Bridging defects in chronic spinal cord injury using peripheral nerve grafts combined with a chitosan-laminin scaffold and enhancing regeneration through them by co-transplantation with bone-marrow-derived mesenchymal stem cells: Case series of 14 patients

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Objective: To investigate the effect of bridging defects in chronic spinal cord injury using peripheral nerve grafts combined with a chitosan-laminin scaffold and enhancing regeneration through them by co-transplantation with bone-marrow-derived mesenchymal stem cells.

Methods: In 14 patients with chronic paraplegia caused by spinal cord injury, cord defects were grafted and stem cells injected into the whole construct and contained using a chitosan-laminin paste. Patients were evaluated using the International Standards for Classification of Spinal Cord Injuries.

Results: Chitosan disintegration leading to post-operative seroma formation was a complication. Motor level improved four levels in 2 cases and two levels in 12 cases. Sensory-level improved six levels in two cases, five levels in five cases, four levels in three cases, and three levels in four cases. A four-level neurological improvement was recorded in 2 cases and a two-level neurological improvement occurred in 12 cases. The American Spinal Impairment Association (ASIA) impairment scale improved from A to C in 12 cases and from A to B in 2 cases. Although motor power improvement was recorded in the abdominal muscles (2 grades), hip flexors (3 grades), hip adductors (3 grades), knee extensors (2–3 grades), ankle dorsiflexors (1–2 grades), long toe extensors (1–2 grades), and plantar flexors (0–2 grades), this improvement was too low to enable them to stand erect and hold their knees extended while walking unaided.

Conclusion: Mesenchymal stem cell-derived neural stem cell-like cell transplantation enhances recovery in chronic spinal cord injuries with defects bridged by sural nerve grafts combined with a chitosan-laminin scaffold.

Keywords: Spinal cord injuries, Paraplegia, Nerve grafting, Stem cell transplantation, Neurorecovery

Introduction

The injured spinal cord has long been considered to have limited regenerative capacity.^{1,2} Stem cell transplantation has partly enhanced its regeneration as reviewed

elsewhere.^{3–5} However, after injury, the spinal cord may be disrupted and extensively defective or scarred. In such a situation, the defect between the cranial and caudal ends of the spinal cord has to be bridged first before contemplating cellular transplantation. Placing peripheral nerve grafts into the spinal cord has been successful experimentally.^{6–12} Clinically,¹³ voluntary

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contractions of the adductors and of the quadriceps have been observed. A chitosan-laminin scaffold has also been found to promote axonal regeneration.^{14–16} Both observations have stimulated us to investigate the possibility of bridging defects in chronic spinal cord injury using peripheral nerve grafts combined with a chitosan-laminin scaffold and enhancing regeneration through them by co-transplantation with bone marrow-derived mesenchymal stem cells.

Materials and methods

Patients

From 2007 to 2008, 14 patients with complete traumatic paraplegia associated with complete cord disruption and cord defects were treated with sural nerve grafting assisted by mesenchymal stem cell-derived neural stem cell-like cell transplantation and a chitosan-laminin scaffold (Table 1). Average age was 22 years (range 9–45 years); 12 were males and 2 were females. All injuries were traumatic in nature. In 11 patients, the injury was caused by a road traffic accident; in three cases, it was caused by a fall from a height. The average duration of injury at the time of surgery was 23 months (range 5 months–7 years). Thirteen patients had been treated previously with posterior internal fixation. Associated injuries included a supracondylar femoral fracture in two cases. Patients were included in the study if they were free of pressure ulcers and had complied for at least 6 months with a physiotherapy program including joint mobilization and assisted standing exercises.

Radiographic evaluation

Radiographic evaluation included plain anteroposterior and lateral radiographs and pre-operative magnetic resonance imaging. These were used to determine the skeletal level of injury (defined as the radiographic level of greatest vertebral damage¹⁷), the presence of cord disruption, and the injury zone¹⁸ (Table 1).

Stem cell and scaffold preparation

Preparation of human mesenchymal stem cells

Using a bone marrow aspiration needle and a heparinized syringe, twenty millilitres (20 ml) autologous bone marrow was aspirated from the iliac crest. This was performed under local anesthesia as an outpatient procedure. The bone marrow aspirate was isolated from the syringe under aseptic conditions, and, following the practice of other authors,^{19–21} transferred to a 50-ml tube filled with 20-ml culture medium (N,N'-Dimethylethylenediamine (DMED) with 10% fetal bovine serum, penicillin G [100 U/ml] and streptomycin (100 U/ml)). The tube was centrifuged at

Table 1 The demographic data of the patients and operative findings

Pt	Age (yrs)/sex (M, F)	Type of trauma	Skeletal level of injury	Presentation/associated injuries	Time of surgery after injury	Previous surgical fixation	Injury zone	Findings at surgery
1	9 M	Rta	No fracture	–	1 yr	–	T1–T2	Tethered dura, cord disruption 2 cm
2	45 M	Rta	T8	–	7 yrs	Post-fix	T5–T8	Tethered dura, cord disruption 2 cm
3	24 F	Rta	T10	–	3 yrs	Post-fix	T9–T12	Tethered dura, cord disruption 2 cm
4	40 M	Rta	T12	–	8 mths	Post-fix	T12–L2	Tethered mutilated dura, cord disruption 4 cm, reropulsed fragments
5	27 M	Rta	T12	Scfrr	1 yr	Post-fix	T12–L3	Tethered mutilated dura, cord disruption 4 cm
6	31 M	Rta	T12	–	10 mths	Post-fix	T11–L1	Tethered mutilated dura, cord disruption 2 cm
7	26 M	Rta	T12	–	10 mths	Post-fix	T12–L1	Tethered mutilated dura, cord disruption 4 cm
8	21 M	Fall	T12	–	2 yrs	Post-fix	T12–L1	Tethered mutilated dura, cord disruption 2 cm
9	24 F	Fall	T12	Bill scfrr	3 yrs	Post-fix	T12–L1	Tethered mutilated dura, cord disruption 4 cm
10	32 M	Rta	T11	–	8 mths	Post-fix	T11	Tethered dura, cord disruption 2 cm
11	29 M	Rta	T11	–	1 yr	Post-fix	T11–T12	Tethered dura, cord disruption 2 cm
12	45 M	Rta	T12	–	2 yrs	Post-fix	T12–L2	Tethered mutilated dura, cord disruption 4 cm, reropulsed fragments
13	21 M	Rta	T12	–	5 mths	Post-fix	T10–L2	Tethered mutilated dura, cord disruption 4 cm, reropulsed fragments
14	27 M	Fall	T12	–	1 yr	Post-fix	T12–L1	Tethered mutilated dura, cord disruption 4 cm, reropulsed fragments

Rta, road traffic accident; fall, falling from a height; scfrr, supracondylar fracture femur; yr, year; mth, month; posterior fixation, post-fix.

2000 rpm for 10 minutes and the cell pellet was resuspended in 40 ml culture medium. A gradient centrifugation method¹⁹ was used to separate bone marrow cells and red blood cells. The cell suspension was loaded on 1.073 g/ml gradient Percoll. The cells were centrifuged at 900g for 30 minutes. The top two-thirds of the total volume were transferred into a tube, centrifuged again at 2000 rpm for 10 minutes, and then washed with phosphate-buffered saline (PBS) to remove the Percoll. This procedure was repeated and the cell pellet was then re-suspended in culture medium. The cells were cultured in DMED with 10% fetal bovine serum, penicillin G (100 U/ml), and streptomycin (100 U/ml). They were incubated for 48 hours and then washed with PBS. The culture medium was changed twice a week for 28 days. Finally, almost all the hematopoietic cells were washed away after several times of medium changing.

Thus, mesenchymal stem cells were grown in culture for enough time to give a suitable number of cells. Mesenchymal stem cell passage number was three.

Identification of undifferentiated human mesenchymal stem cells

Under the inverted microscope, undifferentiated human mesenchymal stem cells were found to be spindle-shaped, attached to the culture dish tightly, proliferated in the culture medium, and were fibrocyte-like. Hematopoietic stem cells were round, did not attach to the culture dish, and were washed away with the culture medium changes. Human bone marrow-derived mesenchymal stem cells showed active proliferative capacity *in vitro* with primary and passage culture.

Having been cultured *in vitro* for 4 weeks, undifferentiated mesenchymal stem cells were identified by fluorescent activated cell sorting by the following superficial markers: CD71⁺ (a cell-surface marker characteristic of mesenchymal stem cells), CD34⁻, CD45⁻ (hematopoietic stem cell markers).^{20,21} The cells stained positively for CD71 (54.5%), but negative or minimally (2.5%) positive for CD34 and CD45.

Differentiation of mesenchymal stem cells into neural stem cell-like cells

For neurogenic induction, subconfluent human mesenchymal stem cells were cultured in the control medium supplemented with 1 mM mercaptoethanol (Sigma, St. Louis, MO, USA) for 24 hours followed by culture in neurobasal medium supplemented with

B27 and 20 ng/ml of brain-derived neurotrophic factor (Invitrogen, Grand Island, NY, USA).²²

Preparation of differentiated cells for transplantation

Cultured cells were dissociated from the culture dishes with 0.25% trypsin (Gibco, Grand Island, NY, USA), neutralized with culture medium, and collected by 2000 rpm centrifugation for 10 minutes at room temperature. The cells were next washed twice with PBS and then suspended in PBS at final concentration of 10⁶/ml for transplantation.²¹

Identification of differentiated mesenchymal stem cells by morphological changes

Under the inverted microscope, the spindle-like undifferentiated mesenchymal stem cells were seen to convert into dendritic-like cells, indicating neurogenic differentiation.

Polymerase chain reaction identification of nestin and S100 β gene expression

Total RNA was extracted from cells using RNeasy Purification Reagent (Qiagen, Valencia, CA, USA), and then a sample (1 μ g) was reverse transcribed with Avian Myeloblastosis Virus (AMV) reverse transcriptase for 30 minutes at 42°C in the presence of oligo-dT primer. PCR was performed using the specific primers

Fw (forward): 5'-TTCCCTTCCCCCTTGCCTAATACC-3'

Rv (reverse): 5'-TGGGCTGAGCTGTTTTCTACTTTT-3' and

5'-AATGTTTCAGTGCAGAGC-3' and

Rv (reverse): 5'-TTGGGATGATGTCGGGAC-3'.

PCR was performed for 35 cycles, each cycle consisting of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, and elongation at 72°C for 1 minute, with an additional 10-minute incubation at 72°C after completion of the last cycle. The PCR product was separated by electrophoresis through a 1% agarose gel, stained, and photographed under ultraviolet light.

There was a marked difference in nestin and S100 β mRNA expression between undifferentiated and differentiated human mesenchymal stem cells.

Preparation of chitosan

Chitosan was deacetylated by heating in 50% NaOH aqueous solution at 120°C for 2 hours. The resulting product was washed in methanol for 1 day. The dried chitosan powder was dissolved in 0.2 mol

acetic acid by stirring to prepare a 1 W/W% chitosan paste.

Preparation of laminin

The chitosan paste was enriched with 50 µg of laminin to produce a tissue-engineered scaffold.

Operative technique

The spine was exposed through the same posterior spinal laminectomy incision used before for posterior fixation. The presence of cord disruption was confirmed. After incising the posterior dura longitudinally and keeping it open using 3/0 Prolene stay sutures, the superior and inferior extents of the injury zone were determined by the absence of fibrosis (gliosis) within the cord, the absence of adhesions between the pia mater, dura, and cord. Next, the dura was expanded and reconstructed using a fascia lata graft (Fig. 1A–1F). This was essential to create ample space for placement of the nerve grafts and scaffold afterwards. The cellular (epimysial) side of the fascia lata was used to create the internal lining of the dura covering the cord-graft construct. This was based on the increasing evidence that leptomeninges had an important role in central nervous system (CNS) restoration.²³ Capacious reconstruction of the dura was performed to allow for cerebrospinal fluid circulation.²⁴

This done, sural nerves were side grafted to the cord,²⁵ especially on its ventral aspect²⁶; both sural nerves served as grafts; a length extending from the lateral malleolus up to middle of the popliteal fossa was obtained (Fig. 1E and 1G–1I). Grafts extended for at least 4 cm both proximal and distal to the superior and inferior ends of the injury zone, respectively.²⁶ At the dorsolumbar level, grafts were extended inferiorly to the cauda equina. Side grafting of the grafts to the ventral aspect of the cord, the area of the cord where motoneurons were found, was meant to induce motoneuron axon sprouting into the grafts. This was carried out experimentally,²⁷ when the avulsed C5–C8 ventral roots in *Macaca fascicularis* monkeys were re-implanted into the ventrolateral part of the spinal cord either immediately or after a delay of 2 months. Clinically, motor function significantly improved after re-implanting avulsed spinal roots directly to the ventral aspect of the spinal cord.²⁸

Before final closure of the dura, a chitosan scaffold containing laminin as a neurite outgrowth promoting factor^{14,29,30} was applied before injecting the autologous stem cells (Fig. 1J). Next, differentiated autologous stem cells were injected into the scaffold superior to the injury

zone, along the graft-cord construct and inferior to the injury zone (Fig. 1K and 1L).

Neurological evaluation

Patients were evaluated at monthly intervals for 2 years

Motor power, sensation, and the American Spinal Impairment Association (ASIA) impairment scale were evaluated using ASIA standards¹⁷ (Tables 2–5). Confounding factors during motor power evaluation included pseudo muscle contractions produced by movements of the trunk and co-contractions between abdominal muscles and different muscle groups. Confounding factors were excluded by testing muscles at three separate instances. Electromyographic studies might have helped, but they were not performed. Optional elements of ASIA neurological impairment assessment were included, because the abdominal muscles and medial hamstrings were the first muscles to regain power.

In testing abdominal muscles, patients with positive Beevor's sign were considered neurological level T8; patients with full abdominal power but weak lower limbs were considered neurological level T12.

In addition, the gluteus maximus and hip abductors were included in the present study, because according to neuromuscular orthopaedics,³¹ patients with a Grade-4 gluteus maximus could stand erect, provided the knee was splinted; with a Grade-4 gluteus maximus and Grade-4 knee extensors, patients could walk unaided.

Components of rectal examination included: touch sensation, pinprick sensation, resting sphincter tone, maximal voluntary contraction, reflex contraction (bulbocavernosus, anal wink), and deep pressure sensation.

The modified Ashworth scale (Table 3) was used to assess muscle spasm.³² Pain was assessed by means of a visual analogue scale from 0 up to 10: 0 no pain, 2 annoying, 4 uncomfortable, 6 dreadful, 8 horrible, and 10 agonizing.

The Lapidus classification³³ was used to assess bladder function. This classification combined the clinical and cystometric findings (capacity, proprioception, contractility, heat and cold sensation, and residual urine). It comprised of the following categories:

1. Sensory neurogenic bladder (due to interruption of sensory afferents): impaired bladder sensation; decompensation with significant residual urine.
2. Motor paralytic bladder (due to destruction of parasympathetic motor innervations): early cystometric filling normal, but bladder contraction absent; large residual urine due to chronic overdistension and decompensation.

Figure 1 This figure illustrates the findings at surgery and the operative technique. (A) Posteroanterior photographic illustration of the surgical site after paravertebral muscle retraction, exposure of the previously inserted vertebral pedicular fixation system, and widespread laminectomy. There is evidence of cord disruption leading to a cord defect, a finding common to all of our 14 patients. Subsequent motor recover in spite of this defect excludes the possibility of spontaneous improvement or improvement by surgical manipulation alone without grafting. (B) Posteroanterior and lateral diagrammatic representation of the previous photographic illustration. At both cranial and caudal ends adjacent to the defect, the dura and the cord are mutilated and adherent to the underlying posterior longitudinal ligament. The red arrow points to the cord defect, but the injury zone is more extensive, as its superior and inferior extents are determined by the absence of fibrosis (gliosis) within the cord, the absence of adhesions between the pia mater, dura, and cord. (C) Photographic illustration showing the extent of cord disruption after releasing the dura from the posterior longitudinal ligament (arrows). (D) Posteroanterior and lateral diagrammatic representation of the photographic illustration in Fig. 1C. (E) The technique of obtaining sural grafts. (F) Posteroanterior and lateral diagrammatic representation showing how the dura is reconstructed. The posterior dura is split and retracted with stay sutures exposing the cord and anterior dura. A fascia lata graft is used to fill the dural defect. It is first sutured to the anterior dura cranially and caudally. (G) Posteroanterior and lateral diagrammatic representation. After anterior dural reconstruction, side grafts are placed at the ventral aspect of the cord. (H) Posteroanterior photographic illustration of the surgical site after side grafting of the cord. The white arrows point to the edges of the dural graft. The yellow arrow points to the sural nerve grafts. (I) Conventional technique of end-to-end grafting. (J) Posteroanterior diagrammatic representation. After side grafting the posterior dura is partially closed and the chitosan paste injected. (K) Posteroanterior diagrammatic representation. To prevent dissipation of the mesenchymal stem cells, they are injected after final closure of the dura. (L) Diagrammatic representation of a sagittal section through the cord showing the final result.

Table 2 Improvement in ASIA impairment scale based on required elements of ASIA neurological impairment assessment

Pt: Skeletal level	Pre-op/post-op	Neurological level	Neurological level	Motor R/L	Neurological level	Voluntary anal contraction	Deep anal sensation	ASIA impairment scale
1: T1-2	Pre-op → post-op	Sensory R/L	T4 → T10	T4 → T8	T4 → T8	None → none	0 → 1	A → B
2: T8	Pre-op → post-op	T8 → L2	T8 → T12	T8 → T12	T8 → T12	None → none	0 → 1	A → C
3: T10 4, 9: T12	Pre-op → post-op	T10 → L2	T10 → T12	T10 → T12	T10 → T12	None → none	0 → 1	A → C (Cases 3, 4), B (Case 9)
5-8: T12	Pre-op → post-op	T11 → L2	T11 → T12	T10 → T12	T10 → T12	None → none	0 → 1	A → C
10, 11: T11	Pre-op → post-op	T12 → L5	T12 → T12	T10 → T12	T10 → T12	None → none	0 → 1	A → C
12-14: T12	Pre-op → post-op	T12 → L5	T12 → T12	T10 → T12	T10 → T12	None → none	0 → 1	A → C

A, complete: no motor or sensory function was preserved in the sacral segments S4-S5; B, incomplete: sensory but not motor function was preserved below the neurological level and included the sacral segments S4-S5; C, incomplete: motor function was preserved below the neurological level, and more than half of key muscles below the neurological level had a muscle grade less than 3; D, incomplete: motor function was preserved below the neurological level, and at least half of key muscles below the neurological level had a muscle grade of 3 or more; E, normal: motor and sensory function were normal.

- Autonomous neurogenic bladder (due to complete sensory and motor separation of bladder from sacral spinal cord): no voluntary initiation of micturition; no reflex bladder activity; no specific sensation; diminished compliance was common.
- Uninhibited neurogenic bladder (due to injury/disease in the “corticoregulatory tract”, leading to loss of inhibition of the sacral micturition reflex centre): voluntary bladder contraction possible; sensation intact; residual urine generally small.
- Reflex neurogenic bladder (due to complete interruption of pathways between brainstem and sacral micturition centre): no bladder sensation; inability to initiate voluntary micturition; presence of hyper-reflexic involuntary bladder contractions; and detrusor-sphincter dyssynergia.

Stiens *et al.*³⁴ described two main types of neurogenic bowel occurring due to dysfunction of the colon caused by lack of nervous control. In the lower motor neuron bowel syndrome or areflexic bowel, due to the destruction of parasympathetic motor innervations, the myenteric plexus coordinated segmental colonic peristalsis, and a dryer, rounder stool shape occurred; the external anal sphincter was denervated. Areflexic bowel was characterized by slow stool propulsion, a dryer, rounder stool shape; there was increased risk for incontinence, constipation, and a significant risk of incontinence due to the lax external anal sphincter. In the upper motor neuron bowel syndrome or hyper-reflexic bowel, there was increased colonic wall and anal tone; there is reflex coordination and stool propulsion. Hyper-reflexic bowel was characterized by constipation, fecal retention at least in part due to the activity of the external anal sphincter.

Results

Complications of surgery

Haematoma formation

Delayed wound healing due to hematoma formation was observed in Cases 2–11.

Seroma formation

Delayed wound healing due to seroma formation was observed in Cases 10–14. This was due to chitosan disintegration.

Infection and myelitis

None of the patients developed infection or osteomyelitis. Cerebrospinal fluid leakage was not observed in any of these patients.

Table 3 Motor and muscle spasm (sp) improvement, timeline of return of function in months (mth)

Pt: Skeletal level	Side (R/L)	Motor power	Lower limb (LL)											Comments	
		Total UL	Hip flexors L2	Knee extensors L3	Ankle dorsiflexors L4	Long toe extensors L5	Ankle plantar flexors S1	Total (R/L)	Both LLs	Total UL and LL	Abd. muscles	Hip adductors	Hamstrings, gluteus maximus, and hip abductors		
1: T1-2	R (pre-op) → (post-op)	50 → 50	0 → 0	0 → 0 sp2 → sp2	0 → 0	0 → 2 mth12	0 → 2 sp2 → sp2 mth9	0 → 0	0 → 4	50 → 54	0 → 4 mth3	0 → 0 sp1+ → sp1+	0 → 0 Hamstrings: sp1+ → sp1+	Co = contractions between ankle dorsiflexion and knee flexion	
	L (pre-op) → (post-op)		0 → 0	0 → 0	0 → 0	0 → 0	0 → 0	0 → 0				0 → 0 sp1+ → sp1+	0 → 0 Hamstrings: sp1+ → sp1+		
2: T8	R (pre-op) → (post-op)	50 → 50	0 → 3 mth9	0 → 2 sp2 → sp1 mth6	0 → 1 mth12	0 → 1 mth12	0 → 0 sp2 → sp1+	0 → 7	0 → 15	50 → 65	3 (+ve Beevor's sign) → 5 mth3	0 → 3 sp1+ → sp1+ mth3	0 → 3 sp1+ → sp1+ mth3		
	L (pre-op) → (post-op)		0 → 3 mth6	0 → 3 sp2 → sp1 mth6	0 → 1 mth12	0 → 1 mth12	0 → 0 sp2 → sp1+	0 → 8				0 → 3 sp1+ → sp1+ mth3	0 → 3 mth6		
3: T10 4-8: T12	R (pre-op) → (post-op)	50 → 50	0 → 3 mth6	0 → 2 sp2 → sp1 mth9	0 → 1 mth12	0 → 1 mth12	0 → 0 sp2 → sp1+	0 → 7	0 → 15	50 → 65	3 (+ve Beevor's sign) → 5 mth3	0 → 3 sp2 → sp1+ mth3	0 → 3 mth9		
	L (pre-op) → (post-op)		0 → 3 mth6	0 → 3 sp2 → sp1 mth6	0 → 1 mth12	0 → 1 mth12	0 → 0 sp2 → sp1+	0 → 8				0 → 3 sp2 → sp1+ mth3	0 → 3 mth9		
9: T12	R (pre-op) → (post-op)	50 → 50	0 → 3 mth9	0 → 0 sp2 → sp2	0 → 0	0 → 0	0 → 0 sp2 → sp2	0 → 0	0 → 6	50 → 56	3 (+ve Beevor's sign) → 5 mth6	0 → 3 sp2 → sp1 mth3	0 → 3 mth9		
	L (pre-op) → (post-op)		0 → 3 mth9	0 → 0 sp2 → sp2	0 → 0	0 → 0	0 → 0 sp2 → sp2	0 → 0				0 → 3 sp2 → sp1 mth3	0 → 3 mth9		
10, 11: T11	R (pre-op) → (post-op)	50 → 50	0 → 3 mth6	0 → 3 sp2 → sp1 mth6	0 → 2 mth12	0 → 2 mth12	0 → 2 sp2 → sp1+ mth12	0 → 12	0 → 24	50 → 74	3 (+ve Beevor's sign) → 5 mth6	0 → 3 sp2 → sp1 mth3	0 → 3 mth6		
	L (pre-op) → (post-op)		0 → 3 mth6	0 → 3 sp2 → sp1 mth6	0 → 2 mth12	0 → 2 mth12	0 → 2 sp2 → sp1+ mth12	0 → 12				0 → 3 sp2 → sp1 mth3	0 → 3 mth6		
12-14: T12	R (pre-op) → (post-op)	50 → 50	0 → 3 mth6	0 → 3 sp2 → sp1 mth6	0 → 2 mth12	0 → 2 mth12	0 → 2 sp2 → sp1+ mth12	0 → 12	0 → 24	50 → 74	3 (+ve Beevor's sign) → 5 mth3	0 → 3 sp2 → sp1 mth3	0 → 3 mth6		
	L (pre-op) → (post-op)		0 → 3 mth6	0 → 3 sp2 → sp1 mth6	0 → 2 mth12	0 → 2 mth12	0 → 2 sp2 → sp1+ mth12	0 → 12				0 → 3 sp2 → sp1 mth3	0 → 3 mth6		

On each side of the body and while the patient was in supine position, five muscles representing the segments of the cervical cord and five muscles representing segments of the lumbar cord were scored on a 5-point muscle grading scale (0, total paralysis; 1, palpable or visible contraction; 2, active movement, full range of motion, gravity eliminated; 3, active movement, full range of motion, against gravity; 4, active movement, full range of motion, against gravity and provided some resistance; 5, active movement, full range of motion, against gravity and provided normal resistance; 5*, muscle able to exert, in examiner's judgment, sufficient resistance to be considered normal if identifiable inhibiting factors were not present; NT, not testable, patient unable to reliably exert effort or muscle unavailable for testing due to factors such as immobilization, pain on effort, or contracture). The sum of all 20 muscles yielded a total motor score for each patient, with a maximum possible score of 100 points for patients with no weakness.

The modified Ashworth scale for muscle spasm evaluation: 0, no increase in muscle tone; 1, slight increase in muscle tone, manifested by a catch and release or by minimal resistance at the end of the range of motion when the affected part(s) is moved in flexion or extension; 1+, slight increase in muscle tone, manifested by a catch, followed by minimal resistance throughout the remainder (less than half) of the ROM (range of movement); 2, more marked increase in muscle tone through most of the ROM, but affected part(s) easily moved; 3, considerable increase in muscle tone passive, movement difficult; 4, affected part(s) rigid in flexion or extension.

Table 4 ASIA standard neurological classification of spinal cord injury; sensory improvement; timeline of return of function in months (mth)

Pt: Skeletal level	Each side (R/L) Light touch (pinprick) Dermatomes	Total (C2–T4) 1–11	T5 12	T6 13	T7 14	T8 15	T9 16	T10 17	T11 18	T12 19	L1 20	L2 21	L3 22	L4 23	L5 24	S1 25	S2–S5 26–28	Total (C2–S5)	Total score (both sides) Pre/ post
1: T1–2	R/L (pre-op) → (post-op)	22 → 22	1 → 2 mth3	1 → 2 mth3	0 → 2 mth6	0 → 2 mth6	0 → 2 mth6	0 → 2 mth6										24 → 34	48 → 68
2: T8	R/L (pre-op) → (post-op)	22 → 22	2 → 2	2 → 2	2 → 2	2 → 2	0 → 2 mth6	0 → 2 mth6	0 → 2 mth6	0 → 2 mth9	0 → 2 mth9	0 → 2 mth9	0 → 1 mth9	0 → 1 mth9				30 → 44	60 → 88
3: T10 4,9: T12	R/L (pre-op) → (post-op)	22 → 22	2 → 2	2 → 2	2 → 2	2 → 2	2 → 2	2 → 2	0 → 2 mth6	0 → 2 mth9	0 → 2 mth9	0 → 2 mth9	0 → 1 mth9	0 → 1 mth9				34 → 44	68 → 88
5–8: T12	R/L (pre-op) → (post-op)	22 → 22	2 → 2	2 → 2	2 → 2	2 → 2	2 → 2	2 → 2	2 → 2	0 → 2 mth9	0 → 2 mth9	0 → 2 mth9	0 → 1 mth9	0 → 1 mth9				36 → 46	72 → 88
10, 11: T11	R/L (pre-op) → (post-op)	22 → 22	2 → 2	2 → 2	2 → 2	2 → 2	2 → 2	2 → 2	2 → 2	0 → 2 mth9	0 → 2 mth9	0 → 2 mth9	0 → 2 mth9	0 → 2 mth9	0 → 2 mth9			36 → 48	72 → 96
12–14: T12	R/L (pre-op) → (post-op)	22 → 22	2 → 2	2 → 2	2 → 2	2 → 2	2 → 2	2 → 2	2 → 2	0 → 2 mth9	0 → 2 mth9	0 → 2 mth9	0 → 2 mth9	0 → 2 mth9	0 → 2 mth9			36 → 48	72 → 96

Sensation evaluation using the ASIA sensory chart included light touch and pinprick discrimination assessment at 28-specific sensory locations on each side of the body specified by designated bony prominences. For the 28 sensory dermatomes on each side of the body, sensory levels were scored on a 0- to 2-point scale, yielding a maximum possible pinprick score of 112 points (for either pinprick or light touch) for a patient with normal sensation (pinprick (sharp/dull): discriminate sharp and dull ends of a standard safety pin; light touch: identify contact with a cotton swab tip; 0 absent – unable to distinguish, 1 impaired – able to distinguish but intensity is abnormal, 2 normal – NT not testable). Deep anal sensation was tested by digital rectal examination and the patient was asked if he felt pressure on the rectal wall (0 absent, 1 present). The findings on pinprick and light touch testing of the sacral segments and anal sensation distinguished complete from incomplete spinal cord injury.

Table 5 Improvement in modified optional elements of ASIA neurological impairment assessment

Pt: Skeletal level	Pre-op/post-op	Joint movement appreciation (proprioception)								Diaphragm	Deltoids	Abdominal muscles	Hip adductors	Hamstrings, gluteus maximus, and hip abductors
		Wrist, thumb IPjoint, small finger proximal IPjoint	Knee	Ankle	Great toe IPjoint	Deep pressure sensation Wrist radial styloid, thumb nailbed, small finger nailbed	Ankle medial malleolus	Great toe nail = bed	Small toe nail = bed					
1: T1–2	Pre-op → post-op	2 → 2	0 → 0	0 → 0	0 → 0	1 → 1	0 → 0	0 → 0	0 → 0	1 → 1	5 → 5	0 → 4	0 → 0	0 → 0
2: T8	Pre-op → post-op	2 → 2	0 → 1	0 → 1	0 → 1	1 → 1	0 → 1	0 → 1	0 → 0	1 → 1	5 → 5	3 (+ve Beevor's sign) → 5	0 → 3	0 → 3
3: T10–4–9: T12	Pre-op → post-op	2 → 2	0 → 1	0 → 1	0 → 1	1 → 1	0 → 1	0 → 1	0 → 0	1 → 1	5 → 5	3 (+ve Beevor's sign) → 5	0 → 3	0 → 3
10, 11: T11	Pre-op → post-op	2 → 2	0 → 1	0 → 1	0 → 1	1 → 1	0 → 1	0 → 1	0 → 0	1 → 1	5 → 5	3 (+ve Beevor's sign) → 5	0 → 3	0 → 3
12–14: T12	Pre-op → post-op	2 → 2	0 → 1	0 → 1	0 → 1	1 → 1	0 → 1	0 → 1	0 → 0	1 → 1	5 → 5	3 (+ve Beevor's sign) → 5	0 → 3	0 → 3

Joint movement appreciation (proprioception) was performed by supporting the proximal portion and moving the distal portion by gripping its medial and lateral edges. This was done at the following joints: wrist, thumb interphalangeal joint, small finger proximal interphalangeal joint, knee, ankle, great toe interphalangeal joint (0, absent – unable to distinguish; 1, impaired – able to distinguish but intensity is abnormal; 2, normal – NT not testable). Deep pressure sensation was tested by applying pressure with the thumb or index finger on the wrist radial styloid, thumb nailbed, small finger nailbed, ankle medial malleolus, great toe nailbed, and small toe nailbed (0 absent, 1 present). The diaphragm was observed under fluoroscopy for movement over two or more interspaces (0 absent, 1 present). Muscle power of the deltoids, abdominal muscles, hip adductors, and hamstrings was graded according to the 0, 1, 2, 3, 4, 5, 5*, NT. The deltoids (C5, 6 ± 4) were examined by testing shoulder abduction.

Abdominal muscles (T6–T12) were tested by observing movement of the umbilicus (Beevor's sign: umbilicus moved up in T9–T11 lesions). In this study, patients with positive Beevor's sign were considered to be neurological level T8; patients with full abdominal power but weak lower limbs were considered neurological level T12. Hip adductors (L2–3) were tested by palpating the adductor longus. The hamstrings (plurisegmental) were examined by testing knee flexion. In addition to the above muscles, the gluteus maximus, and hip abductors were included in the present study, because according to neuromuscular orthopedics,³¹ patients with a Grade 4 gluteus maximus could stand erect, provided the knee was splinted; with a Grade 4 gluteus maximus and Grade 4 knee extensors, patients could walk unaided.

Results of surgery

Determining the ASIA impairment scale

Motor level improved four levels in 2 cases and two levels in 12 cases (Table 2). Sensory level improved six levels in two cases, five levels in five cases, four levels in three cases, and three levels in four cases. A four-level neurological improvement was recorded in 2 cases and a two-level neurological improvement occurred in 12 cases. The ASIA impairment scale improved from A to C in 12 cases and from A to B in 2 cases.

Motor power evaluation

Seven patients showed a 15-grade motor score improvement, improvement occurring in hip flexors (3 grades), knee extensors (2 grades), ankle dorsiflexors (1 grade) and long toe extensors (1 grade) (Table 3). In five patients, a 24-grade motor score improvement was recorded, improvement occurring in hip flexors and knee extensors (3 grades), ankle dorsiflexors (2 grades), long toe extensors (2 grades), and ankle plantar flexors (2 grades). Case 9 showed a six-grade motor score improvement, Case 2 had a four-grade motor score improvement. Improvement in motor power was hampered by an increase of paraplegia in flexion and co-contractions between ankle dorsiflexion and knee flexion in Case 1.

The first muscles to respond were the abdominal muscles, followed, respectively, by the adductors, hip flexors, knee extensors, medial hamstrings [additional file 1], abductors and gluteus maximus, ankle and toe dorsiflexion, and ankle plantar flexion. In 11 cases, the abdominal muscles started to improve at 3 months after surgery, in 3 cases they started to improve at 6 months after surgery. In all but Case 1, improvement in adductor power started at 3 months after surgery; in Case 1, the adductors did not improve. Hip flexion started to improve at 6 months in 12 cases, at 9 months in 1 case; no improvement occurred in Case 1; in Case 2, the left side improved at 6 months, the right side at 9 months. Knee extensors started to improve at 6 months in 12 cases; in 2 cases, these muscles did not improve; in 6 cases improvement was unequal occurring at 6 months in one limb, and at 9 months in the other. In seven patients, improvement in hamstring power occurred at 9 months after surgery, in six cases it occurred at 6 months after surgery; in Case 1, the hamstrings remained spastic with no improvement in motor power. Ankle dorsiflexion started to improve at 12 months in 12 cases; in 2 cases it did not improve. Toe extension started to improve at 12 months in 13 cases; in Case 9, it did not improve at all. Ankle plantar flexion started to improve at 12 months in five cases,

but improved at 9 months in Case 1. At 15 months after surgery, all muscle power improvement had reached a plateau.

Motor recovery was characterized by the following:

- It occurred in spite of the presence of a gap in the spinal cord thus excluding the possibility of spontaneous improvement or improvement by surgical manipulation alone without grafting.
- Proximal muscles improved earlier and better than distal muscles.
- There was differential improvement of different muscle groups.
- Improvement was characterized by starting to occur early, involving several muscle groups and then reaching a plateau.
- Having occurred in Case 2, 7 years after injury, improvement was independent of the time delay between the date of injury and the date of definitive surgery.
- Improvement occurred at the thoracic level; it also occurred in injuries at the thoracolumbar level.
- Improvement at the thoracic level occurred. At this level, the cord defect was 2 cm long, there was no dural mutilation.
- Improvement at the thoracolumbar level occurred although the gliosis and dural mutilation were more extensive than at the thoracic level. In nine cases, the injury was at T12; in all of them, the dura was mutilated and the cord gliotic, in seven cases was the cord defect was 4 cm long, in two cases it was 2 cm (Table 1).
- In the age group (9–45 years) included in this study, improvement was not age-dependent.
- Improvement in muscle power was not affected by delayed wound healing or seroma formation.
- Improvement was affected by the presence of other fractures in the lower limb.

Muscle spasm evaluation

Spasticity was observed in the knee extensors, ankle plantar flexors, hip adductors, and hamstrings (Table 3). Knee extensor spasticity improved from Grade 2 to Grade 1 in 12 cases, but remained Grade 2 in two cases. Ankle plantar flexor spasticity improved from Grade 2 to Grade 1+ in 12 cases, but remained Grade 2 in 2 cases. Hip adductor spasticity improved from Grade 2 to Grade 1 in six cases, from Grade 2 to Grade 1+ in six cases, but remained Grade 1+ in two cases. Grade 1+ hamstring spasticity was observed in Case 1 and was associated with mild pain. Improvement in spasticity started at 3 months after surgery and reached a plateau at 15 months after surgery.

Thus, spasticity was characterized by the following:

- It involved certain groups of muscles.
- It improved, but did not resolve completely.

- It was associated with pain in Case 1. Babinski's sign remained positive (extensor response) in Case 1, in the left lower limb in Case 9, and in the right lower limbs in Cases 5 and 13.

Sensory evaluation

Five cases showed a 24-grade sensory score improvement, four cases a 20-grade sensory score improvement, and four cases a 16-sensory score improvement (Table 4). Case 3 showed a 28-grade sensory score improvement.

Improvement in sensation started at 3 months after surgery in segments T5–T6, at 6 months in segments T7–L1, at 9 months in segments T12–L5; it reached a plateau at 12 months.

Thus, sensory recovery was characterized by the following:

- Direction of recovery was proximal to distal.
- Although the grafts faced the ventral (motor area) of the cord, sensory improvement was superior to motor improvement.

Pain

All patients had annoying pain that was not resolved by surgery.

Optional elements of ASIA neurologic impairment assessment

In 13 cases, deep pressure sensation at the small toe nail bed did not improve; a 1-grade improvement was observed in joint movement appreciation (proprioception) at the knee, ankle and at the great toe interphalangeal joint and in deep pressure sensation at the ankle medial malleolus and great toe nail bed; a 2-grade improvement occurred in abdominal muscles and a 3-grade improvement was recorded in hip adductors, gluteus maximus and hamstrings. In Case 2, proprioception, deep pressure sensation and hip adductors and hamstrings did not improve; abdominal muscles improved by four grades, respectively (Tables 3 and 5).

Components of rectal examination

In all 14 cases, touch and pinprick sensation were zero both before and after surgery. Deep pressure sensation improved from zero to one; maximal voluntary contraction remained zero.

Case 9 reported regaining feeling of uterine contractions during menstruation; erectile function was regained in Cases 3–8 and 10–14.

Bladder and bowel function

These patients had a reflex neurogenic bladder with no bladder sensation; inability to initiate voluntary micturition; hyperreflexic involuntary bladder contractions. Postoperatively, the bladder remained hyperreflexic,

with inability to initiate or control micturition. Sense of bladder fullness was regained in all but three patients (Cases 1, 10 and 11). Patients started to feel bladder fullness at 9 months after surgery. This helped them determine timing for voiding their bladders by catheterization.

Pre-operatively, all of our patients had abdominal distension, constipation, and fecal retention. In Cases 9 and 10, the external anal sphincter was additionally lax with fecal incontinence. Patients with an intact anal sphincter had learned to observe the degree of abdominal distension and to void their bowel by asking their attendant to move their lower limbs. Post-operatively, these conditions remained the same.

Ability to walk

Extension of motor improvement into the lower limbs occurred in Cases 2, 3–8, 9, and 10–14. The abdominal muscles (2 grades), hip adductors, and hamstrings (3 grades) improved in all. Superior motor improvement occurred in Cases 2, 3–8 (hip flexors (3 grades), knee extensors (2 grades), ankle dorsiflexors (1 grade), and long toe extensors (1 grade)), Cases 10–14 (hip flexors (3 grades), knee extensors (3 grades), ankle dorsiflexors (2 grades), long toe extensors (2 grades), and plantar flexors (2 grades)). With motor power Grade 3 hip flexion and 3 in the adductors and hamstrings, these patients could crawl in bed but not walk. Improvement in gluteus maximus and in knee extensors to Grade 3 was insufficient to enable them to stand erect and hold their knees extended while walking unaided.

Discussion

We have presented our clinical experience using mesenchymal stem cell-derived neural stem cell-like cell transplantation to enhance recovery in chronic spinal cord injuries with defects bridged by sural nerve grafts and a chitosan-laminin scaffold. Spinal cord injury is considered chronic months to years after injury.⁵ At this stage, the primary and secondary injuries have ceased; there is no place for neuroprotection, instead, treatment should focus on neurorestoration.

Using peripheral nerve grafts to bridge spinal cord defects

Since the groundbreaking work of David and Aguayo,^{4,6} numerous studies have shown that grafts of peripheral nervous tissue can induce and support axonal outgrowth into the spinal cord.^{35–42} Axons growing within peripheral nerve grafts have been found to retain their physiological properties⁴³ and to make functional synapses with neurons near their point of CNS re-entry.⁴⁴ In a clinical study,¹³ three segments from autologous sural

nerves have been implanted into the right and left antero-lateral quadrant of the cord at T7–8 levels, and then connected to homolateral L2–4 lumbar ventral roots, respectively. Eight months after surgery, voluntary contractions of bilateral adductors and of the left quadriceps have been observed.

In the current study, the spinal cord defect has been bridged by sural nerve side grafts placed in a subpial manner. To allow for increased side neurotization, they have been extended for at least 4 cm along the ventral aspect of both superior and inferior cord segments.^{25,26} Grafting in spinal cord injury differs from grafting of peripheral nerves; conduction of information is the primary aim of peripheral nerve grafting, whereas both conduction and integration of information have to be considered in spinal cord grafting. Thus, peripheral nerve grafting is primarily governed by the theory of axial axonal sprouting through the grafts⁴⁵; end-to-end grafting is therefore the usual procedure in peripheral nerve grafting. Spinal cord regeneration is governed by two other theories. The guidance theory,^{2,46} holds that the glial tissue secreted by astrocytes provides the necessary guidance channels toward axonal sprouting; its minimal stimulation through fine pial incisions during the process of side grafting is favored, while its excessive secretion through freshening of the cord ends during end-to-end grafting (Fig. 1I) would lead to its excessive secretion and would consequently block regeneration. The neural plasticity theory holds that the spinal cord has considerable regeneration reserve. Synaptic plasticity is one form of neural plasticity. It occurs in the motoneuron soma but also in the axon initial segment, which is a highly specialized neuronal subregion that is the site of action potential initiation.⁴⁷ Because of synaptic plasticity, regeneration by axonal side sprouting and side synapses is possible in the spinal cord, a fact that is in favor of side grafting.

In spite of the above, the use of peripheral nerve grafts has been challenged. According to experimental observation, damaged spinal cord axons might grow from the cranial cord into peripheral nerve grafts but would not leave them to enter the caudal cord.⁴ Schwann cells might even promote gliosis and the deposition of non-permissive extracellular molecules, such as chondroitin sulfate proteoglycans.^{48,49} Functional improvement has been reported to be limited both experimentally and clinically.^{50,51}

Co-transplantation by mesenchymal stem cells

Howland *et al.*⁵² have demonstrated the efficacy of embryonic spinal cord transplants on the locomotor

development of kittens that received complete low thoracic spinal cord transections. They have hypothesized several transplant-mediated mechanisms, which might account for enhanced recovery or development of function following spinal cord injury:

1. Transplants could produce neurotrophic factors to rescue axotomized host neurons from injury-induced cell death.
2. Transplants might function as a relay stations (synapses) connecting ascending and descending host neurons.
3. Transplants might bridge defects within axons.

Since that time, various cell types have been used to substitute the cellular components to the injured spinal cord segment and to induce axonal regeneration.³

However, there are still unresolved issues surrounding the use of cellular transplants in spinal cord injury and the type of cell that should be used. Cell types used include activated macrophages, dendritic cells, embryonic stem cells, neural stem cells in the CNS, olfactory ensheathing cells (OEC), mesenchymal stem cells, and Schwann cells.

In a meta-analysis reviewing cellular transplantation strategies in spinal cord injury, Tetzlaff *et al.*,⁵³ have come to the following conclusions. Schwann cells are the most extensively studied cell type, with beneficial effects after transplantation into thoracic spinal cord injury as demonstrated by numerous investigators. However, compared to neural precursors such as oligodendrocyte precursors or neural precursor/stem cells, they provoke a more robust astrocytic reaction, resulting in less-effective integration into the host spinal cord. They require *in vitro* amplification, which may take several weeks and impose a delay on the intervention. In many cases, they appear to require adjuvant treatment to increase efficacy (e.g. Matrigel, rolipram, cyclic adenosine monophosphate (cAMP), neurotrophic factors). The optimal source of Schwann cells, i.e. isolating them from nerves versus other tissues progenitors has yet to be determined.

OECs demonstrate good integration into host spinal cord; claims of axonal sprouting/regeneration have been reported. However, there is no robust evidence of improvement after their transplantation into moderate or severe thoracic contusion injuries. In many cases, they also appear to require adjuvant treatment to increase efficacy (e.g. Schwann cells, Matrigel, rolipram, cAMP, neurotrophic factors).

Neural stem/progenitor cells appear to integrate well into the host spinal cord with improved outcomes in both blunt and sharp models and in large animal models of spinal cord injury. However, they differentiate primarily into astroglial cells, with some

oligodendrocytes seen; neurons are rare. They do not provide optimal bridges for axonal regeneration – hence are less likely suited for axonal repair strategies.

Fate-restricted neural and glial precursor transplantation leads to more white matter sparing and (re-)myelination of host axons at 1 week after thoracic contusions. However, the overall number of studies with these cells is still small and efficacy in chronically injured spinal cords has yet to be demonstrated.

Bone marrow stromal cells have some bridging capacity in sharp transaction models; they demonstrate efficacy in many rodent and large animal and primate studies. However, their integration in the injured spinal cord is very limited. There is no convincing differentiation into neural cells – despite claims to the contrary.

A second unresolved issue is the use of a combination strategy. Bunge⁵⁴ has recommended the use of a combination strategy including Schwann cells. The following combination strategy has been suggested⁵⁴: Schwann cells, neuroprotective agents and growth factors administered in various ways, OEC implantation, chondroitinase addition, or elevation of cAMP.

A third unresolved issue is the number of injections that the patient has to receive. Mackay-Sim *et al.*⁵⁵ have used a single intraoperative injection. However, other authors^{56,57} have performed cellular transplantation in three cycles; in each cycle, five intrathecal injections have been given at 2–11-day intervals.

A fourth unresolved issue is whether to obtain stem cells from patients with spinal cord injury or from healthy volunteers.⁵⁸

Our choice for using autologous mesenchymal stem cells obtained from the iliac crest has been based on the overwhelming experimental evidence^{59–62} in support of the efficacy of mesenchymal stem cells. They are more easily obtained from various tissues such as the bone marrow and adipose tissue⁶³; they have been reported to stimulate neurite outgrowth over neural proteoglycans, myelin-associated glycoprotein, and Nogo-A⁶⁴; they can differentiate both *in vitro* and *in vivo* into cells expressing neuronal markers.^{65–67} Their further differentiation into neural precursors has been recommended,⁶⁸ because transplanted into a neurogenic niche, pluripotent stem cells differentiate into neurons, but transplanted into a non-neurogenic niche, such as the injured spinal cord,⁶⁹ they are limited in differentiation.^{70,71} To avoid this, differentiation into neural stem cell-like cells has been induced by supplementing the culture medium with mercaptoethanol, B27 neurobasal medium, and brain-derived neurotrophic factor, a protocol originally used to produce

neural-restricted progenitors.⁶⁷ Neural differentiation has been confirmed by nestin positivity, a marker common to all neural cell lines.⁷² Neural multipotency has been confirmed by glial cell transformation of a subpopulation, which has become S100 β positive, a marker common to astrocytes,⁷² less so Schwann cells.⁷³ Such a neuromultipotent population is more suitable for transplantation than a fully differentiated one, because terminal differentiated neuronal cells survive detachment and subsequent transplantation procedures poorly.⁶⁷

Combining peripheral nerve grafts with a chitosan-laminin scaffold

The defect within the spinal cord can barely be bridged by nerve grafts alone; besides, the myelin sheath within them is supposed to be inhibitory to axonal growth. This has called for combining them with a synthetic scaffold, in which myelin is absent, both to induce axonal sprouting and to contain the cellular transplants. Chitosan is a synthetic resorbable polysaccharide. Delayed implantation of intramedullary chitosan channels containing nerve grafts has been found to promote extensive axonal regeneration after spinal cord injury.¹⁴ Laminin is a neurite outgrowth-promoting factor^{29,30}; adding it to the viscous fluid mentioned before is of paramount importance for nerve regeneration. A laminin peptide (YIGSR) immobilized on crab-tendon chitosan tubes has been found to promote nerve regeneration.¹⁵ The addition of glial cell-line derived neurotrophic factor (GDNF) and laminin to the chitosan nerve guides enhances both functional and sensory recovery.¹⁶ The biocompatibility of functional Schwann cells induced from bone mesenchymal cells with chitosan conduit membrane has been established.^{74,75}

Results of surgery

As far as results of surgery are concerned, motor recovery has occurred in spite of the presence of a gap in the spinal cord thus excluding the possibility of spontaneous improvement or improvement by surgical manipulation alone without grafting. Although it is not evident from this study whether recovery has been induced by the sural graft-chitosan-cellular transplant construct or by each separately, in view of the increasing evidence that sural grafts would not lead to regeneration alone⁴ and in view of the recognition that scaffolds even if combined with growth factors would not lead to axonal sprouting unless combined with cellular transplants,⁷⁶ we tend to assume that mesenchymal cells transplants have enhanced regeneration through sural nerve grafts.

We have recorded motor power improvement in the abdominal muscles (2 grades), hip flexors (3 grades), hip adductors (3 grades), knee extensors (2–3 grades), ankle dorsiflexors (1–2 grades), long toe extensors (1–2 grades), and plantar flexors (0–2 grades). These results are superior to those of other authors,¹³ who have used sural nerves side grafts alone without stem cell co-transplantation. These authors have observed voluntary contractions of bilateral adductors and of the left quadriceps 8 months after surgery. In both studies, however, proximal muscles have improved earlier and better than distal muscles; differential improvement of different muscle groups has also been recorded. A possible explanation for this is provided by the results of brachial plexus surgery,⁴⁵ where proximal muscles have a better chance of recovery than distal muscles, the motor fibers of which will have become atrophied by the time the axonal growth cone reaches them. Differential improvement of different muscles is also well known in brachial plexus surgery, where the supraspinatus muscle regenerates far better than the infraspinatus muscle, although both are supplied by the suprascapular nerve.

Peripheral nervous system regeneration is held to be superior to CNS regeneration. In this study, however, motor improvement started to occur early (3 months), involving several muscle groups and then reaching a plateau. By way of contrast, in brachial plexus surgery, although the supraspinatus starts to improve at 3 months, the deltoid, biceps, and triceps improve late (at about 8 months after surgery) but progress to Grade 4 rapidly. After that, forearm and hand functions are rarely regained. Superior central compared to peripheral nervous system regeneration is supported by the neural plasticity theory and by the fact that even a small number of intact axons traversing the injury zone, as few as 5–10% in small animal experiments, may be sufficient to support significant functional recovery.⁷⁷ The regeneration plateau may be explained by the excessive gliosis. The phenomenon of early widespread improvement followed by limited further regeneration might provide an explanation for the seemingly miraculous improvement of all subjects; some converting from ASIA A to C, although none of them has become a walker. More evidence has yet to be adduced to confirm this phenomenon, possibly through electromyographic studies.

Improvement has been independent of the time delay between the date of injury and the date of definitive surgery. Motor and sensory improvement has occurred in Case 2 although he was operated upon 7 years after injury. This is supported by the observations made by

Li and Raisman,⁷⁸ who have noted that sprouts from cut corticospinal axons persisted despite the presence of astrocytic scarring in long-term lesions of the adult rat spinal cord. Put into clinical practice, this means spinal cord transplantation might be instituted in late cases.

Improvement has occurred both at the thoracic and thoracolumbar levels. Recovery at the thoracic level is strongly correlated with the extent of cord and dural damage, which has been less than at the dorsal lumbar level. By way of contrast, improvement at the thoracolumbar level has occurred although the extent of cord damage and dural mutilation has been more extensive than at the thoracic level. However, recovery has been possible because of extension of the grafts to the cauda equina. Considered altogether, these results emphasize the role of scaffolds and are in accordance with the clinical observation of superior regeneration of the cauda equine.⁷⁹ They are also consistent with the experimental observation that damaged spinal cord axons might grow from the cranial cord into peripheral nerve grafts but would not leave them to enter the caudal cord.⁴ Since the cauda equina belongs to the peripheral nervous system, axons could leave the peripheral nerve grafts and enter it.

Although nerve grafts have been placed at the motor area ventrally, more improvement in sensation has been recorded, sensory level having improved six levels in two cases, five levels in five cases, four levels in three cases, and three levels in four cases. Synaptic neural plasticity might provide an explanation for this. Other possible explanations might be the contribution of the cellular side of the dural graft to neurogenesis²³ or the capacious reconstruction of the dura allowing for cerebrospinal fluid flow.²⁴

Although the grafting scheme is similar in all cases motor level has improved four levels in 2 cases and two levels in 12 cases. Why improvement has been remarkable in 12 cases in this study but inferior in 2 cases might be related to subtle gliosis of the proximal and distal cord ends, which cannot be assessed during surgery. Out of the 12 cases, who have improved remarkably, motor power improvement has been superior in 5 cases compared with the other 7 cases. This could be explained by interobserver and intraobserver variability in recording lesser degrees of motor power.

Recovery of bladder and bowel functions has been inferior to motor and sensory recovery. This is in keeping with experimental evidence.⁸⁰

Considered altogether, our results are comparable with those studies using cellular transplantation alone in spinal cord injuries with gliosis yet with no defect.

In a study including 171 spinal cord injury patients injected at the time of operation with OECs,⁸¹ motor scores have increased by 5–8, light touch scores by 13–25, and pinprick scores by 11–25. Motor score increases of 4–6 and a sensory score increase of 20 have been reported in a further study.⁸² Improvement to ASIA Grades B and C has also been observed.^{82–84} In contrast to the findings by other authors,⁸¹ in the age group (9–45 years) included in this study, improvement has not been age-dependent. However, it has been affected by the presence of other fractures in the lower limb, however.

There is marked variability reporting results in these studies, however. Sensory but no motor improvement, e.g. has been found in a clinical trial of autologous injection of OECs into the spinal cord in six patients with complete, thoracic paraplegia.⁵⁵ This is confirmed by other reports.⁸⁵ To account for these contradictory reports, it is hypothesized that improved transmission in intact fibers subserving the zone of partial preservation leads to early improvements.⁸⁶ Another possible explanation is the source,^{87,88} culture conditions, and passage number^{89–92} of the mesenchymal stem cells used, as all are known to influence mesenchymal stem cell potential. A third possible explanation is differential efficacy of mesenchymal stem cells from different donors.⁹³ A fourth possible explanation is that mesenchymal stem cells obtained from spinal cord injured patients not from healthy volunteers might be associated with inferior results.⁵⁸ A fifth possible explanation is spontaneous or treatment-induced anatomical neural plasticity, the adaptive reorganization of the neural pathways occurring after injury, and acting to restore some of the lost function.⁹⁴

Complications in this study include haematoma and seroma formation. Recovery has not been affected by seroma formation. Complications reported by other authors include meningitis and transient post-operative hypotonicity.⁸⁵ One objection to chitosan is its rapid disintegration leading to seroma formation. Combining chitosan with co-polymerized lactic and glycolic acid and loading it with nanoparticles containing the above components might be a solution.¹⁶

Future recommendations

Although improvement has been recorded in this study, none of our patients has become a walker. Further attempts should be made to improve motor power in the gluteus maximus and knee extensors, so that the patients might be able to walk. Axonal regeneration following spinal cord injury is limited partly because injured axons encounter a series of inhibitory factors

that are non-permissive for growth.^{46,48,94,95} These include myelin inhibitors (Nogo-A, MAG108 (myelin-associated glycoprotein), and OMgp109 (oligodendrocyte myelin glycoprotein)); chondroitin sulfate proteoglycans (neurocan, versican, aggrecan, brevican, phosphacan, and NG2); semaphorins; and ephrins. Future efforts should therefore be directed to more lysis of these proteoglycans by adding chondroitinase ABC microinjection systems^{42,96,97}; this should be thermostabilized beforehand.⁹⁸ Furthermore, evidence points to the superiority of Type I versus Type II astrocytes in migrating into host tissue and mixing with host glia while suppressing scar formation, and in promoting regeneration of sensory axons and improving locomotor function.⁴⁹ Thus, future efforts should also be directed to charting growth of part of the cell transplant to Type I astrocytes. Multiple instead of single injections should also be resorted to, as multiple subarachnoid administrations of bone marrow mesenchymal stem cells promoted the restoration of injured spinal cord and improved neurological functions.⁵⁶ At the time of surgical nerve grafting, the implantation of an indwelling intrathecal catheter for several months might be necessary for continuous delivery of gliolytic factors, growth factors, and cellular transplants.

Conclusions

We have presented our clinical experience using mesenchymal stem cell-derived neural stem cell-like cell transplantation to enhance recovery in chronic spinal cord injuries with defects bridged by sural nerve grafts combined with a chitosan-laminin scaffold. Chitosan disintegration leading to post-operative seroma formation has been a complication. Motor power improvement has been recorded in the abdominal muscles (2 grades), hip flexors (3 grades), hip adductors (3 grades), knee extensors (2–3 grades), ankle dorsiflexors (1–2 grades), long toe extensors (1–2 grades), and plantar flexors (0–2 grades). These results are superior to those of other authors,¹³ who have used sural nerves side grafts alone without stem cell co-transplantation and who observed voluntary contractions of bilateral adductors and of the left quadriceps 8 months after surgery. However, this improvement is insufficient to enable them to stand erect and hold their knees extended while walking unaided. Further attempts should be made to improve motor power in the gluteus maximus and knee extensors, so that the patients might be able to walk. Such attempts should include the establishment of continuous spinal drug delivery systems, lysing the gliosis possibly by chondroitinase ABC, re-evaluation of the combination of cells and of the scaffold, and

supplying the scaffold with cell adhesion molecules and trophic factors.

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