Therapeutic Effect of Cell Transplantation and Chondroitinase in Rat Spinal Cord Injury

Abstract

Background: Spinal cord injury (SCI) leads to permanent functional deficits because the central nervous system lacks the ability for spontaneous repair. Cell therapy strategies offered a hope in neurological repair. The clinical use of human embryonic stem cell transplantation is hampered by scientific and ethical controversies. Olfactory ensheathing cells (OECs)/bone marrow mesenchymal stem cell (MSC) is a promising cell source for autologous neurotransplantation devoid of ethical concerns. Aim: This study aimed to evaluate the combined therapeutic effect of OEC, MSC, and chondroitinase in SCI rat models. Materials and Methods: Adult female albino Wistar rats were divided into ten groups, n = 6 rats in each group and control (n = 11). T10 level laminectomy was done in anesthetized rats to create drop-weight SCI. Both OEC and MSC were transplanted on the 9th day following SCI as a combined therapy with different dosage of 2 × 10⁶, 5 × 10⁶, 10 × 10⁶, and >10 × 10⁶ at a ratio of 1:1 with/without chondroitinase (0.2 U). One group of SCI rats was treated with chondroitinase alone 0.2 U. Dulbecco’s Modified Eagle medium was injected in control rats. The outcome of transplantation was assessed using Basso, Beattie, Bresnahan (BBB) scale and motor-evoked potential studies. Results: All the treated groups showed hindlimb motor recovery in BBB score except control group (P < 0.05). All the three combinations showed better results than OEC + MSC groups in hindlimb motor recovery. In dose–response relationship, 5- and 10-lakh combinations elicited increased functional recovery than 2- and more than 10-lakh combinations. However, chondroitinase alone demonstrated a highest BBB score than any other groups. Conclusions: Chondroitinase/cell combinations have a therapeutic beneficial effect in SCI.

Keywords: Basso, Beattie, Bresnahan, chondroitinase, electromyography, mesenchymal stem cells, olfactory ensheathing cells, spinal cord injury, transplantation

Introduction

Spinal cord injury (SCI) comprises complex orchestrated pathophysiological events characterized by neuronal death, demyelination, and glial scar formation. Following injury, central nervous system (CNS) axons fail to regenerate because of axonal growth inhibitors such as chondroitin sulfate proteoglycans (CSPGs), Nogo-A, myelin-associated glycoprotein, oligodendrocyte-myelin glycoprotein,[1] reduced intrinsic growth-promoting gene expression,[2,3] lack of trophic support,[4] and inflammatory response.[5]

Bone marrow-mesenchymal stem cells (BM-MSCs) demonstrate neuroprotection by reducing cell death,[6,7] promoting endogenous cell proliferation,[8] inducing angiogenesis,[9] enhancing axonal remodeling,[10] and promoting functional recovery after CNS injury.[11] MSCs secrete various cytokines, neurotrophins, and growth factors such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF),[12] vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (FGF),[13] which might create a favorable environment for regeneration.

Olfactory ensheathing cells (OECs) facilitate and guide the regeneration of olfactory axons from the peripheral nasal mucosa to the olfactory bulb in the brain.[14] OECs have a dual characteristic of astrogial (reside in CNS) and Schwann cells (axonal ensheathment and neurotrophic support).[15] Thus, OEC has shown to promote regeneration after SCI.[16-19] The inhibitory cues of the glial scar are rich in CSPG, which is the major obstacle. To overcome this obstacle, chondroitinase ABC was employed in SCI.[20-26] Targeting

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a single factor can increase regeneration and axonal sprouting into or around the lesion site to a limited extent. Instead, an approach combining multiple targets can act synergistically to stimulate robust regeneration.

Individual treatments of OEC, MSC, and chondroitinase prove moderate efficacy in functional recovery after SCI. Hence, there is a need for combination strategies to overcome multiple factors that limit axonal growth. The current study aimed to evaluate the combined effect of OEC and MSC with/without inhibitory cues of the glial scar dissolving enzyme chondroitinase ABC in SCI rat models.

Materials and Methods

Adult albino Wistar rats were used for the study. The animals were obtained from the animal house of the institution (Christian Medical College, Vellore, Tamil Nadu, India). The study was approved by an Institutional Review Board and Institutions’ Animal Ethical Committee.

Cell culture/enzyme

Adult rat OECs from olfactory mucosa and rat BM-MSC from femur were cultured as described. Chondroitinase ABC protease free from Proteus vulgaris, catalog: 100332, Seikagaku BioBusiness Corporation, Japan, was purchased and used for the experiments.

Animal experiments

Experimental design

Sixty-five adult female albino Wistar rats were divided into ten groups (n = 6 rats in each treatment group and n = 11 rats in control). Both OECs and BM-MSCs were transplanted on the 9th day following SCI as a combined therapy with different dosage of 2 lakhs (2 × 10⁶), 5 lakhs (5 × 10⁵), 10 lakhs (10 × 10⁵), and 10 lakhs (>10 × 10⁵) at a ratio of 1:1. Chondroitinase alone 0.2 U was treated in one group of SCI rats. Combination treatment of OECs, MSCs, and chondroitinase was done with different dosages of 2-, 5-, 10-, and more than 10-lakh cells at a ratio 1:1 with a constant dose of chondroitinase (0.2 U). In the control group (n = 11) after SCI, only DMEM was injected without cells on the 9th day.

Laminectomy and spinal cord injury

Female albino Wistar rats, 100–250 g in body weight, were anesthetized 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. Tegaderm™ was applied over it to prevent fur contamination during surgery. 2.0-cm incision was made over the lower thoracic area, muscle and connective tissues were bluntly dissected to expose the T6-T9 vertebrae. A T10 level laminectomy was completed using a microsurgery bone rongeur, taking care not to damage the spinal cord. The drop-weight injury was performed by using 10-g weight rod fall from 25-cm height on the exposed spinal cord. Absorbable suture (Vicryl, Johnson-Johnson Pvt Ltd, India) was used to ligate the incised muscle and skin. Meloxicam 1 mg/kg as analgesic, enrofloxacin 2.5 mg/kg as antibiotic, and ringer lactate 5 ml/100 g were administered subcutaneously as postoperative care. Animals had free access to food and water throughout the study.

Postoperative care

Following the surgery, rats were placed in cages and monitored until they recovered from anesthesia. Rats were monitored twice a day throughout the postinjury survival period for general health and mobility within the cage. Bladder was manually expressed twice daily. Ringer lactate 5 ml/100 g was administered subcutaneously twice daily after each bladder expression for the first 7 postoperative days. Meloxicam 1 mg/kg as analgesic and enrofloxacin 2.5 mg/kg as antibiotic were administered for the first 7 postoperative days. Inspection for skin ulcers or evidence of autophagia was carried out daily. Bedding (paddy husk) was changed every alternate day.

Chondroitinase/cell transplantation

Following the drop-weight SCI, injection of chondroitinase/cell transplantation was done on the 9th day in spinal cord-injured rats. Behavioral assessment (Basso, Beattie, Bresnahan [BBB]) was conducted prior to the cell transplantation/chondroitinase treatment as described below. Rats were re-anesthetized (intraperitoneal ketamine/xylazine: 90:10 mg/kg), and the original incision was re-opened to expose the injured cord. Under a surgical microscope, the wound was explored and the injured spinal cord segment as well as a few millimeters above and below normal spinal cord was exposed. On the day of transplantation, second-passage OECs and MSCs were harvested from cultures and transferred into the 25-µl Hamilton syringe (approximately 100,000 cells/µl). All injections were made with the aid of a sterile Hamilton syringe. Both OEC and BM-MSCs were transplanted as an allogeneic combined therapy with different dosages of 2 lakhs, 5 lakhs, 10 lakhs, and more than 10 lakhs at a ratio of 1:1. Chondroitinase alone 0.2 U was treated in one group of SCI rats. Combined treatment of OEC, MSC, and chondroitinase was done with different dosages of 2 lakhs (2 × 10⁶), 5 lakhs (5 × 10⁵), 10 lakhs (10 × 10⁵), and more than 10 lakhs of cells at a ratio of 1:1 with the constant dose of chondroitinase (0.2 U). 2–50 µl of cell suspensions was injected at multiple sites, in and around at the site of injury into the spinal cord. The injured site was re-opened in the control rats and DMEM alone was injected on the 9th day. Following enzyme/cell transplantation, the surgical wound was closed and routine postoperative care was given.
Behavioral assessment – Basso, Beattie, Bresnahan score

The BBB scale\(^{[33]}\) 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 includes categorical combinations of rat hindlimb joint movements, trunk position and stability, stepping, coordination, paw placement, toe clearance, and tail position, representing sequential recovery stages that rats attain after SCI. The motor assessment will be done up to 8–10 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 (45 cm × 60 cm rectangular tray) and video-recorded for assessment. All rats were assessed for BBB before transplant, i.e., on the 9\(^{th}\) day after SCI and every week posttransplant onward up to 8 weeks.

Motor-evoked potential studies

Transcranial stimulation of motor cortex was done in the anesthetized rats and the electromyography (EMG) signals were recorded from the lower-limb muscles to indicate the functional integrity of the spinal cord. Bipolar superficial electrode was used to stimulate the motor cortex and the responses were recorded from the gastrocnemius muscles. Recording was done from control as well as cell/enzyme-transplanted rats at 8–10 weeks postspinal injury/transplantation. Recorded EMG signals were analyzed for amplitude.

Histology

At the end of the study, control and treated rats were anesthetized by intraperitoneal injection of ketamine/xylazine 90:10 mg/kg and transcardially perfused with 4% paraformaldehyde solution. A few centimeter length of spinal cord centered on the injury epicenter was removed and postfixed in 30% sucrose/phosphate-buffered saline at 4°C overnight. Longitudinal and cross-sections of spinal cord were cut and stained with hematoxylin and eosin. Representative tissue section was visualized in the light microscope.

Statistical analysis

Hindlimb motor recovery-BBB scores and amplitude of motor-evoked potential were statistically analyzed using SPSS version 16 (Apple computer Inc, Chicago, USA) one-way ANOVA post hoc Tukey’s test to compare significances with different groups. The paired \(t\)-test was also done to compare within groups. Data for each group were represented as mean ± standard deviation \(P < 0.05\) was considered statistically significant in this study.

Results

Basso, Beattie, Bresnahan

Olfactory ensheathing cell + mesenchymal stem cell (1:1)

Sequential hindlimb motor recovery was elicited in all the treated groups except control [Figure 1a]. Before transplantation, all the groups showed BBB (0.00 ± 0.00), but after transplantation BBB of 2 lakhs (4.0 ± 2.82), 5 lakhs (6.1 ± 4.62), 10 lakhs (5.1 ± 2.56), more than 10 lakhs (5.3 ± 2.50), which showed statistical significances (\(P < 0.05\)) in hindlimb motor functional recovery [Figure 2].

All the treated groups exhibited functional recovery with variation in BBB scores at the end of the 8\(^{th}\) week as compared to the control group (0.09 ± 0.30), which shows statistical significance (\(P < 0.05\)) [Figure 1b]. Different dosage elicits different outcome in motor recovery, but on statistical analysis showed no significant difference (\(P > 0.05\)) among the transplanted groups [Figure 1b]. However, in a dose–response relationship, 5 lakhs of OEC + MSC show promising maximum mean BBB score of 6.1 as compared to other dosages.

Olfactory ensheathing cell + mesenchymal stem cell + chondroitinase (1:1 + 0.2 U)

From the 1\(^{st}\) week posttransplantation onward, the hindlimb motor recovery progressed steadily in all the treated groups except control [Figure 3a]. Before transplantation, all the groups showed BBB (0.00 ± 0.00), but after transplantation BBB of 2 lakhs (4.8 ± 2.31),
Different cell dosages with chondroitinase-treated groups elicited differences in BBB score, but there was no statistically significant difference ($P > 0.05$) among the treated groups. When all the treated groups were compared with the control group, only chondroitinase group, 5 lakhs of OEC + MSC + chondroitinase group, and 10 lakhs of OEC + MSC + chondroitinase group showed statistical significance ($P < 0.05$) in functional recovery [Figure 2]. A dose–response relationship study showed that 5- and 10-lakh combinations expressed similar and better motor recovery than 2-lakh and more than 10-lakh combinations. Chondroitinase alone-treated group showed better results in hindlimb motor recovery compared with the other combinations [Figure 3].

**Electromyography**

**Olfactory ensheathing cell + mesenchymal stem cell (1:1)**

Although transplanted cells are OEC + MSC (1:1), but differ in dosage, these doses have an impact on recovery analyzed by the motor-evoked potential in amplitude. All the transplanted groups’ EMG amplitude of 2 lakhs ($0.8 \pm 0.54$), 5 lakhs ($1.6 \pm 0.87$), 10 lakhs ($1.2 \pm 0.54$), and more than 10 lakhs ($1.3 \pm 0.19$) was statistically analyzed with a control group ($0.2 \pm 0.18$), which showed statistical significance ($P < 0.05$), except 2-lakh group ($P = 0.17$). The low dose of 2 lakhs exhibited decreased amplitude, which indicates less amount of regeneration after SCI. Five-lakh group demonstrated maximum amplitude similar to BBB score, but above 5 lakhs of dosages decline in amplitude. Among the treated groups, the amplitude of 2-lakh compared with 5-lakh groups elicited statistical significance ($P < 0.05$), but the remaining groups did not show statistical significance ($P > 0.05$) [Figure 4].

**Histology**

Spinal cord tissue shows contused injury epicenter [Figure 6a]. There is a marked increase in degenerative cavity size of 2100.9 µm in control group [Figure 6b]. However, the cavity size was reduced to 1679 µm in treated (OEC + MSC + chondroitinase) SCI groups [Figure 6c] as...
compared to that of control group. Longitudinal section of the spinal cord [Figure 6d-g] showed degeneration of white matter fibers, which was increased in control group as compared to treated groups. Cross-section of the spinal cord showed dissolved and degenerated cells in gray matter [Figure 6 h-k]; there is an increased degenerative cavity in control group as compared to treated groups.

Discussion

OEC has unique properties of both CNS glial cell astrocyte and peripheral nervous system glial Schwann cells. OEC can reside in a CNS environment like astrocytes and facilitate axonal regrowth like Schwann cells.[14,34-37] OECs were found to secrete BDNF, neuregulins, NGF[38] GDNF,[39] NT-3, and NT-4.[39] Numerous studies have demonstrated that OECs have the ability to remyelinate axons in vivo models.[15,40] Both trophic/tropic actions of OEC and changes to the permissive environment of the glial scar/lesion site ultimately resulted in decreased glial fibrillary acidic protein reactivity and cavity formation.[41] These special qualities of OEC attract the researchers to consider these cells for nervous system repair. MSC contribution to tissue repair by paracrine effects (BDNF, NGF, FGF2, VEGF, transforming growth factor-β, and insulin-like growth factor 1), transdifferentiation, and neuroprotection create a favorable environment for endogenous cell proliferation and thus the functional recovery achieved after CNS injury.[42] Chondroitinase reduces inhibitory cues of CSPG and enhances functional recovery.[26-29]

The current study evaluates the effect of OEC, MSC, and chondroitinase in SCI with various parameters in consideration such as route of administration, dosage of cells with enzymes, and therapeutic window period. Varieties of spinal injury models such as complete transection,[43] hemisection,[44] tract lesion,[45] contusion,[46] and demyelination[47] were experimented in various centers. The drop-weight injury was created in the current study to mimic road traffic accident. MSC transplanted 1 week after impact SCI at T8 level in female Sprague-Dawley rats showed cell survival, differentiation, and remarkable improvement in locomotor recovery of SCI rats. The effect of transplanted MSC in injured cord shows reduced lesion cavitation and white matter loss.[48] The therapeutic window period for transplantation is also a key issue, as evidence suggests that cell engraftment and improved functional outcome if transplanted after a week, but < 14 days after injury.[49] In this study, we have chosen 9th day post-SCI for transplantation to avoid loss of cells due to the inflammatory response during acute trauma. We have transplanted cells/enzymes in and around at the site of injured spinal cord as a therapeutic strategy rather than as an infusion or injecting in the lumbar.

The combination of OEC+MSC+chondroitinase of 2-lakh group showed better motor recovery than 2-lakh groups of OEC+MSC analyzed by BBB and EMG. Similarly, all three combinations of 10-lakh groups showed increased BBB and EMG amplitude than 10-lakh of OEC+MSC group, whereas 5-lakhs of OEC+MSC and OEC+MSC+chondroitinase group exhibit approximately similar outcome. However, higher dosage of more than 10-lakhs of OEC+MSC and OEC+MSC+chondroitinase group did not increase in recovery than 5-lakhs or 10-lakhs, which may be due to saturation or unable to accommodate in the cord. In the dose-response relationship study, 5 to 10-lakh combinations have the maximum therapeutic effects of SCI of the rat models, weighing about 100-200 g. As expected, all the three combination strategies had the maximum therapeutic effects to address multiple obstacles after SCI. However, chondroitinase alone demonstrated higher BBB score than that of cell combinations. Although the sample size is small in the current study to confirm chondroitinase as the best
candidate. Histological studies demonstrated lesser amount of degenerating cells in treated cord as compared to Control. Extensive evidence of regeneration by histological method was limited in the current study. Future studies may address the in-depth mechanism of regeneration after SCI.

Conclusions

The combination of OEC, MSC, and chondroitinase shows promising therapeutic effect in SCI for future clinical applications.

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Conflicts of interest

There are no conflicts of interest.

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