5596			0.896
						0.9959	0.3065			0.2557	0.1845	0.005
								1.2231	0.5596	0.2557	0.1845	0.000

Amplitude of control group (0.25±0.1845) compared with 5 lakh of GBC group (1.22±0.5596), 5 lakh of OEC+MSC+Chondroitinase group (0.99±0.3065) shows P=0.000, P=0.005 respectively in significant. In treated group, 5 lakh of OEC group (1.20±0.4167) versus 5 lakh of MSC group (0.41±0.1662) as difference in amplitude (P=0.010). When 5 lakh of MSC group (0.41±0.1662) analysed with 5 lakh of OEC+MSC group (0.83±0.5467), 5 lakh of OEC+MSC+Chondroitinase group (0.99±0.3065) and control group (0.25±0.1845) gives P=0.406, P=0.104, P=0.955 respectively, Which denotes there is no statistical difference in amplitude. When 5 lakh of GBC group (1.22±0.5596) compared with 5 lakh of MSC group (0.41±0.1662) shows significant (P=0.008) in peak amplitude. 5 lakh of OEC+MSC group (0.83±0.5467) shows significant in amplitude (P=0.04) with that of control group (0.25±0.1845).

Table 6.9.8 statistical analysis of motor evoked potential -amplitude of different groups of 10Lakh cells after transplantation

OEC 10L (n=6)		MSC 10L (n=6)		O+M 10L (n=6)		O+M+C 10L (n=6)		Control (n=11)		P value
Mean	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	
1.2117	0.4876	1.2361	1.1002							1.000
1.2117	0.4876			1.2328	0.5404					1.000
1.2117	0.4876					1.4593	1.1929			0.976
1.2117	0.4876							0.2557	0.1845	0.103
		1.2361	1.1002	1.2328	0.5404					1.000
		1.2361	1.1002			1.4593	1.1929			0.984
		1.2361	1.1002					0.2557	0.1845	0.090
				1.2328	0.5404	1.4593	1.1929			0.983
				1.2328	0.5404			0.2557	0.1845	0.091
						1.4593	1.1929	0.2557	0.1845	0.023

There is a significant in amplitude (P=0.023) between control group (0.25±0.1845) and 10 lakh of OEC+MSC+Chondroitinase group (1.45±1.1929). 10 lakh of OEC group (1.21±0.4876), 10 lakh of MSC group (1.23±1.1002), 10 lakh of OEC+MSC group (1.23±0.5404) compared with control group (0.25±0.1845) yield P=0.103, P=0.090, P=0.091, which shows no differences in amplitude. As shown in (Table 6.9.8) among the treated groups, there is no statistical significances.

Table 6.9.9 statistical analysis of motor evoked potential -amplitude of different groups of more than 10 lakh cells after transplantation

OEC >10L (n=6)		MSC>10L (n=6)		O+M>10L (n=6)		O+M+C>10L (n=6)		Control (n=11)		P value
Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	
0.5695	0.0494	0.6733	0.3349							0.981
0.5695	0.0494			1.8010	0.6268					0.000
0.5695	0.0494					0.9587	0.2574			0.263
0.5695	0.0494							0.2557	0.1845	0.344
		0.6733	0.3349	1.8010	0.6268					0.000
		0.6733	0.3349			0.9587	0.2574			0.564
		0.6733	0.3349					0.2557	0.1845	0.114
				1.8010	0.6268	0.9587	0.2574			0.001
				1.8010	0.6268			0.2557	0.1845	0.000
						0.9587	0.2574	0.2557	0.1845	0.002

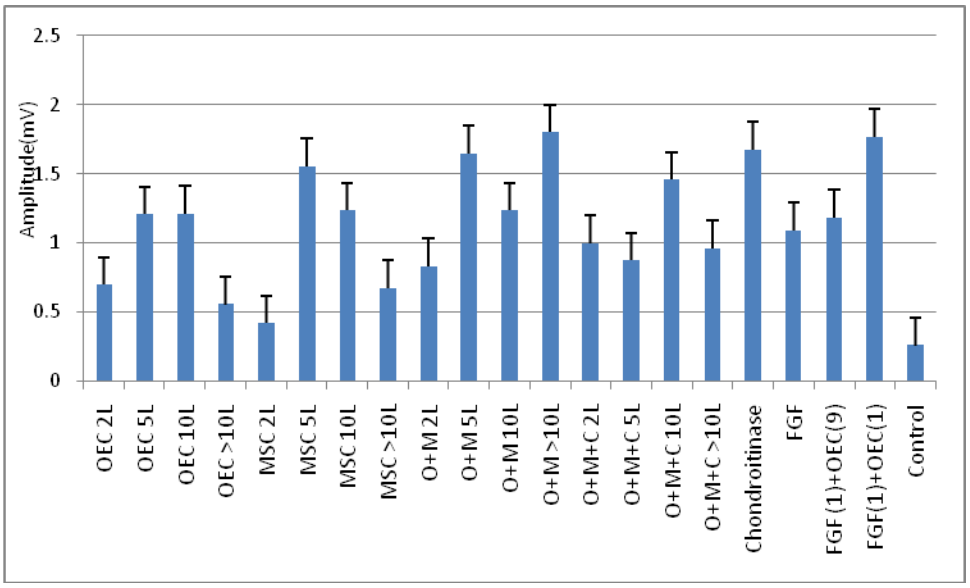
Amplitude of combinational treatment of more than 10 lakh of OEC+MSC+ Chondroitinase group (0.958±0.2574) shows remarkable increases in action potential (P=0.002) on comparison with control group (0.255±0.1845). More than 10 lakh of OEC with MSC group (1.801±0.6268) proves significant (P=0.000), when analysed with control group (0.255±0.1845). Individual cells, more than 10 lakh of OEC group (0.569±0.049), more than 10 lakh of MSC group (0.673±0.3349) shows no significant (P=0.344) and (P=0.114) respectively, when compared with control group (0.255±0.184). More than 10 lakh of OEC+MSC group (1.801±0.6268) statistical significant was seen (P=0.000), on comparison with more than 10 lakh of OEC (0.569±0.0494), more than 10 lakh of MSC (0.673±0.3349), more than 10 lakh of OEC+MSC+Chondroitinase (0.958±0.2574). However, combination therapy proves effective on evaluation by motor evoked potential studies than individual cells (Table 6.9.9).

Table 7.0.0 Motor evoked potential studies of all groups

The characteristics of motor responses following cortical stimulation after transplantation and among controls are shown below.

Cells/Dosage/Enzymes/Growth factor	Sample size	Onset time (ms)	Peak time (ms)	Amplitude (mV)
OEC 2L	n=6	53	66	0.6967
OEC 5L	n=6	59	71	1.2072
OEC 10L	n=6	51	66	1.2117
OEC >10L	n=6	61	75	0.5695
MSC 2L	n=6	60	72	0.4172
MSC 5L	n=6	63	75	1.5553
MSC 10L	n=6	51	64	1.2361
MSC >10L	n=6	40	51	0.6733
O+M 2L	n=6	44	55	0.8316
O+M 5L	n=6	46	58	1.6485
O+M 10L	n=6	39	49	1.2328
O+M >10L	n=6	56	68	1.801
O+M+C 2L	n=6	47	59	0.9959
O+M+C 5L	n=6	59	72	0.8711
O+M+C 10L	n=6	46	57	1.4593
O+M+C >10L	n=6	45	59	0.9587
Chondroitinase	n=6	36	46	1.6745
FGF	n=6	40	52	1.0889
FGF(1)+OEC(9)	n=6	44	58	1.1847
FGF(1)+OEC(1)	n=6	52	64	1.7691
Control	n=11	35	44	0.2557

Graph 7.0.1 MEP-amplitude of different group



(Graph 7.0.1) shows peak amplitude differences in different cell/combinations, most of the groups secure more than 0.5mV in amplitude, expect two group 2 lakh of MSC transplant group and control group. Action potentials are generated based on the spinal tracts continuation and regeneration. This clearly shows that transplantation of cell/enzyme/growth factor has therapeutic effects.

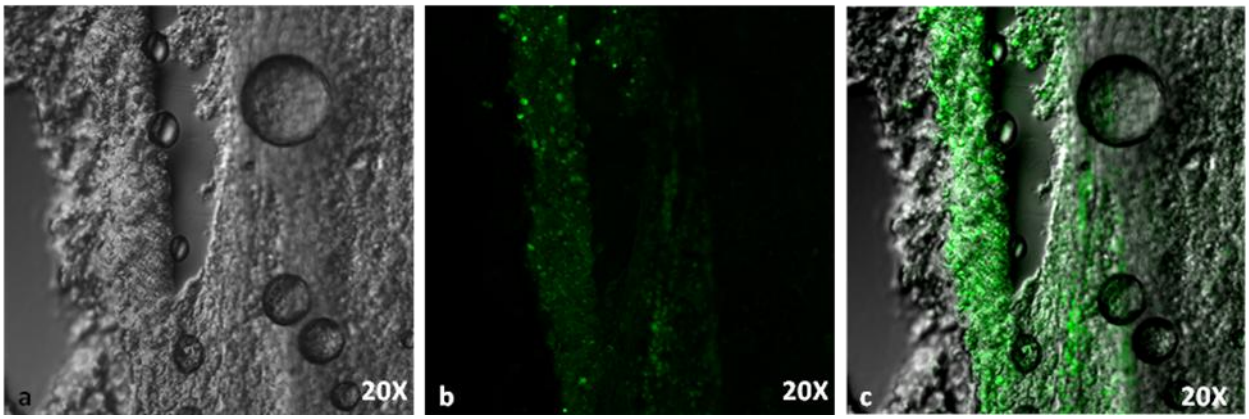


Figure 7.0.2 20µm thick cryosection of GFP labeled OEC in transplanted spinal cord.
a. Phase contrast
b. GFP positive cells in spinal cord around injury epicenter
c. Merged

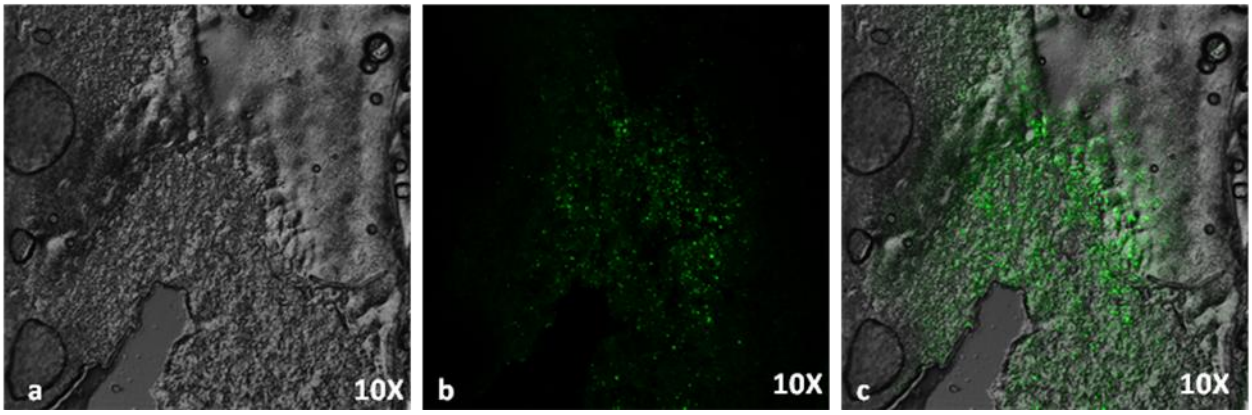


Figure 7.0.3 20µm thick cryosection of GFP labeled MSC in transplanted spinal cord.
a. Phase contrast
b. GFP positive cells in spinal cord around injury epicenter
c. Merged

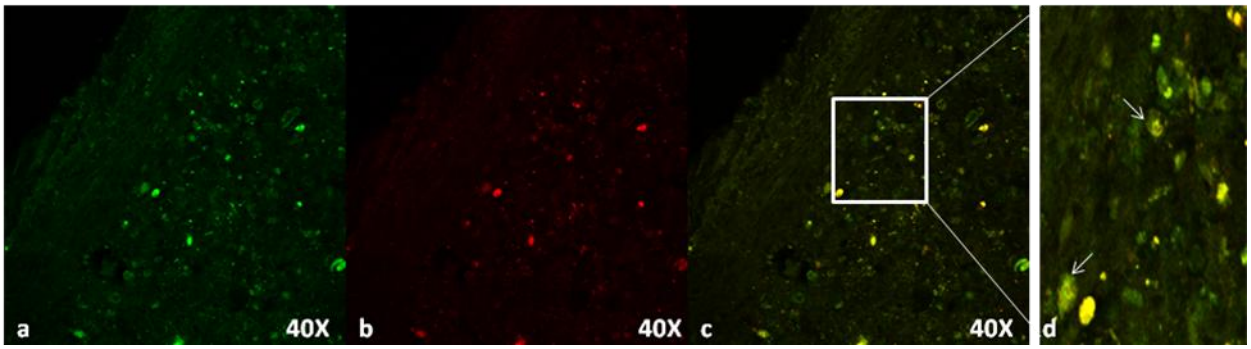


Figure 7.0.4 20µm thick cryosection of GFP labeled MSC differentiated in transplanted spinal cord
(a) MSC GFP label cells in spinal cord (green).
(b) βIII tubulin-PerCp positive cells in spinal cord (red).
(c) Merged of (a) and (b).
(d) Yellow colour (white arrow) shows differentiated neurons in spinal cord.

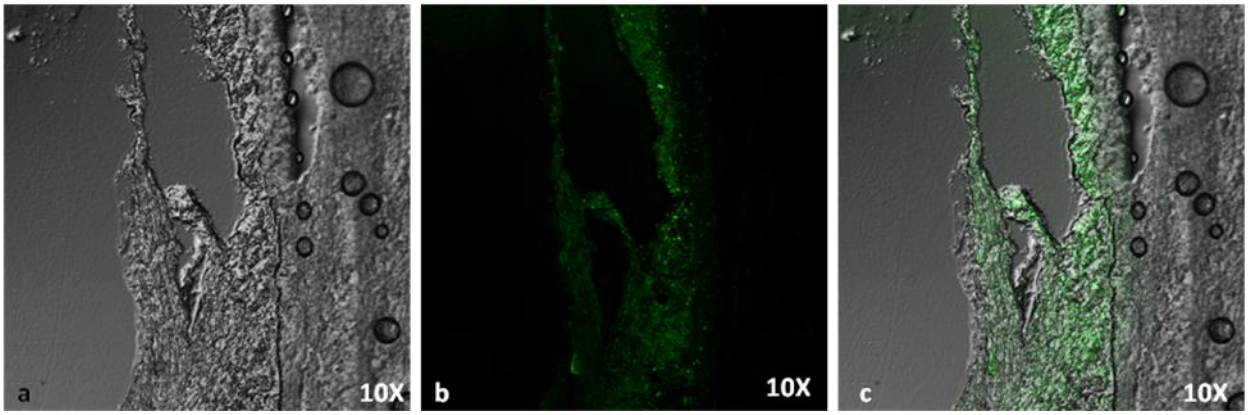


Figure 7.0.5 20µm thick cryosection of GFP labeled GBC in transplanted spinal cord.
(a) Phase contrast
(b) GFP positive cells in spinal cord around injury epicenter
(c) Merged

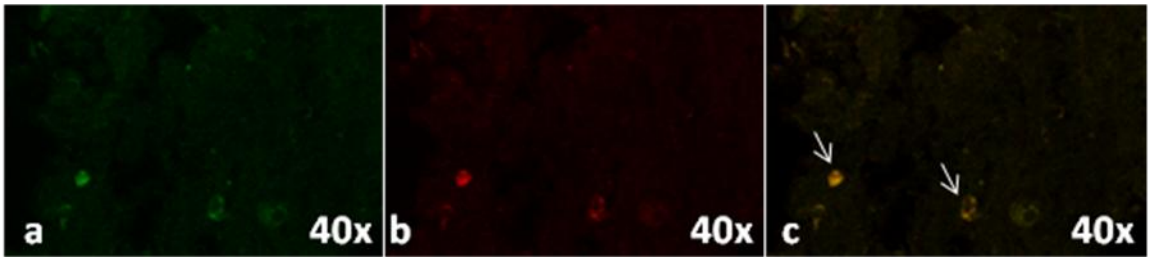


Figure 7.0.6 20µm thick cryosection of GFP labeled GBC differentiated in transplanted spinal cord.

(a) GFP labeled GBC in spinal cord (green).

(b) βIII tubulin-PerCp positive cells in spinal cord (red).

(c) Merged (a) and (b), yellow colour (white arrow) shows GBC differentiated into neurons in spinal cord.

Table 7.0.7 Statistical analysis of retrograde labelled cell bodies

	OEC		Control		P value
	Mean	Std Dev	Mean	Std Dev	
Caudal	310	14.1	280	14.1	0.33
Cranial	125	7.0	21	1.4	0.33

1.2mm caudal to the injury epicenter and 1.2mm cranial to the epicenter of both treated and control rat longitudinal section was assessed for fast blue labelled cell bodies. The number of labelled cell bodies in caudal was not significant (P=0.33) different between control (280±14.1) and transplant (310±14.1). But in cranial side, the number of labelled cell bodies was more in OEC treated (125±7.0) cord when compared to control (21±1.4), but statistically does not shows significant P=0.33. This shows that neuronal tracts are regenerated in treated groups. This information is important to interpret the results of (FB) dye tracing as a marker of regeneration in spinal cord injury.

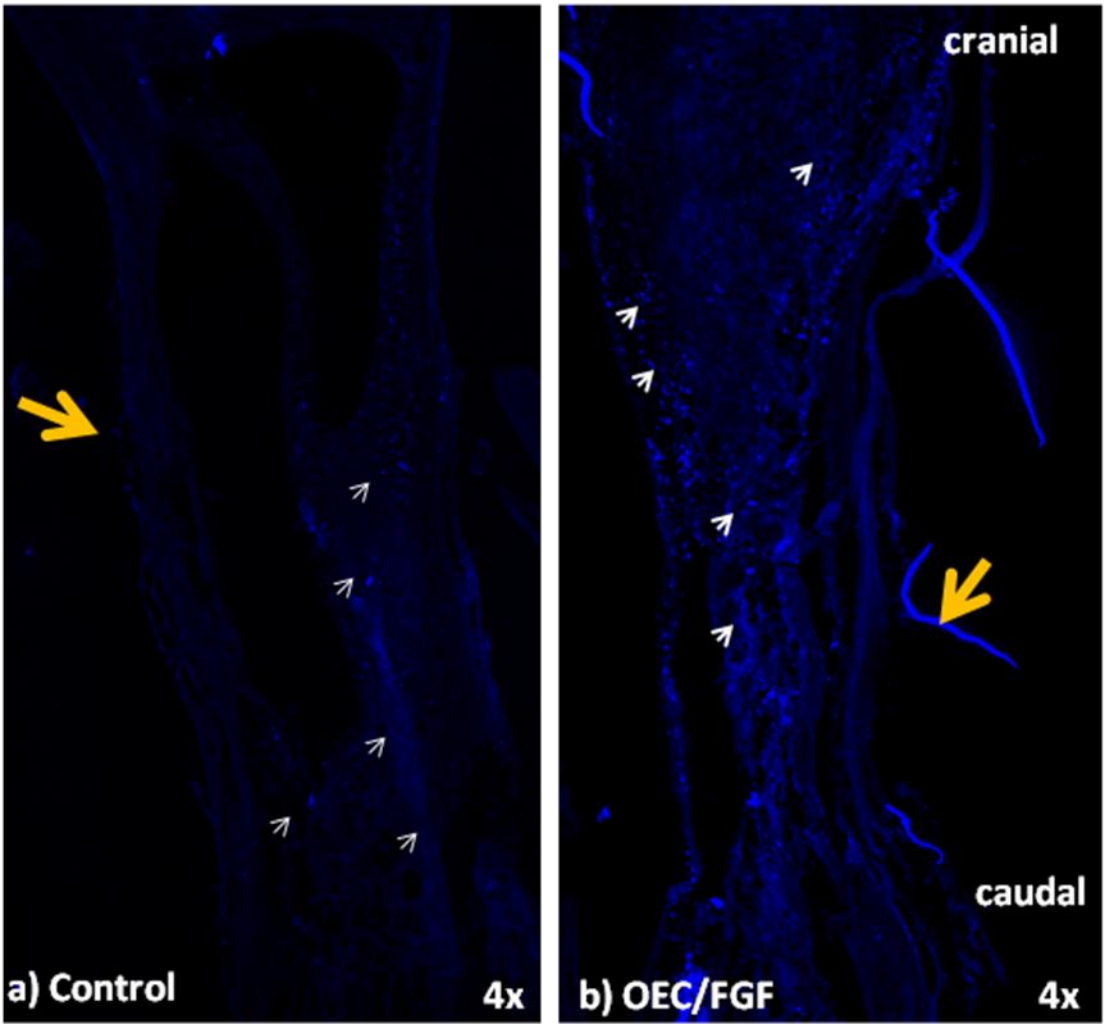


Figure 7.0.8 20µm thick cryosection of retrograde labelling of fast blue in rat spinal cord.

(a) control

(b) OEC and FGF treated rat spinal cord. (White arrow shows blue fluorescent cell bodies and yellow arrow shows injury epicenter).

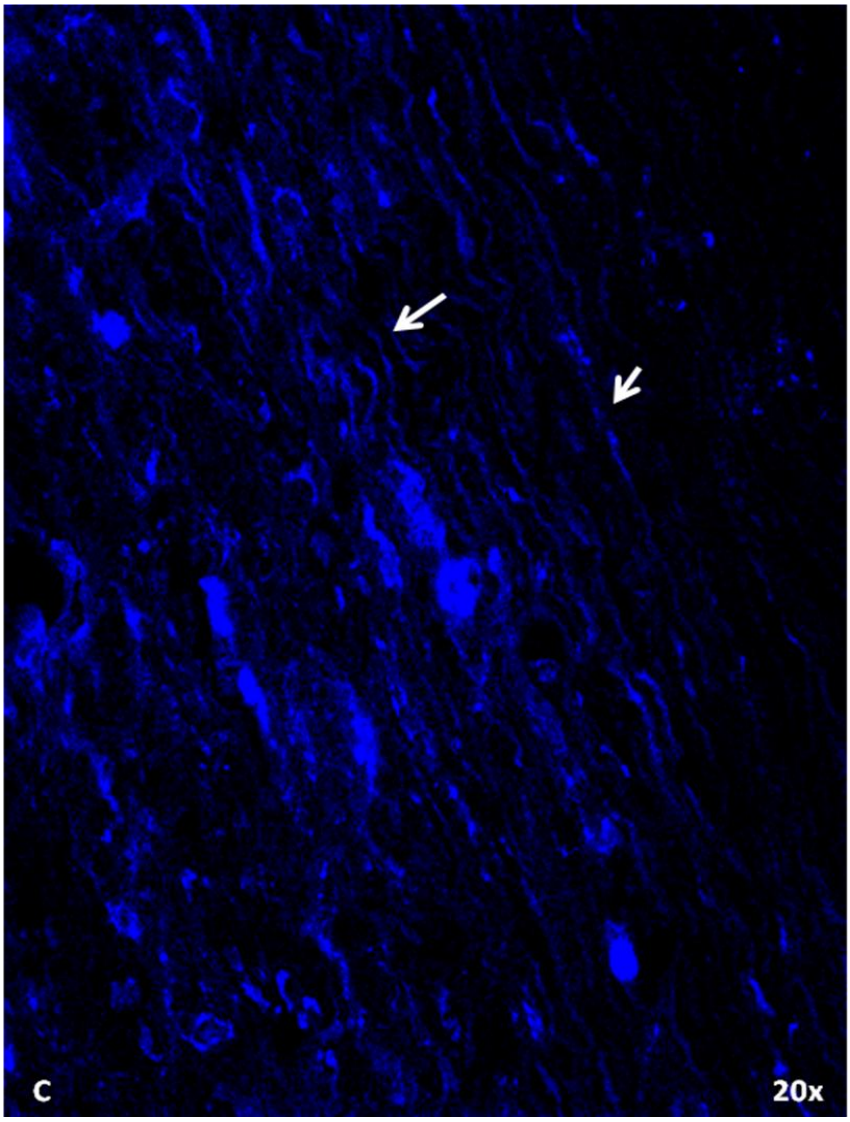


Figure 7.0.9 20µm thick longitudinal cryosection of rat spinal cord, retrograde tracer fast blue labeled axons (white arrow).

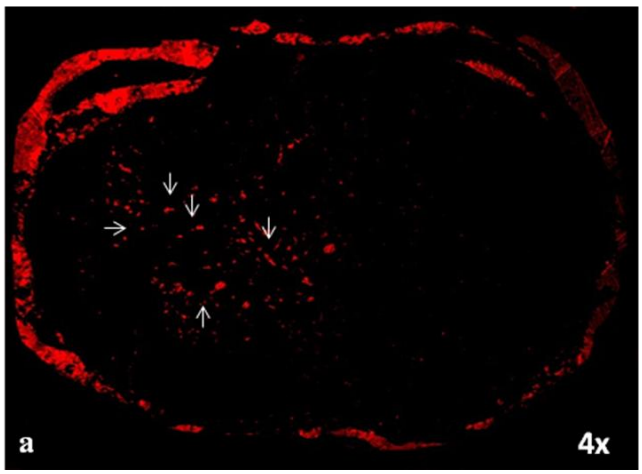


Figure 7.1.0 20µm thick cryosection of spinal cord below injury epicenter. Anterograde tracer Biotinylated dextran amine (BDA) shows labeled axons on the contralateral side (white arrow).

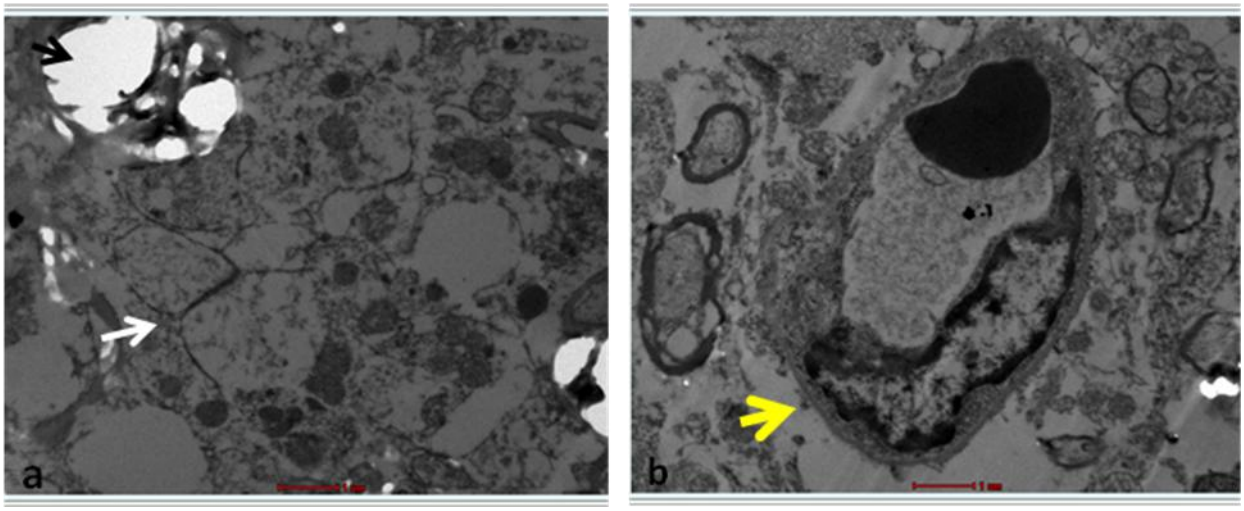


Figure 7.1.1 Electron microscopic (EM) photomicrographs showing (a) demyelinated (white arrow) and dissolved axons (black arrow) of contusion injured spinal cord (control). (b) OEC transplanted cord shows remyelination of OEC (yellow arrow). All demyelinated spinal cords that received rat OEC injections showed clear evidence of remyelination.

Histology

GFP labeled MSC surviving in the spinal cord around injury epicenter (Figure 7.0.3) and invivo differentiated into neurons, expressing β III tubulin (Figure 7.0.4). The result shows the homing of mesodermal origin MSC converted into ectodermal neuronal lineage, which is responsible for spinal cord regeneration. Similarly, Olfactory epithelial globose basal stem cells after transplanted into injured spinal cord shows homing (Figure 7.0.5) and differentiated into neurons (Figure 7.0.6). This favoured the recovery of hind limb motor function in spinal injured rats. GFP labeled olfactory ensheathing cells/olfactory nerve fibroblast surviving in the cord (Figure 7.0.2) and electron microscopic shows remyelination of demyelinated spinal cords that received rat OEC injections after contusion injury (Figure 7.1.1), which is responsible for functional recovery after SCI.

Retrograde tract tracing

Fast blue is a diamidino dye, taken up by cells, is transported over long distances and stays in the cytoplasm. Fast blue injected caudal to injury site, migrated upto the injury epicenter in control spinal cord, whereas in OEC+ FGF treated cord dye expression was seen in cranial to injury epicenter. Contol cord shows degenerative cavities and fast blue dye expressed upto injury epicenter, and beyond the injury towards the cranial side the dye expression was almost less (Figure 7.0.8). This shows that spontaneous regeneration is absent, and the tracts are not regenerated after spinal cord injury. But in OEC and FGF

treated spinal cord shows dye expression predominant in the cranial side beyond the injury epicenter. Neurons are identified by axons (Figure 7.0.9) and visible cell body.

The number of labelled cell bodies in caudal was not significant (P=0.333) different between control (280±14.1) and transplant (310±14.1). But in cranial side, the number of labelled cell bodies was more in OEC treated (125±7.0) cord when compared to control (21±1.4), but statistically does not shows significant P=0.33. This strongly suggests that tracts are regenerated after spinal cord injury in treated rats. This information is important to interpret the results of (FB) dye tracing as a marker of regeneration in spinal cord injury.

Anterograde tract tracing

Biotin-Dextran amine (BDA) injected in the motor cortex of right hemisphere of rat brain. The dye migrated and expressed on contralateral side of spinal cord (left side of cord), below the injury epicenter (Figure 7.1.0). This strongly proves that, corticospinal tract are regenerated in treated rats.

Electron microscopy

All demyelinated injured spinal cord that received OEC/ONF alone or with other combinations shows remyelination. Remyelination was characterized by large cytoplasmic and nuclear region surrounding the axons. Electron microscopy indicated that virtually all demyelinated injured spinal cord after transplantation showed clear

evidence of remyelination like peripheral type of remyelination by Schwann cells or OEC transplantation in CNS (Figure 7.1.1).

In final, all treated rats as define recovery in motor recovery scale (BBB score), and motor evoked potential shows increased amplitude as compared to control group to suggest axon continuation after injury. To support transplant-mediated repair, survival of transplanted cells in the cord by histological and electron microscopically shows positive results. In addition, anterograde and retrograde tract tracing shows tracts are regenerated in treated rat spinal cord after injury. The results strongly suggest, OEC, MSC, GBC, FGF acidic, and Chondroitinase have therapeutic beneficial effects in rat spinal cord injury model, and can be applied in human spinal cord injured patients.

Discussion

As the scientific knowledge stands today there is no treatment method available to repair the damaged spinal cord. This is contributed by multiple factors. There are two distinct aspects to failure of regeneration after CNS injury: (i) Limited intrinsic regenerative potential of the neurons. (ii) Inhibitory extrinsic environment after CNS injury. On the intrinsic side, several efforts have been undertaken to characterize the molecular signals required to stimulate growth of axons following injury. Interestingly, the axons injured in the peripheral nervous system are capable of regeneration and restoring the lost functions. Regeneration associated genes like *L1*, *c-fos*, *c-jun* and 43KD growth-associated protein are upregulated after axonal injury in PNS. This is considered to be major factor responsible for PNS regeneration, in contrast to CNS. Further it has been observed that a decrease in intracellular cAMP in mature neuron, in contrast to developing neurons, is a limiting factor for regenerative response in axotomized CNS. Phosphodiesterase inhibitor (Rolipram) can elevate intracellular cAMP level and axonal sprouting can be achieved after CNS injury. On the extrinsic side, myelin inhibitors and glial scar make nonpermissive environment for axonal growth. Activation of guanosine triphosphatase Rho, which causes growth cone collapse, resulting in poor axonal regeneration. Nogo, MAG, and OMgp established through NgR pathway are known to be potential axonal growth inhibitors within the CNS. Chondroitin sulfate proteoglycan (CSPG) at the site of injury is also another glial scar inhibitor. Since the factors inhibiting axonal regeneration in spinal cord are multifactorial a wide range of strategies have been experimented in several labs across the world. These include cell transplants like Schwann cells, pre-oligodendrocytes, Bone marrow stem cells, activated

macrophages, olfactory ensheathing cells, and other stem cells. In addition, trophic factors, growth factors like fibroblast growth factor, Brain derived neurotrophic factor, enzymes like chondroitinase, antibodies like IN-1 Nogo antibody and agents like Cethrin for inactivation of Rho, Minocycline have been used.

In this thesis it has proposed to address the inhibition of axonal regeneration in the central nervous system by three main strategies

1. Enzyme administration -Chondroitinase.
2. Growth factor administration (FGF acidic).
3. Cell transplantation (OEC, BMSC, GBC)

Chondroitinase enzyme for spinal cord regeneration

Following spinal cord injury there is a proliferation of astrocytes at the site of injury, which generate significant quantity of chondroitin sulfate proteoglycan (CSPG). This acts as a mechanical barrier for axonal regeneration. It has been observed in the literature that degradation of CSPG promotes axonal interaction between transplant and host, and enhances functional recovery following transplantation in SCI rats. Chondroitinase may be responsible for transplant-mediated axonal remodeling and recovery of functions (197,198). Chondroitinase removes carbohydrate residue molecules from the CSPG leaving lesser amount of de-glycosylated proteoglycans that are very less inhibitory to axon growth. Chondroitinase digestion of the perineuronal extracellular matrix can promote plasticity in hippocampus and visual cortex (199,200). Enhancement of plasticity in spinal cord injury may be one mechanism of regeneration. Furthermore,

plasticity and regeneration may work in concert to have better recovery after injury, but critical in modulation of corticospinal tract in spinal cord plasticity (201). Schwann cell transplantation along with chondroitinase resulted in neuronal re-growth in rat transection injury (202). Bradbury et al (2002) illustrated that chondroitinase treatment in rat dorsal column lesion improved beam walk, grid walk, paw placement and corticospinal tract conduction of rat models (184). Modulation of the host environment by CSPG may provide favorable environment for transplanted cell with host targets for axonal remodeling and functional recovery. Several studies have indicated that CSPG are abundantly expressed around the lesions on 7-8 days after CNS injury (178,203,204).

In this thesis as mentioned in the results, rats treated with chondroitinase showed highest BBB score of 7.1 at the end of 8^{th} week after administration and also demonstated higher amplitude of action potential in lower limbs following transcortical motor evoked potential studies, which is statistically significant. To observe any additive effect of chondroitinase along with cell transplantation rats were administered with chondroitinase along with 5 lakh and 10 lakh concentration of OEC and MSC. The BBB score of these rats which received cell transplantation in addition to chondroitinase showed BBB score of 5.8, suggesting no beneficial effect in increasing cell doses.Further increase in dose of cell (more than 10 lakh) with constant dose of chondroitinase showed decline in BBB score of 4.1. In low dose of cell (2 lakh) with constant dose chondroitinase declined in BBB score of 4.8, when compared to chondroitinase alone treated rats. Similarly, EMG amplitude was lesser than Chondroitinase group. The additive effect of chondroitinase

was highest, when cell transplantation was administered in dosages of 2 lakhs and 10 lakhs. This shows that chondroitinase alone or in combination with cells has therapeutic effects in spinal cord injury.

Acidic Fibroblast Growth Factor for spinal cord regeneration

The molecular mechanism of using aFGF in SCI is to attenuate the secondary injury after primary mechanical insult, provide neuroprotection and support of axonal regeneration (205). aFGF translocates into nucleus, triggers transcription and protein synthesis in invitro experiments. This intrinsic activity plays a crucial role in neuron for axonal regeneration after SCI. Neuroprotective effects are mediated by the inactivation of glycogen synthase kinase 3β (GSK3β) pathway and activation of phosphatidylinositol 3 kinase (PI3K) and Akt cascades (206–208). aFGF combined with peripheral nerve graft has been proven beneficial after complete cord transection in animal models. aFGF prevents inflammatory response and thus reduces death of neurons after SCI (173,174,176,209–211).

In this thesis as mentioned in the results, rats treated with FGF alone immediately after spinal cord injury attained highest in motor recovery of 9.6 in BBB score, when compared to FGF+ OEC treated rats. Rats which received FGF on the first day after injury and OEC on 9^{th} day showed better recovery of 8.3. Similar response was seen, when OEC and FGF was administred immediately after injury in rat's with the score of 8 in BBB scale. This clearly indicates that acidic Fibroblast growth factor inhibits

secondary inflammatory cascade, which is considered to be responsible for extensive neurological damage after spinal cord injury. This suggests that acidic FGF has neuroprotective effect in rats, which is responsible for improved functional recovery in SCI rats.

Cell transplantation for spinal cord injury

The cell based therapeutic potential strategies for SCI is on two concepts: (i) Replacement of dead cells (neuron or oligodendrocytes) after injury. (ii) Enhance or support axonal regeneration by influencing favourable environment, neuroprotection or both.

Olfactory ensheathing cells for spinal cord regeneration

In the peripheral nerves axons are myelinated by Schwann cells which permit and promote regeneration of axons and functional restoration following injury. In contrast with in the CNS the axons are myelinated by oligodendrocytes which exert an inhibitory influence for axon regeneration through OMgp(Oligodendrocyte-myelin glycoprotein), Nogo, MAG (Myelin-associated glycoprotein). With in the CNS axons of olfactory pathways are capable of regeneration, which is facilitated by its accompanying cells known as olfactory ensheathing cells. The ability of OEC to facilitate neural regeneration in central nevous system is explored in this thesis. Both olfactory ensheathing cells (OEC) and Schwann cells create an favorable environment for axonal regeneration, when transplanted into the injured CNS. Transplanted cells exert an influence on host tissue for regenerating axon beyond the transplanted area. Studies have shown that

schwann cell transplantation resulted in increase in glial fibrillary acidic protein (GFAP) expression than OEC transplant. Further inhibitory chondroitin sulfate proteoglycan (CSPG) expression was increased in Schwann cell transplantation than OEC transplantation. Schwann cell transplant exacerbate the nonpermissive CSPG expression by the host tissue, so that axon to grow beyond the injury is difficult (212).

This shows that for axonal regeneration in addition to the type of cell one has to consider the host environmental effects like CSPG, Rho, Nogo.

OEC has favourable properties of regeneration promoting environment than with schwann cells transplantation (213). Purified OEC transplant found poor survival of OEC in spinal cord lesion, Li Y et al used mixture of 50% OEC and 50% olfactory nerve fibroblast for better recovery (214). Another group achieved function recovery by using semi-purified cells (215) and 98% pure culture of OEC from olfactory bulb (216). Strikingly, when regrowing axons crosses the lesion gap, injured axons were reported to preferentially use the surface of the spinal cord in the presence of meningeal fibroblasts (216). This suggests that a cooperation of fibroblasts with transplanted olfactory ensheathing cells. Acute transplantation of purified OEC has stimulated very few injured axons to regrow around the large lesion gaps (217), but mixed OEC/ONF are involved in long-distance axon regeneration (218)

In this thesis OEC cultured from rat olfactory mucosa was characterized by flow cytometry which showed a combination of pure OEC (40%) mixed with ONF (35%).

These cells were administered in different dosages and cell combinations into rat models of spinal cord injury and responses observed. As mentioned in the results it has observed that, 5 lakh dose of OEC yields better recovery of 7.1 in BBB score as compared to other groups. Both low and high dosages of OEC transplant demonstrated lower BBB score. Transplantation of 2 lakhs cell showed 3 in BBB score and in more than 10 lakh treated rats showed in BBB score of 6.7.

EMG amplitude of rats transplanted with more than 10 lakh OEC showed lesser amplitude as compared to 5 lakh and 10 lakh of OEC treated group. The increase in BBB of more than 10 lakh group may be due to spasticity.

In two lakh dosage category, OEC in combination with MSC and OEC+MSC+Chondroitinase shows better improvement in BBB score of 4 and 4.8 respectively, in comparison with individual OECs. But in higher dosage category of OEC, 5 lakh and more than 10 lakh of OEC individual cells without MSC/Chondrotinase shows higher BBB score of 7.1 and 6.7 respectively, when compared to other individual or combination of cells. However, on assessment of all the groups of OEC transplanted rats it was observed that OEC transplant individually or in combinations with MSC, Chondroitinase, FGF shows promising results in comparison with control. Normally, OEC do not form myelin, but when they are transplanted into demyelinated cord, they have capability to remyelinate the axons like Schwann cells in the peripheral nervous system (194,219). All demyelinated injured spinal cord that received OEC/ONF alone or with other combinations shows remyelination. Remyelination was characterized by large cytoplasmic and nuclear region surrounding the axons. Electron microscopy indicated

that virtually all demyelinated injured spinal cord after transplantation showed clear evidence of remyelination like peripheral type of remyelination by Schwann cells or OEC transplantation in CNS (Figure 7.1.1). This remyelination is responsible for motor recovery (BBB score) in treated rats, and in impulse propagation from motor cortex to hind limb during EMG shows increased amplitude in rats.

Mesenchymal Stem Cells for spinal cord regeneration

Bone marrow derived mesenchymal stem cell (MSC) is a suitable cell for transplantation for regeneration of spinal cord, since it is easily obtainable for allogenic or autologous transplant, expand quickly and differentiate into types of neural cells both in vitro (220–224) and invivo (225–227). To address the true neuronal differentiation patch-clamp techniques was tried in MSC and neuronal induced MSC.

The mechanism of action is still controversial; the probable action of MSC may be through paracrine effect by secreting soluble trophic factors around the injured region. This bioactive molecules enhances homing and proliferation of endogenous stem cells in the injured location (228–230). MSC are non-immunogenic in nature,since it lacks HLA-DR B7 co-stimulatory molecules, CD80 and CD86, leading to inhibition of memory T-cell responses and attenuation of antibody production by B-lymphocyte (231,232). Allogenic bone marrow MSC transplanted in human did not elicit immune response in the host tissue (232–235). MSC injected immediately after injury showed very low or no improvement in motor function, probably because of high inflammatory response resulting in cell death. But transplantation of MSC on 7^{th} day post-injury showed better

recovery (236,237). The route of administration may vary like intravenous (238,239), Lumbar puncture (237) and directly at the site of injury through intraspinal injections. Injection at the site of injury may assure the cells are implanted into the lesion site.The only disadvantage is re-exploration and surgical risks. It has been suggested that transplanted MSC into a spinal cord lesion site enhance axonal regeneration and promote functional recovery in animal models (234,240–245). The overall effect of mesenchymal stem cell was to rescue neuronal cells by immunomodulation, neuroprotection, and possibly remyelination and neuroregeneration (246,247).

In this thesis mesenchymal stem cell were collected from rat bone marrow and they were cultured and characterized through flow cytometry as well as immunohistochemistry. Mesenchymal stem cells were administrated in different dosage and combinations to rat models of spinal cord injury and the outcome evaluated.

Cells were transdifferentiated into neural cells and characterized by immunohistochemistry. In order to determine the functional characteristics of these differentiated cells, patch-clamp studies were carried out to detect the presence of voltage-gated sodium channels.

It has been reported that mesenchymal stem cells patched during second to fourth passage exhibit fast activating delayed rectifier currents in almost all cells (196). In 4 out of 4 cells that were patched, inward sodium or calcium currents were not observed. However Li *et al* (196) have reported that 19% of rat MSCs had voltage-gated sodium currents and

8% had voltage-gated calcium currents. Mesenchymal stem cells from different days after the second passage express fast- activating delayed rectifier potassium channel and 12 day after neuronal induction, the profile of outward currents was observed, all patched cells did not express inward sodium or calcium currents. Voltage-gated sodium channel expression will be confirmatory for functional excitable cell neurons. A different method of neuronal induction has to be tried in future to get voltage-gated sodium channel expression in patch-clamp technique.

Considering different dosage of mesenchymal stem cell transplantation it was observed that a dose of 5 lakh cells treated rat's showed highest score of 6 in BBB scale, compared to 10 lakh of MSC and more than 10 lakh of MSC treated groups. In correlation with BBB score and EMG of MSC alone with different dosage treated rats exhibit similar outcome. Combination of OEC with MSC, 2 lakh and more than 10 lakh showed better effect than with individual MSC of 2 lakh and more than 10 lakh group. When MSC combined with OEC and Chondroitinase showed promising effect in BBB score than with individual mesenchymal stem cells groups. The dosage of 5 lakh showed best response with MSC or MSC and OEC or OEC+MSC+Chondroitinase group. Motor evoked potential study showed increase in amplitude of OEC+MSC group and OEC+MSC+Chondroitinase group than MSC alone treated group. However, MSC transplanted individual or in combination shows therapeutic beneficial outcome as compared to control group. This recovery could be due to transdifferentiation of MSC

into neural cells as well as paracrine effect providing neuroprotection and immunomodulation in spinal cord injured transplanted rats.

Globose basal stem cell for spinal cord injury

Neural stem cells located in sub-ventricular zone and hippocampus of brain is in fact inacessable without major invasive neurosurgical methods. Globose basal stem cell (GBC) expresses SOX2, nestin, Neural cell-adhesion molecule (NCAM), which are the markers for neural stem cell in the brain. It has the properties of bone marrow mesenchymal stem cells by expressing the markers CD54, CD29, and CD73, CD90 and CD105.

Pandit et al., demonstrated that olfactory stem cells exhibit multipotency after transplantation in mice model of cochlear damage, and showed progress in auditory function (248). Nivet.E et al., reported human olfactory ecto-mesenchymal stem cell (OE-MSC) injected into mice hippocampal lesions site, showed that the transplanted cells differentiated into neurons and exhibited restoration of learning and memory (249).

In this thesis globose basal stem cells were cultured from rat olfactory epithelium were characterized by IHC and flow cytometry. These cells were administered intra-spinally at the dose of 5 lakh cells to rat models of spinal cord injury and outcome was evaluated. It was observed that 8 weeks after transplantation BBB score improved to 7.3. In the 5 lakh doses category GBC showed best response, when compared to other 5 lakh group of

OEC, MSC, OEC+MSC, OEC+MSC+Chondroitinase. Only five lakh of GBC tried in this experiment, without any combinations with growth factor or enzymes. These transplanted cells differentiated into neurons in injured rat spinal cord. In this thesis GBC were differentiated into neural cells and characterized by immunocytochemistry and flow cytometry. Ectodermal origin globose basal stem cell (GBC) shows promising than mesodermal origin bone marrow mesenchymal stem cell (MSC). These results show promising therapeutic beneficial effects in spinal cord injury.

In principle, fuctional recovery after SCI could be achieved by two ways of axonal regrowth: sprouting of spared uninjured axons to form new circuits compensating for the lost functions and lesioned axons can be regenerated that can potentially re-form the lost connections (250,251). The long projecting descending tracts, the corticospinal tract controls voluntary movements and it is very important for functional recovery after spinal injury (252). Delivery of neurotrophic factor promotes optic nerve regeneration from retinal ganglion (253). With this background, the experiment was tried in spinal cord injured treated rats. Biotin-Dextran amine (BDA) injected in the motor cortex of right hemisphere of rat brain. The dye migrated and expressed on contralateral side of spinal cord (left side of cord), below the injury epicenter (Figure 7.1.0). Thus, contralateral neural network from the motor cortex of the brain to the spinal cord was presumably formed after the spinal injury. These data demonstrate that the contralateral projection from the motor cortex to the hind limb muscles was formed after spinal cord injury. Our present data strongly support the effect of individual therapies can be appropriately

assessed by formation of the restorative neural network that is corticospinal tract, identified in the present study.

There is a marked increase in Fast blue dye expression on cranial side of treated rats as compared to control. These data demonstrate that neuronal tracts are regenerated in treated groups. This information is important to interpret the results of (FB) dye tracing as a retrograde marker of regeneration in spinal cord injury (Table 7.0.8).

Olfactory mucosa can serve as a unique source of specialized glial (OEC), as well as globose basal stem cell and requires less invasive intervention for autologous or allogenic transplantation in human spinal cord injuries. In clinical application autologous transplantation will be advantage to overcome GVHD, with no ethical concern and genetic conflicts and no donor requirement. In allogenic cell transplantation, genetic constituents differ necessitating immunosuppressive agents to minimize the GVHD. Bone marrow MSC injected rat's shows promising motor recovery, when compared to control group. In addition combination of OEC with Chondroitinase improves in BBB score and increased action potential of MEP suggesting the recovery after transplantation. Bone marrow is an alternate source of stem cell, patient own marrow can be collected with less invasive methods from iliac crest and then transplanted. A major difference is that the olfactory mucosa is a neurogenic tissue developed from ectoderm and used for ectoderm spinal cord regeneration; whereas bone marrow is developed from mesoderm and transplanted for ectoderm spinal cord.

Summary &Conclusion

This thesis was a explorative study to evaluate potential possibilities for spinal cord regeneration. At present there is no medical treatment available to cure spinal cord injury and the person remain paralysed and incontinent for life. Based on the literature three major strategies were evaluated namely cell therapy, administration of growth factor and enzyme. All these studies were conducted in rat model of spinal cord injury.

Rat olfactory mucosa isolated from the posterior region of nasal septum, and lamina propria was enzymatically separated from the epithelium. Lamina propria was enzymatically dissociated to yield olfactory ensheathing cells (OEC) and olfactory nerve fibroblast (OEC). The cells were cultured, characterized by IHC and flow cytometry to test p75NTR (OEC), fibronectin (ONF). Fresh second passage cells were labeled with lentiviral-GFP and transplanted on the 9^{th} day following spinal cord injury with different dosage/combination into injured cord.

Olfactory epithelium enzymatic digested to yield cells. These cells were cultured and Globose basal stem cells (GBC) were isolated by GBC III antibody and characterized immunohistochemically and flow cytometry methods for expression of neural stem cell marker (nestin, SOX2, NCAM), bone marrow MSC marker (CD90, CD54, CD29,CD105, CD73) and haematopoietic marker (CD45, CD34). The result showed that GBC has the properties of both neural stem cell and mesenchymal stem cell. In addition, GBC formed neurosphere in culture condition, which is the characteristic of neural stem cell in brain. These cells were neuronally induced and characterized for neuronal marker (βIII-tubulin, MAP2, NeuN, Neurofilament). The result showed invitro

differentiation of GBC into neurons, and its multipotency. So this is considered as an alternative source of transplantation for spinal cord injury. Second passage GBCwere transplanted into spinal cord on day 9 following injury.

Bone marrow collected from rat femur and tibia, and then isolated MSC were cultured. Cultured MSC were characterized for MSC marker (CD90, CD54, CD29, CD105, CD73) and haematopoietic marker (CD45,CD14, CD34) by flow cytometry and IHC to prove purity of MSC, not contaminated with haematopoietic cells. These cells neuronally induced and characterized for positive marker of neurons and glia (MAP2, NF, NeuN,βIII tubulin, O4 and GFAP). Mature neuron express voltage-gated sodium channel, which is the hallmark of functional excitable cells. To address this issue, voltage-gated sodium channel (Nav1.1) expression was seen by IHC and patch-clamp studies was done to prove the existence of gated sodium channel; but only K^+ channel was expressed. Multipotent bone marrow mesenchymal stem cells (MSC) were lentiviral GFP labeled and then transplanted on 9^{th} day after spinal cord injury in rat.

Acidic fibroblast growth factor was administered to minimize the initial damage after spinal cord injury and inhibit secondary inflammatory cascade. The results show neuroprotective effects of aFGF.

Chondroitinase enzyme was injected into injured spinal cord to address the inhibitory, glial scar and the results showed promising effects in rat model.

OEC, MSC, GBC may differs in characteristics, but these cells introduced into spinal cord injured area for one purpose i.e., regeneration of spinal cord in order to restore the lost functions by enhancing the axons to regrow, remyelinate the demyelinated axons,

immunomodulation, replacement of neurons/glia by differentiation or transdifferentiation, neuroprotection. OEC/MSC/GBC/chondroitinase/aFGF tried individually as well as combination with different dosage in order achieve maximum beneficial therapeutic effects in spinal cord injury rat model. The outcome of transplantation was evaluated by motor recovery scale (BBB score), transcranial stimulation of motor evoked potential studies, and by histlogical methods. All spinal cord injured treated rats showed improvement in motor recovery scale (BBB score) in comparison with untreated (control) group. Similarly, EMG study shows delayed onset time of action potential followed by peak amplitude, the increase or decrease in amplitude shows the degree of regeneration of injured spinal cord after transplantation. Demyelinated injured spinal cord that received OEC/ONF alone or with other combinations shows remyelination. This remyelination is responsible for motor recovery (BBB score) in treated rats, and in impulse propagation from motor cortex to hind limb during EMG shows increased amplitude in rats. Based on the number of fibres regenerated the action potential of EMG varies. MSC and GBC showed differentiation in injured cord after transplantation. Retrograde tracer (FB) injected below the injury epicenter and the dye migrated beyond the injury to state axons are regenerated in transplanted rats. Similarly, anterograde tracer BDA injected in the hind limb homunculus motor cortex and the tracer migrated to below the injured area in treated rats. The BDA expression seen on contralateral side of spinal cord, this indicates corticospinal tract as regenerated after transplantation. There was extensive remyelination of demyelinated axons in the treated rat spinal cord. This remyelination

helps in conduction of axons and elicits functional recovery after transplantation. The data suggest that after transplantation, stem cells differentiated into neurons, Olfactory glials remyelinate the demyelinated axons, enhances the axons to grow and these regeneration was assessed by BBB, and motor evoked potential studies, anterograde and retrograde tract tracing, which shows promising effects in spinal injury rat model.

These observations indicate that cell therapy has moderate therapeutic effects. Rats treated with cell transplantation in all the groups had beneficial therapeutic effects in comparison to control group. Rats treated with Fibroblast growth factor and Chondroitinase enzyme showed better motor recovery. In dose response relationship, this study showed that 5 lakh cells have promising effects when compared to other dosages.

The study showed that the stem cell transplantation therapy for spinal cord injury treatment, though moderate in outcome in the given time period (eight weeks post-transplantation), has a definite and promising effect on the motor recovery. The cofactors, such as dose of cells delivered into the injured site, the length of motor recovery assessment period and the age of the animals used for the experimental study, have to be taken into consideration while banking on the cell therapy option for human application. It is also evident that enzymatic dissolution of the scar tissue in the vicinity of SCI with chondroitinase and the creation of suitable environment for regrowth and remyelination of damaged axons with fibroblast growth factor gave better motor recovery effect than with cell transplantation therapy. Hence, it is concluded that the combinatorial treatment for CNS injury, taking into consideration of the therapeutic value of each stem cell type

tried in this study, may not provide complete cure as expected. Nevertheless, cell therapy could give a definite relief to the patient suffering from spinal cord injury. Prolonged assessment of motor recovery for a year or so after cell transplantation is beyond the scope of this study

Importantly; OEC, olfactory epithelial cells can provide an accessible source compared to intra-cranially located neural stem/progenitors for autologous neurotransplantation, eliminating the need for immunosuppression thus reducing GVHD problems. While olfactory bulb derived OECs shows remarkable regenerative potential, but practically olfactory bulb tissue harvesting is invasive for therapeutic autologous transplantation strategies. Bone marrow mesenchymal stem cell can also be isolated less invasively from iliac crest and could be a source of stem cell. Autologous neurotransplantation for spinal cord injury should be preceded by further studies in larger animals models for future clinical practice.

Scope for further study

CNS regeneration is a multi-faceted problem, therapeutic strategies would involve combinatorial approaches neuroprotective, neuroregenerative and rehabilitative for optimizing the recovery of SCI patients.

There is a enormous progress in research, hoping to cure spinal cord injury by

1. Minimising the initial damage- Neuroprotection

Many investigations are tried by many researchers to reduce the spread of secondary damage, soon after the injury occurred. So, that minimizes the scale of injury and long-time consequences.

2. Counteracting with inhibitor- Regrowth

Enhancing the intrinsic ability of axons to reinnervate and grow as well as neutralize the plethora of inhibitors in their path (Nogo, MAG, and OMgp).

3. Removing glial scar barrier- Chondroitinase

Glial scar is a major obstacle for regrowing axons, acts as a impenetrable barrier.

4. Nurturing regrowth- Olfactory ensheathing glia

Adult neurogenesis takes place in olfactory system because of OEC, in SCI OEC enhances the growth of nerve fibres and myelinates.

5. Tissue engineering

Biocompatible scaffold could be used as guidance channel to bridge the lesion gap, so that neurons and glia can grow and does its functions.

6. Replacing damaged cells-Stem cells

Stem cells have a greater potential to replace dead neuron and glia. Whether autologous or allogenic stem cells provide immense therapeutic potential.

7. Gene therapy

PTEN deletion enhances axonal growth.

For human application following to be addressed

1. Dose.
2. Efficacy.
3. Route of administration.
4. Ethical concern.
5. Mode of action.
6. Adverse effects.

Appendix

BASSO, BEATTIE, BRESNAHAN (BBB) LOCOMOTOR RATING SCALE

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0 No observable hind limb (HL) movement.

1 Slight movement of one or two joints, usually the hip &/or knee.

1 Extensive movement of one joint or Extensive movement of one joint and slight movement of one other joint.

2 Extensive movement of two joints.

3 Slight movement of all three joints of the HL.

4 Slight movement of two joints and extensive movement of the third.

5 Extensive movement of two joints and slight movement of the third.

6 Extensive movement of all three joints of the HL.

7 Sweeping with no weight support or Plantar placement of the paw with no weight support.

8 Plantar placement of the paw with weight support in stance only(i.e. when stationary) or Occasional, frequent or consistent weight supported dorsal stepping and no plantar stepping.

9 Occasional weight supported plantar steps, no FL-HL coordination.

10 Frequent to consistent weight supported plantar steps and no FL-HL coordination.

11 Frequent to consistent weight supported plantar steps and occasional FL-HL

coordination.

12 Frequent to consistent weight supported plantar steps and frequent FL-HL coordination.

13 Consistent weight supported plantar steps, consistent FL-HL coordination; and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance or frequent plantar stepping, consistent FL-HL coordination and occasional dorsal stepping.

14 Consistent plantar stepping and consistent FL-HL coordination; and No toe clearance or occasional toe clearance during forward limb advancement predominant paw position is parallel to the body at initial contact.

15 Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs frequently during forward limb advancement predominant paw position is parallel at initial contact and rotated at lift off.

16 Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs frequently during forward limb advancement predominant paw position is parallel at initial contact and lift off.

17 Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs consistently during forward limb advancement predominant paw position is parallel at initial contact and rotated at lift off.

18 Consistent plantar stepping and consistent FL-HL coordination during gait; and toe clearance occurs consistently during forward limb advancement predominant paw position is parallel at initial contact and lift off; and tail is down part or all of the time.

19 Consistent plantar stepping and consistent coordinated gait; consistent toe clearance; predominant paw position is parallel at initial contact and lift off; and trunk instability, tail consistently up.

20 Consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, consistent trunk stability; tail consistently up.

Definitions

Slight: Partial joint movement through less than1/2 the range of joint motion.

Extensive: Movement through more than half of the range of joint motion.

Sweeping: Rhythmic movement of HL in which all three joints are extended, then fully flex and extend again; animal is usually side-lying and plantar surface of paw may or may not contact the ground; no weight support across the HL is evident.

No weight support: No contraction of the extensor muscles of the HL during plantar placement of the paw; or no elevation of the hindquarter.

Weight support: Contraction of the extensor muscles of the HL during plantar placement of the paw; or elevation of the hindquarter.

Plantar stepping: The paw is in plantar contact with weight support then the HL is advanced forward and plantar contact with weight support is re-established.

Dorsal stepping: Weight is supported through the dorsal surface of the paw at some point in the step cycle.

F-HL coordination: For every FL step a HL step is taken and the HLs alternate.

Occasional: less than or equal to half≤50%

Frequent: More than half but not always; 51 – 94%

Consistent: Nearly always or always; 95-100%

Trunk instability: Lateral weight shifts which cause waddling from side to side or a partial collapse of the trunk.

Publications

George Tharion, K. Indirani, **M. Durai**, M. Meenakshi, Suresh R. Devasahayam, Nadipi Reddy Prabhav, Cassandra Solomons, Suranjan Bhattacharji**. Motor recovery following olfactory ensheathing cell transplantation in rats with spinal cord injury**. Neurology India, Jul-Aug 2011, Vol 59, Issue 4, 566-572.

Avinash Thakur, **DuraiMurugan Muniswami**, George Tharion, Indirani Kanakasabapathy. **Isolating globose basal stem cells from Albino wistar rats using a highly specific monoclonal antibody**. Journal of Clinical and Diagnostic Research, 2013 Nov, Vol-7(11): 2419-2422.

Avinash Thakur, **DuraiMurugan Muniswami**, George Tharion, Indirani Kanakasabapathy. **Induction protocols for neuronal transformation of globose basal stem cells.** International Journal of Current Research and Review, July 2013, Vol 5(14): 16-23.

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