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Safety and possible outcome assessment of autologous Schwann cell and bone marrow mesenchymal stromal cell co-transplantation for treatment of patients with chronic spinal cord injury

SAEED ORAEE YAZDANI[1,2], MARYAM HAFIZI[2], ALI-REZA ZALI[1], AMIR ATASHI[2], FARZAD ASHRAFI[1], AMIR-SAEED SEDDIGHI[1] & MASOUD SOLEIMANI[3]

[1]*Functional Neurosurgery Research Center, Department of Neurosurgery, Shohada Tajrish Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran,* [2]*Stem Cell Biology Department, Stem Cell Technology Research Center, Tehran, Iran, and* [3]*Hematology Department, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*

Abstract

Background aims. Cell replacement therapy has become a promising issue that has raised much hope in the regeneration of central nervous system injury. Evidence indicates that successful functional recovery in patients with spinal cord injury will not simply emphasize a single therapeutic strategy. Therefore, many recent studies have used combination strategies for spinal cord regeneration. *Methods.* We assessed the safety and feasibility of a bone marrow mesenchymal stromal cell and Schwann cell combination for the treatment of patients with chronic spinal cord injury. Eight subjects who received a complete traumatic spinal cord injury (American Spinal Injury Association [ASIA] classification A) enrolled in this study. The patients received this autologous combination of cells directly into the injury site. The mean duration of follow-up was approximately 24 months. *Results.* No magnetic resonance imaging evidence of neoplastic tissue overgrowth, syringomyelia or psuedomeningocele in any of the patients was seen during the study. There was no deterioration in sensory or motor function in any of the patients during the course of the study. Three patients had negligible improvement in ASIA sensory scale. No motor score improvement and no change in ASIA classification was seen. The patients had widely subjective changes in the course of the study such as urination and defecation sensation and more stability and trunk equilibrium in the sitting position. *Conclusions.* There were no adverse findings at least 2 years after autologous transplantation of Schwann cell and mesenchymal stromal cell combination into the injured spinal cord. It appears that the use of this combination of cells is safe for clinical application to spinal cord regeneration.

Key Words: *bone marrow, cell transplantation, combination therapy, mesenchymal stromal cell, Schwann cell, spinal cord repair*

Introduction

Functional recovery from spinal cord injury (SCI) remains one of the major goals in recent decades. SCI is a devastating clinical condition that causes permanent disabilities and economic burden on patients and societies. We know that compared with peripheral nerve injuries, damage to the central nervous system (CNS) has a low capacity for self-regeneration because neurogenesis rarely occurs. Currently, only methylprednisolone and thyrotropin-releasing hormone have been shown to have limited effectiveness for regeneration of the spinal cord (1). Cell replacement therapy has become a promising issue that has raised much hope in the regeneration of CNS injuries (2), and advances in translation of experimental animal research into clinical trials are developing.

The Schwann cell (SC) is a major cellular component of the peripheral nerve, which, during development, derives from neural crest cells. They surround peripheral axons with a spirally wrapped myelin sheath that facilitate electrical massage transmission along the axons. In addition, these cells can express and secrete various growth factors such as nerve growth factor, brain-derived neurotrophic factor, neurotrophin 3, ciliary neurotrophic factor, glial cell line–derived neurotrophic factor and fibroblast growth factor, thereby creating a permissive environment for axonal regeneration. It has been well documented that endogenous SCs migrate into the spinal cord after injury (3).

Many experimental and clinical studies have suggested that the transplantation of bone marrow

Correspondence: **Ali-Reza Zali**, MD, Functional Neurosurgery Research Center, Department of Neurosurgery, Shohada Tajrish Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran. E-mail: dr_a_zali@yahoo.com; **Masoud Soleimani**, PhD, Hematology Department, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran. E-mail: soleim_m@modares.ac.ir; **Farzad Ashrafi**, MD, Neurosurgery Research Center, Department of Neurology, Shohada Tajrish Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran. E-mail: farzad.ashrafi@gmail.com

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mesenchymal stromal cells (MSCs) could promote functional improvements after SCI (4,5). In addition, MSCs can produce neuroprotective and immunomodulatory cytokines, which rescue the neurons from cell death after injury. These cells have the ability to replace lost cells in the injured tissue through differentiation into various cell types (6,7).

The safety of MSC and SC transplantation alone in patients with SCI has been established. Many clinical trials about the application of these cells in such disorders have been completed or are now ongoing. Although the results range widely, and efficiency of these cells is controversial (8,9).

To date, it is clear that different factors, including spreading secondary damage, neuronal and axonal loss, scar formation and the presence of inhibitory molecules, account for the lack of axonal regeneration after SCI. As well, evidence indicates that successful functional recovery in patients with SCI will not simply emphasize a single therapeutic strategy. It appears that a combination strategy will be needed to cover the different mechanisms of injury (10,11). Recently, studies have used combination strategies for spinal cord regeneration, and the safety application of these approaches has been demonstrated in animal models (12–15). Few clinical studies have been conducted to assess the possible outcome of combination therapy for the treatment of patients with SCI (9,16,17). In this study, we assessed safety of co-transplantation of autologus bone marrow MSC and SC in patients with chronic, complete, traumatic SCI.

Methods

Study design and selection criteria

This study was designed in accordance with the Declaration of Helsinki and was approved by the Ethics in Medical Research Committee of the Shahid Beheshti University of Medical Sciences and Stem Cell Technology Research Center. All procedures were performed after written informed permission was obtained. Written consent included procedures of culture and analyses of biopsies from the tissue. Patients were fully aware of the experimental process of the treatment, unexpected outcomes and possible or unforeseen adverse effects such as spasticity, neuropathic pain, autonomic dysreflexia, worsening of motor or sensory function and infection.

Eight patients (four men and four women) were enrolled in this study, with a mean age of 30.50 ± 8.15 years (range, 15–45 years). All patients who were selected, had chronically (mean time between the injury and the operation was 39.80 ± 19.07 months), completed (ASIA classification A), cervical (one case) or thoracic (seven cases) lesions. Lesions resulted from road traffic accidents in six patients, fall from height in one patient, and diving in one patient (Table I).

Inclusion criteria of the study were absence of additional serious medical problems, brain disease or psychological disturbance; absence of lower motor neuron disease on electromyography; no compression, stenosis or tethering in the magnetic resonance (MR) images of the spinal cord taken in the beginning of the study; successful decompression or stabilization of SCI at least >1 year before transplantation; and demonstration of persistent complete paralysis below the level of injury. Three of eight patients had received posterior spinal fixation with instrumentation. The remainder of the patients had received only decompression without instrumentation. The patients were followed over the course of approximately 2 years (mean follow-up period was 24.37 ± 6.54 months).

Rehabilitation was considered for all patients before surgery (at least 6 months) and after surgery (by the end of study for each patient) as a clear, constant and regular program that included physical therapy strategies for encouraging motor function below the injury site.

Preoperative and postoperative neurological status of the patients was determined by American Spinal Injury Association (ASIA) score (motor and sensory), developed by the American Spinal Injury Association according to advice presented by International Campaign for Cure of spinal cord Paralysis (ICCP) panel. All patients were independently

Table I. Demographic and clinical features of patients.

Patient	Sex	Age (years)	Time from SCI (months)	ASIA classification	Level of injury	Zone of partial preservation	Follow-up (months)
1	M	17	49	A	T9	T10	38
2	F	35	13	A	T6	T7	43
3	F	45	15	A	T3	T5	27
4	F	26	51	A	T12	L1	36
5	F	26	59	A	T11	T12	27
6	M	15	21	A	T8	T9	26
7	M	25	23	A	T1	T2	36
8	M	34	63	A	C4	C5	36

examined by two trained expert clinicians, and scores were recorded at each examination time (before surgery and every 6 months after transplantation by the end of study).

MR images of the patients were taken before enrolment and also carried out to examine any changes in the spinal cord and surrounding tissues at the 6, 12 and 18 months of follow-up after transplantation.

Electromyography (EMG) was performed in special cases when the patients reported any changes in motor function to differentiate voluntary muscle contraction from reflex or involuntary spontaneous limb movement.

Cell isolation, characterization and transplantation

Patients were hospitalized for harvesting of SCs and MSCs; they were discharged after the procedure on the next day and remained ambulatory before cell transplantation surgery. Sham operations were not considered in such a pilot safety and feasibility study.

To harvest SCs according to a modification in the protocol that was set up previously in our laboratory (18), the sural nerve of the patient (12 cm in length), under general anesthesia, was taken from the calf region. The specimens were placed in Dulbecco modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) and transferred to the cell culture laboratory in a sealed, cold container under sterile conditions. In the laboratory, after removal of adjacent soft tissue from sural nerves, tissue was sliced into 1- to 2-mm pieces and treated with collagenase (1.4 U/mL; Sigma, St. Louis, MO, USA) and Dispase (2.4 U/mL; Sigma) for 3 h in 37°C. After elimination of collagenase by twice washing with DMEM/F12 and mesh filtering, the cells were incubated in DMEM/F12, not including fetal bovine serum (FBS) for 5 d (37°C, 5% CO_2). After the fasting period, the concentration of FBS (Gibco) in culture gradually increased during the period of 1 week up to 10%. Characterization and determination of the isolated cells was carried out by S-100 immunocytological staining. We double-checked the cell population with P-75 immunocytological staining, which is more specific than S-100 for SCs. In brief, after fixation of the isolated cells in 4% paraformaldehyde (Sigma), they were permeabilized with 0.1% Triton X-100 (Sigma) for intracellular antigen staining and then blocked with 5% goat serum (Gibco). The cells were then incubated at 4°C overnight with the anti-S100 or anti-P75 (both from Chemicon, Temecula, CA, USA) antibody. They were then incubated with appropriate fluoresceine-isothiocyanate–conjugated secondary antibody (Abcam, Cold Spring Harbor, NY, USA) for 3 h at room temperature. At the end of process, the cells were visualized by means of a fluorescent microscope (TE2000-S; Nikon-Eclipse, Tokyo, Japan) (18).

Bone marrow blood (100–150 mL) was aspirated from the iliac bone and diluted in Hanks balanced salt solution (HBSS, Sigma) at a ratio of 1:1. After the samples went through a density gradient by Ficoll (1.077 g/L, Sigma) at a ratio of 1:3, bone marrow blood was centrifuged (400*g* for 40 min); the mononuclear cell layer was recovered from the gradient interface and washed with HBSS. After Ficoll was removed, the cells were centrifuged three times with less gradient and time to separate platelets, and mononuclear cells were isolated. To confirm that isolated cells were MSCs, we assessed the differentiation ability of these isolated cells to adipogenic and osteogenic cells. In addition, we analyzed surface markers of the cells through flow cytometry. For osteogenic induction, the growth medium was supplemented with 100 nmol/L dexamethasone (Sigma), 50 μg/mL ascorbic acid 2-phosphate (Sigma) and 10 mmol/L β-glycerol phosphate (Merck, Rahway, NY, USA) for 21 d. To signify the calcium mineralization, samples were fixed and stained with Alizarin red (Sigma) . For adipogenic differentiation, the cells were cultured for 3 weeks in the presence of 0.5 mmol/L hydrocortisone (Sigma), 0.5 mmol/L isobutyl methyl xanthine (Sigma) and 60 mmol/L indomethacin (Gibco). Oil red O staining was performed to confirm the accumulation of oil droplets and adipogenic differentiation (19). A total of 1×10^5 cells were allocated into 2-mL microtubes with 100 μL phosphate-buffered saline (PBS, Sigma) for flow cytometry analysis. The cells were stained with monoclonal antibodies against human CD45, CD73 (all fluorescein isothiocyanate conjugated), CD105 and CD90 (both PE-conjugated; all from Abcam) at 4°C for 30 min. In every test, the appropriate isotype matching the antibody was used as control to cover nonspecific binding. Cells were then washed twice with PBS and fixed with 1% paraformaldehyde in PBS. After fixation, flow cytometry analysis was performed on FACS Calibur cytometer (Becton Dickinson) by means of CellQuest software. Win MDI 2.8 software was used to create the histograms (19).

All procedures were performed in a clean room equipped with 10,000 class facilities (Bahar Clean Room, Iran) and class 100 vertical laminar flow cabinets (Jal, Iran).

After general anesthesia was induced, the patient underwent intubation and was carefully placed into the prone position. The level of operation was determined with the use of a C-arm apparatus. After drape and preparation, the skin incision was made on the proposed area. Two subperiosteal partial laminectomies were performed; the dura was opened in

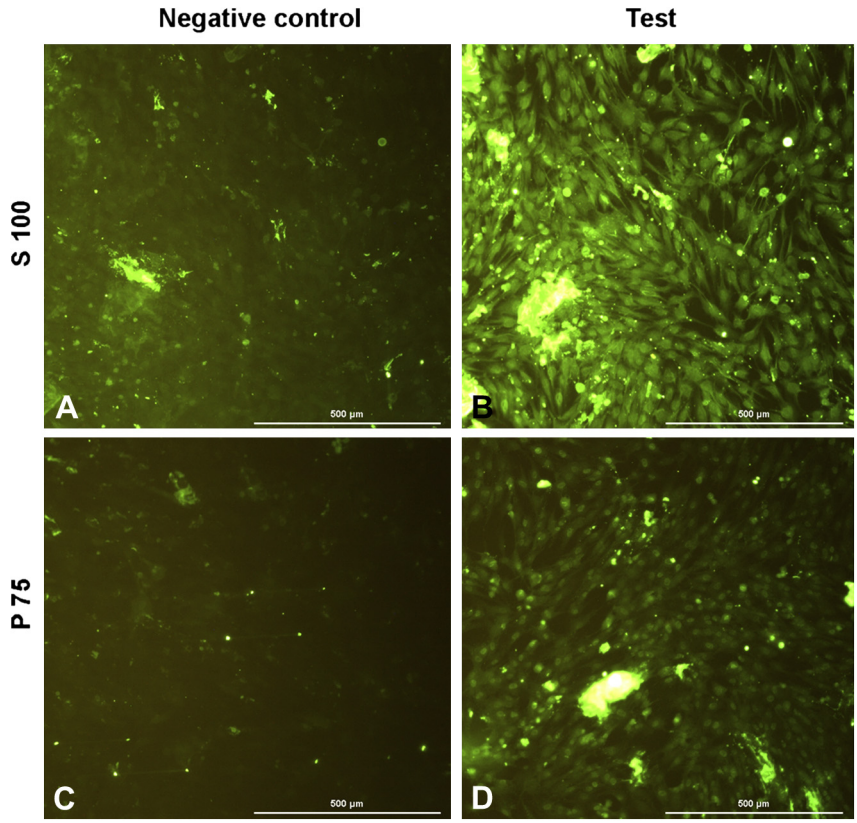

Figure 1. Immunocytochemical staining of isolated human Schwann cells. Cells were stained with S-100 and P75 antibody. (A) Negative control for S-100 (staining only with secondary antibody). (B) Staining with S-100 antibody. (C) Negative control for P-75 (staining only with secondary antibody). (D) Staining with P-75 antibody. Scale bar is 500 µm.

the midline over the injured cord, and cord exposure was attained.

In the meantime, the cell culture was washed with HBSS and treated with 1 mL trypsin–ethylenediamine tetra-acetic acid (Sigma). The cells were examined under an inverted microscope, and, after 5–10 min, the remainder of trypsin was neutralized by addition of 2 mL DMEM plus 10% FBS. The cells were then detached through subsequent pipetting and washed twice with lactated Ringers serum and were resuspended with PBS with concentration of 10^6 cells in 1 mL. Each stage was performed for the cells (MSCs and SCs) separately. At the end of preparation, the cells collected, and cell viability assay with propidium iodide (PI; Sigma) was performed. Briefly, PI intercalates into double-stranded nucleic acids and is applied as an indicator of cell viability. It can penetrate cell membranes of dying or dead cells, but viable cells remove it. To adjust flow cytometer settings for PI, we added 5–10 µL of PI staining solution to tubes, each containing 1×10^4 cells/100 µL. After gentle mixture and incubation for 1 min in the dark, PI fluorescence was determined with a FACSCanTM instrument, and data were analyzed by Win MDI 2.8 software; results were illustrated by Density plot diagrams (19). After that, a mixture of isolated cells with concentration of 10^6 cells in 1 mL (50:50 the ratio of MSC: SC) was prepared, and they were transplanted into the injured spinal cord.

After the dura was opened, cells were injected into the spinal cord at three sites throughout the damaged cord and into the proximal and distal ends of the intact cord with the use of an insulin syringe connected to a 30.5-gauge needle by the free-hand method over 2 min to limit cord damage under microscopic view. Mild bulging during injection in the site of injury confirmed the injection. The needle was kept in place for additional 1 min in every site of injection to prevent leakage and was withdrawn slowly after injection. No leakage was seen during injection and after withdrawal of the needle. The dura opening was closed with the use of nonabsorbable sutures (4–0 silk) and then covered with Gelfoam. A wound drain was positioned and connected to a collection bag. The wound was closed in anatomical layers. The patients underwent regular postoperative care, including vital sign recording, routine clinical laboratory tests, adverse events monitoring such as neurological decline and meningitis signs or symptoms, and were closely observed.

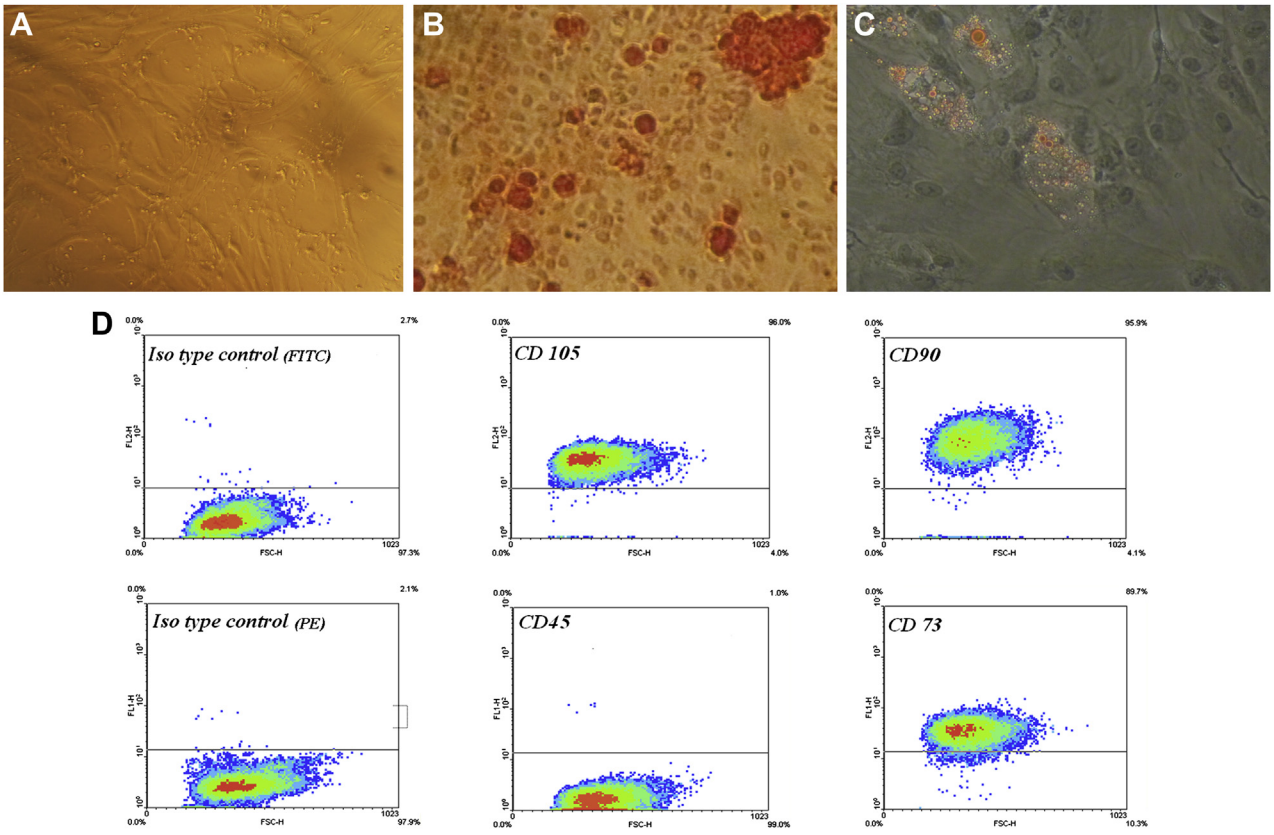

Figure 2. Characterization of isolated human mesenchymal stromal cells. (A) Phase-contrast microscopic image of undifferentiated MSCs, (B) osteogenic and (C) adipogenic differentiation of MSCs. (D) Determination of cell surface antigens by flow cytometry. The cells were positive for CD 73, CD90 and CD105 and negative for CD45, which is compatible with properties of MSCs.

Results

The cells that were harvested from the sural nerve by means of the fasting method were positive for S-100 and P-75, which showed that isolated cells had properties of SCs (Figure 1).

Bone marrow cells could differentiate to adipogenic and osteogenic cells. In addition, flow cytometry analysis revealed that these cells were positive for CD73, CD90 and CD105 and negative for CD45 (Figure 2). Data confirmed that isolated cells were MSCs.

All the procedures were performed under sterile conditions, and a test for sterility was carried out at every stage of the procedure. No contamination was detected (data not shown).

We applied the cells in second passage. Abnormal behavior of cells such as overgrowth was not observed *in vitro*. Cytogenetic analysis (karyotype) was performed. No abnormality was detected (data not shown).

Cell viability assessment was performed with the use of PI. The results showed only 2% dead cells in the cell population (cell viability was approximately 98%) (Figure 3).

There was no mortality in patients participated in study. One patient (patient 6) had a hematoma and pain at the surgery site. The hematoma had developed in soft tissue and produced no neurological deficit. At first, the hematoma was aspirated with an angiocatheter through the two edges of the sutured skin and later was managed conservatively. The hematoma resolved 4 weeks after operation. No meningitis or any infectious complication related to the transplant procedure was detected.

Some of the worst effects on visibility of images in three patients with instrumentation were seen (not shown). In the remainder of the transplant recipients, the radiological findings were unchanged, and no MRI evidence of neoplastic tissue overgrowth, syringomyelia or psuedomeningocele in any of the patients was seen. The preoperative sagittal alignment of the spine was maintained with no new external compression of the spinal cord (Figure 4).

All eight patients were evaluated at 6 months before transplantation (at the beginning of rehabilitation program), at the time of transplantation and 6, 12 and 18 months after surgery by means of the ASIA scale. After 6 months of rehabilitation (before transplantation), no improvement was seen in ASIA scale. There was no deterioration in sensory or motor function in any of the patients during the course of the study. There was no worsening of respiratory

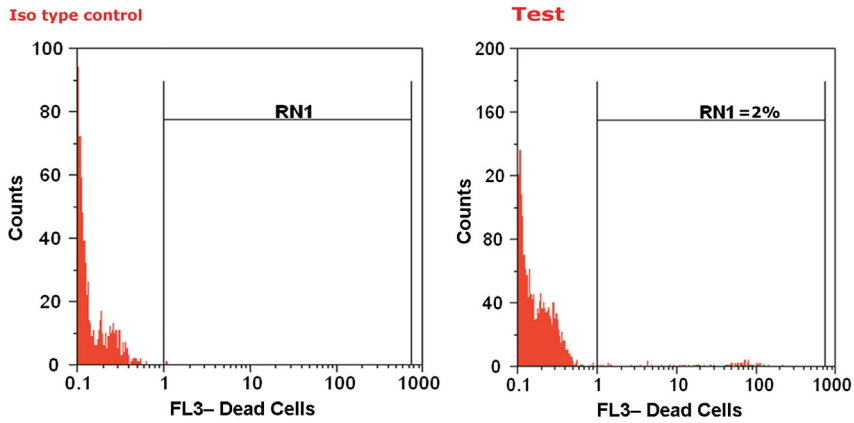

Figure 3. Viability assay of mixture of SCs and MSCs with the use of propidium iodide (PI), before (isotype control) and after (test) treatment with PI. PI fluorescence was determined with a FACScanTM instrument. Data revealed approximately 2% dead cells in the cell mixture.

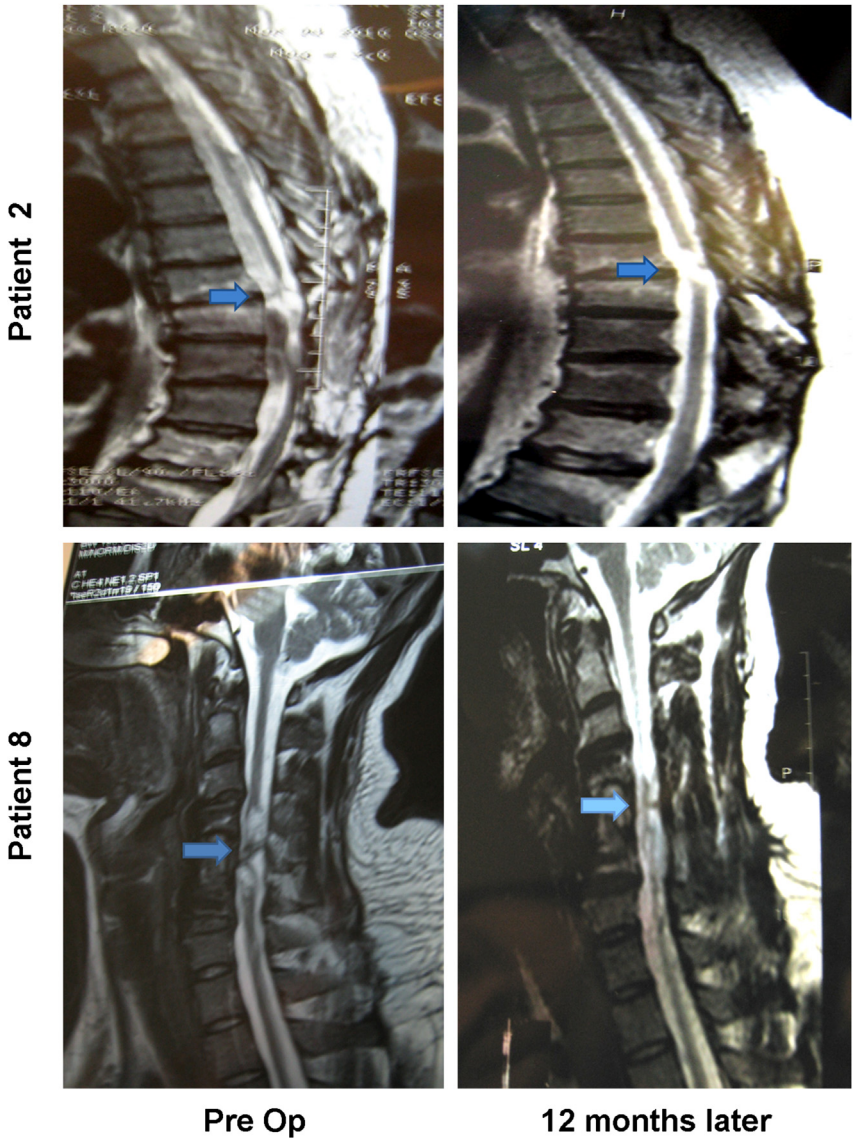

Figure 4. Magnetic resonance images of thoracic (patient 2) and cervical (patient 8) spinal cord before and 12 months after transplantation. No evidence of neoplastic tissue overgrowth, syringomyelia or psuedomeningocele was seen.

Table II. Motor and sensory levels before and 24 months after transplantation.

Patient	6 Months before Operation (S/M)	At the time of Operation (S/M)	6 Months after Op (S/M)	12 Months after Op (S/M)	18 Months after Op (S/M)	24 Months after Op (S/M)
1	66/50	66/50	74/50	70/50	70/50	70/50
2	54/50	54/50	54/50	54/50	54/50	54/50
3	44/50	44/50	44/50	44/50	44/50	44/50
4	78/50	78/50	78/50	78/50	78/50	78/50
5	74/50	74/50	74/50	74/50	74/50	74/50
6	64/50	64/50	64/50	64/50	70/50	70/50
7	48/50	48/50	48/50	52/50	52/50	52/50
8	10/17	10/17	10/17	10/17	10/17	10/17

function. Three patients (patients 1, 6 and 7) had negligible improvement in ASIA sensory scale. The changes from before transplant to 18 months after transplant in ASIA scoring for sensory light-touch and pin prick for patients 1, 6 and 7 were 6, 8 and 6, respectively. No motor score improvement was seen among the cases. One of the cases (patient 6) had changes in motor function in which the EMG demonstrated involuntary spontaneous movement and no voluntary muscle contraction (data not shown). Table II provides the estimated mean change of patient scores on the ASIA scale. All the patients were selected from ASIA classification A. Overall, no improvement was observed in ASIA classification of the patients.

The patients reported widely subjective changes during the course of the study. Three of the patients (patients 4, 5 and 8) (approximately 37%) had neuropathic pain and received the required treatment. Two of the patients (patients 2 and 7) reported an increase in lower-extremity muscle spasm that required antispasmodic medications. Paresthesia was reported by patients 2 and 6, which decreased over time and disappeared 6 months after operation. Before transplantation, all the patients had no sphincter control or urination and defecation sensation. After that, patient 2 reported urinary sensation and patients 6 and 7 were feeling defecation in addition to urination, although none achieved sphincter control. More stability and trunk equilibrium in the sitting position was reported by five of the patients (patients 1, 2, 3, 6 and 7).

Table III shows changes in sensory, motor and other aspects in the patients.

Discussion

Because of the varied and numerous changes in spinal cord tissue subsequent to injury, successful treatment for repair may involve strategies combining neuroprotection, axonal regeneration promotion and rehabilitation (10). Indeed the multi-faceted inhibitory nature of the CNS lesion suggests that therapies used in combination may be more effective (20).

Because no practical spinal repair strategies have yet been generally accepted, little surgery has been undertaken to date to promote regeneration of the human spinal cord. Clinicians and scientists in the field of spinal cord research and medicine are poised to begin translating promising new experimetal finding into treatments for people (21). Cell therapy is one of the attractive new experimental findings for spinal cord regeneration, and many trials with the use of this method have been performed (1).

Previously, SCs and MSCs were applied in some trials alone, and safety application was documented (8,9,22). MSCs have been in the spotlight of cell therapy because of their various remarkable properties, such as lack of immunologic rejection, having strong secretory properties and plasticity to develop into different cell types (6,23). SCs are known to secrete several growth factors; they can deposit growth-promoting proteins in the extracellular matrix and guide and myelinate axons (24,25).

We used the fasting method to harvest SCs, according to a protocol that was set up previously in our laboratory (18). With this method, the cells were incubated in DMEM/F12 (not including FBS) for 5 d, and, after concentration of FBS, gradually increased in the culture medium. In regard to fibroblast sensitivity to glucose deprivation (26,27), after the fasting period, most were absent from the culture. Characterization of the isolated cells was carried out by S-100 immunocytological staining, and the cell population was double-checked by means of P-75 staining. The results confirmed the presence of SCs in culture and low probability of fibroblast contamination. With regard to common results, we did not stain the cells further with P0 antibody (specific antibody for SCs).

Recently, combination therapies have been applied for spinal cord regeneration (10,11,20). Application of two types of effective cells for spinal cord regeneration is one of these combination methods. In animal models, the safety of this combination has been recognized (13,28). Although it appears that co-transplantation of olfactory

Table III. Summary of subjective and objective changes in patients.

Patient	Change in sensory level	Change in motor level	Neuropathic pain	Paresthesia	Muscle spasm	Urination sensation	Defecation sensation	Equilibrium in sitting position
1	Yes	No	No	No	No	NO	No	Yes
2	No	No	No	Yes	Yes	Yes	No	Yes
3	No	No	No	No	No	No	No	Yes
4	No	No	Yes	No	No	No	No	No
5	No	No	Yes	No	No	No	No	No
6	Yes	No	No	Yes	No	Yes	Yes	Yes
7	Yes	No	No	No	Yes	Yes	Yes	Yes
8	No	No	Yes	No	No	No	No	No

ensheathing glia and mesenchymal stromal cells does not have synergistic effects after SCI in the rat (29), Ban *et al.* (28) showed that a combination of SCs with bone marrow MSCs is one of the best cell strategies for repair after SCI in rats.

There were no adverse findings at least 2 years after autologous transplantation of combination of SCs and bone marrow mesenchymal stromal cells into the injured spinal cord. The intervening MR imaging at the end of study showed no change from before surgery for any evidence of tumor, cyst, posttraumatic syringomyelia or other adverse radiological findings. No evidence of medical problems caused by cellular transplantation was reported, which documents the safety of the use of this cell combination.

The application of ICCP panel guidelines for selecting patients for SCI trials in the safety phase led to the selection of patients with complete lesions (ASIA classification A), which are found more often at the thoracic level (30). It recommends that phase 1 trials include randomly assigned control subjects but that it is not essential. Indeed, the main purpose of this study was to assess safety and possible outcomes of SC and MSC co-transplantation for the treatment of patients with chronic SCI. In view of this topic and in accordance with the ICCP panel recommendation, consideration of randomly assigned control subjects is useful but not necessary (31). Selection of patients with at least 20 months' history of stable and steady-state neurological status before cell treatment decreases the likelihood of spontaneous recovery to a very negligible extent (32).

Although some improvement in light touch and pinprick sensation was observed, no improvement in ASIA classification was seen. These findings are in accordance with the results of Yoon *et al.* (9), which showed no improvement in ASIA scale in patients with chronic spinal injuries.

In accordance with the fact that no improvement was seen in ASIA scale after 6 months' rehabilitation (before transplantation), it appears that rehabilitation cannot improve sensory and motor scores by itself in patients with chronic spinal injuries. It does suggest that improvements are independent of rehabilitation programs.

Some subjective reports in relation to urinary and defecation sensation and stability of the trunk in the sitting position were registered. An increase in muscle spasm, neuropathic pain and paresthesia was reported by some patients.

Neuropathic pain is one of the serious complications after SCI. It might be related to the aberrant regeneration of damaged axons (33). Furthermore, some studies have reported that the cell transplantation strategy increased the risk of neuropathic

pain after surgery (34,35). In our survey, approximately 37% of patients reported neuropathic pain, which is similar to that in the results of Yoon *et al.* (9) (33% in subacute and chronic group) and Lima *et al.* (36) (29%). Therefore, we could not say with certainty that combination cell therapy increases the risk of neuropathic pain development. Studies with larger volume sizes in phase II are required for final comment on this subject.

In brief, Schwann cells are known to secrete several growth factors; deposit growth-promoting proteins in the extracellular matrix; and guide and myelinate axons. MSCs have been in the spotlight of cell therapy because of their various remarkable properties, such as lack of immunologic rejection, having strong secretory properties and their plasticity to develop into different cell types. With regard to the different capacities of these cells and the different mechanisms through which these cells make their effects, most probably harnessing the whole potential of both of these cell types has enough value to see if the mentioned cells have merit to be considered as suitable candidates for cell therapy approaches of SCIs.

This study suggests the safety of SC and MSC combination therapy in damaged human spinal cord. This combination caused no new deficit or adverse effect in patients and appeared to be safe in short- and longer-term assessments. Although limited improvement was seen in this safety phase for the assessment of efficacy of this combination, controlled trials with a large number of patients is necessary.

Conclusions

It appears that the use of an SC and MSC combination is safe for clinical application to spinal cord regeneration. Although no significant improvement was seen after transplantation and some degree of spasticity and neuropathic pain was reported, study with lager volume sizes in phase II is required to access feasibility, efficacy and side effects of combination therapy.

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