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CLINICAL TRIALS

An approach to personalized cell therapy in chronic complete paraplegia: The Puerta de Hierro phase I/II clinical trial

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Abstract

Background aims. Cell transplantation in patients suffering spinal cord injury (SCI) is in its initial stages, but currently there is confusion about the results because of the disparity in the techniques used, the route of administration, and the criteria for selecting patients. *Methods.* We conducted a clinical trial involving 12 patients with complete and chronic paraplegia (average time of chronicity, 13.86 years; SD, 9.36). The characteristics of SCI in magnetic resonance imaging (MRI) were evaluated for a personalized local administration of expanded autologous bone marrow mesenchymal stromal cells (MSCs) supported in autologous plasma, with the number of MSCs ranging from 100×10^6 to 230×10^6. An additional 30×10^6 MSCs were administered 3 months later by lumbar puncture into the subarachnoid space. Outcomes were evaluated at 3, 6, 9 and 12 months after surgery through clinical, urodynamic, neurophysiological and neuroimaging studies. *Results.* Cell transplantation is a safe procedure. All patients experienced improvement, primarily in sensitivity and sphincter control. Infralesional motor activity, according to clinical and neurophysiological studies, was obtained by more than 50% of the patients. Decreases in spasms and spasticity, and improved sexual function were also common findings. Clinical improvement seems to be dose-dependent but was not influenced by the chronicity of the SCI. *Conclusion.* Personalized cell therapy with MSCs is safe and leads to clear improvements in clinical aspects and quality of life for patients with complete and chronically established paraplegia.

Key Words: *cell therapy, mesenchymal stromal cells, paraplegia*

> "In scientific research, topics are not depleted. What usually happens is that there are men exhausted on the topics"
>
> *Santiago Ramón y Cajal, 1897.*

Introduction

Spinal cord injury (SCI) is one of the most devastating diseases and often causes permanent disability in young patients. In seeking a cure, these patients often undergo treatments that lack scientific and methodological rigor.

At present, cell therapy is a therapeutic promise in this field of research [1–9] but still subject to many uncertainties, with significant confusion due to the disparity of protocols, selection of subjects, cell type, dose and routes of administration used.

In experimental studies, it is noteworthy that the functional recovery of paraplegic animals after mesenchymal stromal cell (MSC) transplantation starts before

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tissue regeneration occurs to allow the passage of ascending and descending axons. Therefore, it is obvious that after MSC transplantation into injured central nervous system (CNS) must exist various repair processes, including the release of neurotrophic factors by the transplanted stem cells, or the activation of endogenous mechanisms of the spinal cord, able to partially restore neurological functions previously abolished [10–18].

Considering previous experimental studies suggesting the advisability of transplanting enough cells, our efforts must be aimed at achieving the highest possible survival of MSCs, once transplanted, and suggest that administration of repeated doses of cell therapy could be beneficial. In this regard, various experimental studies showed that MSCs can reach areas of SCI after being deposited in the subarachnoid space [19–23], an observation that must be taken into account when applying these techniques in patients. Moreover, our preclinical experience with adult paraplegic pigs shows that local cell therapy for SCI requires attention to numerous technical details, such as the morphology of the lesion, an adequate cell suspension medium, caliber of the injection needle, rate of cell administration in the injured tissue, and achieving a high concentration of cells in the smallest possible volume, in order not to produce added damage to the spinal cord [13,24].

On the other hand, the prospect of achieving human cell therapy medicaments using allogeneic cells to the treatment of SCI obviously is a legitimate objective by pharmaceutical companies, but we believe any kind of cell therapy requiring immunosuppression is unacceptable due to repeated urinary infections that often plague these patients.

These considerations lead us to develop the present clinical trial, aimed at evaluating the safety and efficacy of personalized cell therapy with autologous MSCs obtained from bone marrow, in patients with complete and chronic paraplegia.

Methods

Study design

The present clinical trial (ClinicalTrials.gov identifier: NCT01909154; EudraCT identifier: 2010-023285-46) included 12 patients (male/female: 9/3) suffering chronic complete paraplegia (ASIA A) due to traumatic SCI in the dorsal (thoracic) region. Mean age was 40.5 years (SD, 8.75 years), and time from SCI to treatment ranged from 3.17 to 26.75 years (mean, 13.86 years; SD, 9.36 years). Clinical and demographic data and the flow chart of the patients can be seen in Supplementary Table S1 and Supplementary Figure S1.

The trial protocol was approved by the ethics committee of Puerta de Hierro-Majadahonda Hospital and by the Spanish Agency for Medicaments and Health Products (AEMPS), and was conducted in accordance with the principles of the Declaration of Helsinki [25] and good clinical practice guidelines [26]. Adverse events were collected throughout the follow-up and classified according to the Medical Dictionary for Regulatory Activities (MedDRA v. 18.1).

Clinical scores were obtained from each patient, prior to cell therapy and at 3, 6, 9 and 12 months after treatment by means of the following scales: the scale provided by the American Spinal Injury Association (ASIA) [27], the SCI functional rating scale of the International Association of Neurorestoratology (IANR-SCIFRS scale) [28], the Functional Independence Measure (FIM) scale [29] and the Barthel scale [30] for the study of functional independence in the activities of daily life (ADL), the Visual Analog Scale (VAS) [31] for the evaluation of neuropathic pain, the Penn [32] and the modified Ashworth [33] scales for the evaluation of spasms and spasticity, respectively, the Geffner scale [34] for the study of bladder function, and the Neurogenic Bowel Dysfunction (NBD) scale [35] for the evaluation of symptoms related to neurogenic bowel dysfunction. Neurophysiological, urodynamic and magnetic resonance imaging (MRI) studies were also performed before and after treatment. Additional details are provided in the Supplementary Appendix, available online at doi:10.1016/j.jcyt.2016.05.003.

Cell therapy medicament

We used a cell therapy medicament (NC1) based on our preclinical experience and currently approved as a medicament under clinical investigation by the AEMPS (PEI No. 12–141). It consists of autologous MSCs and autologous plasma as its excipient. Prior to NC1 preparation, a sample of peripheral blood was retrieved from each patient for genomic studies to rule out chromosomal abnormalities that could discourage cell expansion and to obtain a genetic fingerprint (KaryoNIM Stem Cells and KaryoNIM STR test, respectively; NIMGenetics; additional information is provided in the Supplementary Appendix). For obtaining the excipient, as a first step in the preparation of the NC1, we start with the removal of 500 cc of peripheral blood from each patient. In our cleanroom, blood was centrifuged at 900*g* for 8 min to obtain the plasma fraction, which is aliquoted in 15 mL tubes and stored at -80°C until the medicament formulation.

Culture of MSCs

Approximately 2 weeks later, 50 mL of bone marrow was aspirated under aseptic conditions from the iliac bones of each patient, immediately anticoagulated by a 5 mL solution composed of 100 IU/mL sodium heparin Chiesi (Chiesi España) and 10^4 IU/10^4 µg penicillin-streptomycin (BioWhittaker-Lonza) and sent

to our cleanroom for culture and expansion under good manufacturing practice (GMP). Mononuclear cells (MNC) were separated by density gradient using an automated cell-processing system (SEPAX, BioSafe). Then, they were plated at a density of 16×10^4 to 20×10^4 cells/cm^2, in 175 cm^2 flasks on Alpha-Minimum Essential Medium (MEM) with Earle's Balanced Salt Solution (BSS), and supplemented with 20% Australian prion-free fetal bovine serum (FBS) (Lonza; Lot Number, 9SB006), 200 mmol/L L-glutamine (BioWhittaker-Lonza) and 10^4 IU/10^4 µg penicillin-streptomycin (BioWhittaker-Lonza).

The cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere for 3 days, after which nonadherent cells were removed by replacing the medium. When the cultures approached confluence (90–100%), adherent cells were detached by treatment with trypsin/ethylenediamine tetraacetic acid (EDTA) solution (BioWhittaker-Lonza). Neutralization of trypsin and subsequent washing was performed with Alpha-MEM medium supplemented with 10% FBS and 2 mmol/L L-glutamine, centrifuging at 1250 rpm for 10 min. After study of viability, cells were cultured to obtain the required number according to the plan previously made for each patient. Cells were replated at a density of 3000–5000 cells/cm^2 in factory farming of 4 floors with free-antibiotic Alpha-MEM medium supplemented with 10% FBS and 2 mmol/L L-glutamine, and the culture was maintained renewing the medium every 3–4 days until a confluence of 90–100%. Once the culture reached confluency, it was prepared to obtain the bulk of MSCs. At this time, MSCs were detached with trypsin/EDTA and washed with Hank's BSS medium (BioWhittaker-Lonza) supplemented with 5% albumin (20% albumin, Grifols). After that, MSCs were resuspended with the previously obtained autologous plasma to remove traces of the washing medium. After cell counting, MSCs for the second dose were separated and then cryopreserved, at a concentration of 2.2×10^6 cells/mL in a FBS solution, in dimethylsulphoxide (DMSO; Miltenyi Biotec). For this, we used a liquid nitrogen-free controlled rate freezer (EF 600, Grant-Asymptote). Finally, the MSCs for surgical administration were formulated, according to the number scheduled for each patient, after a new centrifugation at 1250 rpm for 10 min.

To prepare the second dose, cryopreserved MSCs were thawed in a thermostatic bath at 37°C, washed with antibiotic-free Alpha-MEM medium supplemented with 10% FBS and 2 mmol/L L-glutamine, and centrifuged at 1250 rpm for 10 min. After this, a cell count was performed and MSCs were plated at a concentration of 10000–15000 MSCs/cm^2 in 175 cm^2 culture flasks with antibiotic-free Alpha-MEM medium supplemented with 10% FBS and 2 mmol/L L-glutamine to reach a confluence of 90–100% over a period of 4–5 days, and then we proceeded in the same way as with the first MSCs dose.

Formulation and packaging

After obtaining the MSCs for surgical or second dose administration, they were resuspended in the autologous plasma at a cell concentration of 100,000 cells/µL. After formulation, the cell therapy medicament was packaged in sterile and endotoxins-free 1-mL Hamilton microsyringes, with a 20-guage needle. Subsequently the needle was removed and a sterile luer plug nut was placed on the end of each preloaded syringe. Microsyringes with the medicament were placed inside a sterile metal box, which was also double bagged before being transported to the operating room for cell transplantation.

Phenotypic characterization of MSCs

For phenotypic characterization of MSCs, monoclonal antibodies conjugated with different fluorochromes (Fluorescein [FITC]/Phycoerythrin [PE]/Alexa-647 [AL-647]), which combine a number of both positive and negative MSCs membrane markers, were used. Positive markers used were CD105 FITC (R&D Systems); CD90 AL-647 (AbD Serotec, OX5 1GE); HLA Class I FITC (Cytognos); CD73 PE (BD Bioscience) and CD166 PE (R&D Systems). Negative markers used were CD34 PE (BD Bioscience); HLA class II PE (Cytognos); CD80 AL-647 (AbD Serotec); CD45 FITC (Cytognos); and CD31 FITC (Cytognos). Furthermore, suitable isotopic controls for FITC, PE (Cytognos) and AL-647 (AbD Serotec), respectively, were used as controls for specificity of the monoclonal antibodies. The labeled cells were acquired with a flow cytometer FC500 MPL Cytomics (Beckman Coulter) using the MXP software (Beckman Coulter). Nonviable cells were discarded using the labeling reagent LIVE&DEAD (Invitrogen), and the collected data were analyzed with the CXP analysis software, version 2.1 (Beckman Coulter). Criteria for the administration of MSCs in our present clinical trial included a viability >95%, absence of microbial contamination (bacteria, fungus, virus or mycoplasma), expression of CD105, CD90, HLA I, CD73 and CD166 for >90% of cells and absence of CD34, CD80, HLA II, CD45 and CD31 (expression of each < 5%), as assessed by flow cytometry (Supplementary Figure S2).

Surgical planning

In all patients, basal neuroimaging studies were used to make a surgical plan for each patient so that a greater

number of cells could be administered into the suspected cystic areas and fewer cells could be administered into the areas of normal appearance, to prevent additional damage to the spinal cord. As a result of this planning, the number of injections of the cell therapy medicament in areas of SCI ranged from 3–7 (average, 4 microinjections/patient), and the volume of medicament in each microinjection ranged from 50–1500 µl (average, 360 µL/microinjection). Given that our cell therapy medicament had a concentration of 10^5 cells/µL, the amount of administered cells per microinjection ranged from 5×10^6 MSCs to 150×10^6 MSCs (average, approximately 36×10^6 MSCs). In each patient, in addition to injections into the injured spinal cord tissue, 30×10^6 MSCs (300 µL) were administered in the perilesional subarachnoid space by means of an intrathecal catheter (mod BOC 8711, Medtronic Inc.). Intramedullary injections of MSCs were performed through a laminectomy and durotomy, and we used a microinjection pump (mod 310, Stoelting Co.) connected to a 100-µL Hamilton syringe with a 20-gauge needle, at a microinjection rate of 10 µL/min, which was modified depending on the morphological characteristics of the lesion and the tissue consistency. After cell administration, the dura mater was hermetically closed, to prevent leakage. Three months after surgery, all patients received another 30×10^6 MSCs into the subarachnoid space by lumbar tapping through a 20-gauge needle. The total number of MSCs administered to each patient ranged from 130×10^6 to 260×10^6 (mean, 202.5×10^6; SD, 46.73×10^6). Surgical planning in the patients of the series is provided in the Supplementary Appendix (See Supplementary Figures S3 to S14).

Statistical analysis

Wilcoxon rank test was used to assess the differences between scores in clinical scales and urodynamic parameters at different time points. Hypothesis testing between categorical variables were obtained through Cochran *Q* test. Effect sizes (ES) were computed using the G*Power software (v. 3.1.9.2, Statistical Power Analysis Software). Correlations were obtained using Spearman rank correlation coefficient. Differences were considered significant when $P < 0.05$. SPSS (v. 21.0, IBM) and Prism software (v. 5.04, GraphPad Software) were used.

Results

In our present clinical trial, the cell expansion process did not involve any alteration in the genome of the cells in any of the cases, according to the results obtained after analysis by Array-CGH platform.

Adverse events

During the study, 69 adverse events (AEs) were collected, but only 22 of them (31.8%) were related to the surgical procedure or cell therapy administration, and they were considered to be of minor (79.1%) or moderate (20.9%) intensity. Generally, they consisted of postoperative pain, transient hyperthermia or subcutaneous collection in the area of the surgical wound. Most AEs collected during the study were urinary tract infections (78.57%) in patients with recurrent urinary infections. Details of collected AEs are provided in the Supplementary Appendix (Supplementary Tables S2–S4).

ASIA scale

A significant recovery of sensitivity to pin prick (PPS) and light touch sensitivity (LTS) has been found after cell therapy (Table I). Figure 1 shows the dermatomes in which our patients recovered sensitivity, regardless of type or intensity, at 12 months after surgery.

Prior to cell therapy our patients had absence of motion in the lower limbs, except for one patient who had a SCI at D11–D12 and a motor score (MS) of 52. The evolution of MS in the series showed a significant difference comparing baseline and 12 months of follow-up (ES, 0.64; $P = 0.026$). At that time, six patients showed recovery in the hip flexor muscles. Two patients showed recovery in the hip flexor and knee extensor muscles, and one patient showed improvement in the hip flexor, knee extensor, ankle dorsiflexor, long toe extensor and ankle plantar flexor muscles, but only reached active movement against gravity in the hip flexor muscles.

In Total ASIA (TA) scores, a progressive improvement was observed, without correlation with patient age, level of SCI or chronicity of paraplegia. However, there was a high correlation between TA improvement and number of cells administered (at 3 months $P = 0.002$, $r = 0.807$; at 6 months $P = 0.005$, $r = 0.749$; and at 9 months $P = 0.013$, $r = : 0.691$; Figure 2). Furthermore, throughout the follow-up, 3 patients moved from ASIA A to ASIA B, and 1 patient moved from ASIA A to ASIA C, so, therefore, more than 30% of patients in the study improved from a complete lesion to an incomplete lesion ($P = 0.029$, Supplementary Table S6). Additional details of the evolution in ASIA scores are provided in the Supplementary Appendix (See Supplementary Figures S15–S28).

IANR-SCIFRS scale

The IANR-SCIFRS scale evaluates spinal cord function through nine sections, with a final section that

Table I. ASIA scores at different time points.

Score subject	Time	Mean	SD	*P*	ES
Motor score	Before surgery	50.17	0.58	-	-
	3 months after surgery	50.17	0.58	1	-
	6 months after surgery	50.33	0.78	0.317	-
	9 months after surgery	50.92	2.07	0.102	-
	12 months after surgery	52.75	4.16	**0.026***	0.64
Pin prick score	Before surgery	57.83	11.17	-	-
	3 months after surgery	65.67	10.88	**0.005****	0.81
	6 months after surgery	70.25	13.88	**0.003****	0.85
	9 months after surgery	75.33	16.36	**0.002****	0.88
	12 months after surgery	81.00	16.84	**0.002****	0.88
Light touch score	Before surgery	57.92	9.42	-	-
	3 months after surgery	65.42	9.83	**0.005****	0.81
	6 months after surgery	69.25	10.23	**0.003****	0.85
	9 months after surgery	74.50	10.52	**0.002****	0.88
	12 months after surgery	79.50	13.92	**0.002****	0.88
Total ASIA score	Before surgery	165.92	22.83	-	-
	3 months after surgery	181.25	22.90	**0.005****	0.81
	6 months after surgery	189.83	27.83	**0.003****	0.85
	9 months after surgery	200.75	34.40	**0.002****	0.88
	12 months after surgery	213.25	37.19	**0.002****	0.88

Significant *P* values are in **bold**.

only applies to men and assesses sexual function (Supplementary Table S7).

In our patients, the mean global IANR-SCIFRS scale score before treatment was 29.92 points (SD, 2.64), and at end of the study was equal to 37.92 points ($P = 0.002$; ES, 0.89). Before treatment, all our patients showed a "medium degree of functional disability," whereas at 12 months after surgery, all patients show a "mild degree disability." Furthermore, there was a correlation between the improvement in overall scores of the IANR-SCIFRS scale and total number of cells administered (at 12 months $P = 0.017$, $r = 0.673$), which suggests a dose-dependent effect of the treatment. Similarly, there was a high correlation between improvement in TA scores and improvement in overall IANR-SCIFRS scores (at 3 months $P = 0.002$, $r = 0.804$; at 6 months $P < 0.0001$, $r = 0.851$; at 9 months $P = 0.001$, $r = 0.845$; and at 12 months $P = 0.001$, $r = 0.821$). Table II shows the scores at each follow-up interval in the sections of the IANR-SCIFRS scale showing improvement. Additional information is provided in the Supplementary Appendix (see Supplementary Table S9 and Supplementary Figures S30–S47).

FIM and Barthel scales

The FIM scale showed significant improvement at the 6-month follow-up in both overall score and sphincter control. At the end of the monitoring study, the difference with the baseline overall score showed an ES of 0.64 ($P = 0.026$). In sphincter control, the difference showed an ES of 0.66 ($P = 0.023$). In the Barthel scale, no significant differences were observed. Additional information is provided in the Supplementary Appendix (See Supplementary Table S10).

VAS scale

The analysis showed a decrease in neuropathic pain during the follow-up that reached statistical significance at 12 months (effect size, 0.59). Additional details are provided in the Supplementary Appendix (Supplementary Figure S48).

Penn and Ashworth scales

In the Penn scale, a significant improvement was observed soon after surgery (ES, 0.71 at 3 months; $P = 0.014$), and was maintained at 6 months (ES, 0.75; $P = 0.009$), 9 months (ES, 0.75; $P = 0.010$) and 12 months (ES, 0.74; $P = 0.010$). The modified Ashworth scale showed improvement in spasticity with statistical significance at 3 months (ES, 0.69; $P = 0.017$), 6 months (ES, 0.85; $P = 0.003$), 9 months (ES, 0.85; $P = 0.003$) and 12 months (ES, 0.85; $P = 0.003$). Additional details are provided in the Supplementary Appendix (See Supplementary Figures S49 and S50, and Supplementary Table S11).

Geffner scale

The analysis of this scale confirmed the bladder function improvement over baseline values, which reached statistical significance at the end of the study period (ES, 0.66; $P = 0.023$). Additional details are provided

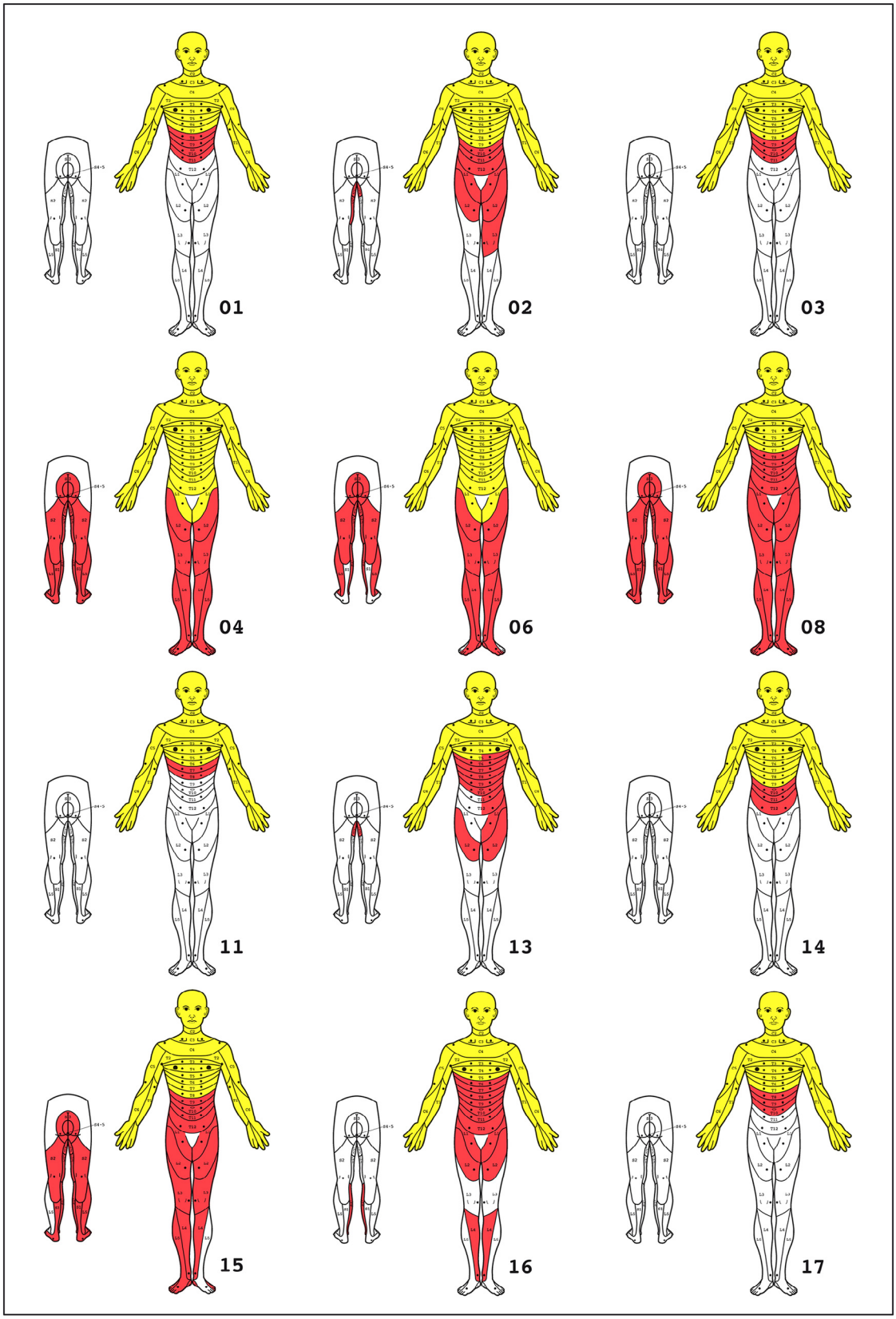

Figure 1. Recovery of sensitivity 12 months after surgery in patients in the study. Dermatomes in red indicate some degree of sensitivity. Those in yellow indicate preserved sensitivity prior to cell therapy. Blanks indicate total anesthesia. Recovery of sensitivity ranged from 2–14 levels (dermatomes) with a mean of 6.8 (SD, 4.0).

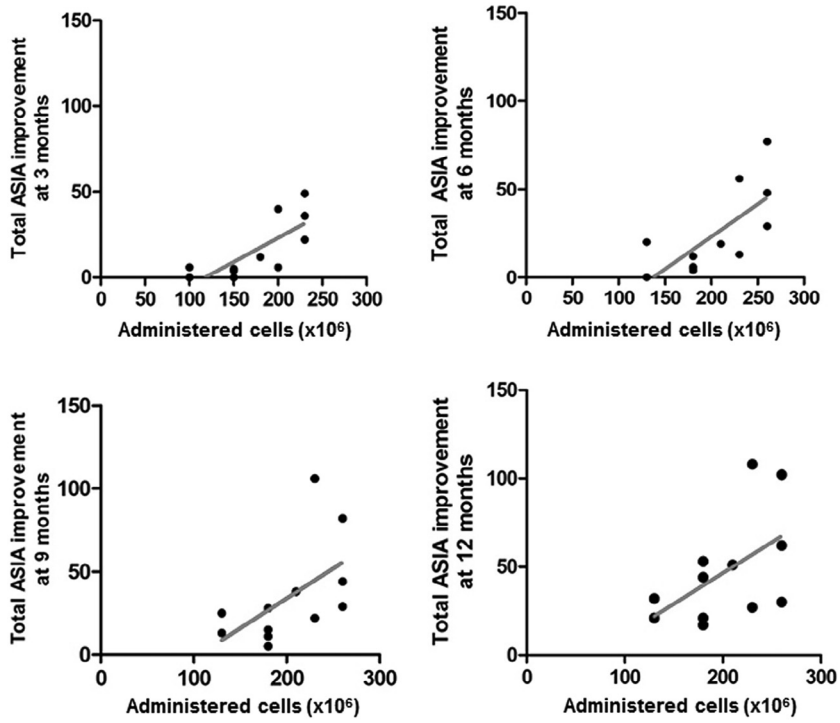

Figure 2. Spearman correlation study, showing a dose-dependent efficacy in terms of total number of administered cells and improvement in total ASIA scores.

in the Supplementary Appendix (See Supplementary Table S12 and Supplementary Figure S51).

NBD scale

After surgery, patients noticed peristalsis, and defecation was modified in terms of frequency and time needed. Improvement was generally immediate and described as extremely rewarding. According to the NBD scale, before cell therapy 7 patients had severe neurogenic bowel dysfunction, 3 patients had moderate dysfunction and 2 patients had mild dysfunction. At the end of the follow-up, 7 patients had minimal dysfunction, 1 patient had mild dysfunction and 4 patients had moderate dysfunction (ES, 0.81; $P = 0.005$). See additional details in the Supplementary Appendix (Supplementary Table S13 and Supplementary Figures S52 to S56).

Neurophysiological studies

All patients showed neurophysiological improvement during the follow-up period. Previous to cell therapy, none of our patients recorded somatosensory evoked potentials (SSEPs) nor motor-evoked potentials (MEPs). The neurophysiological study performed 6 months after surgery showed the appearance of SSEPs in 5 patients (41.67%) and 2 more 12 months after surgery (58.3%; ES, 0.41; $P = 0.008$). Regarding MEPs, 2 patients showed an appearance 6 months after surgery, and an additional patient at 12 months (overall, 3 patients, 25%; $P = 0.097$). At 6-month follow-up, EMG studies with concentric needle showed voluntary muscle contractions below the level of the SCI in three patients (25%). At 12 months, the EMG showed voluntary muscle contraction in muscles below the level of the SCI in 7 patients (58.3%; ES, 0.44; $P = 0.005$; Figure 3, Supplementary Table S14, Supplementary Figures S58 to S62 and Supplementary videos). Furthermore, at 12 months, polyphasic muscle action potentials in infralesional muscles, suggesting a process of active muscle reinnervation, were recorded in 10 patients (83.3%; ES, 0.58; $P = 0.001$). In 7 patients, this was induced by voluntary muscle contraction, and, in the other 3, it was induced by reflex or passive contraction. Additional details are provided in the Supplementary Appendix.

Urodynamic studies

Urodynamic studies showed improvement in 10 patients (83%) 12 months after surgery. It consisted of the possibility of voluntary micturition in the flowmetry test or in the study of pressure/flow in 5 patients (41.6%), increased bladder capacity at filling in 8 patients (66.6%), decreased detrusor pressure at bladder filling in 9 patients (75%) and increased bladder compliance in 10 patients (83.3%). When these data were compared with baseline studies, the statistical analysis showed significant improvements in detrusor

Table II. Scores in the sections of the IANR-SCIFRS scale showing improvement.

Score subject	Time	Mean	SD	*P*	ES
IANR-SCIFRS overall score	Before surgery	29.92	2.64		
	3 months after surgery	34.33	1.97	**0.003****	0.85
	6 months after surgery	36.08	2.39	**0.002****	0.89
	9 months after surgery	36.75	2.42	**0.002****	0.89
	12 months after surgery	37.92	2.23	**0.002****	0.89
Lower limb movement	Before surgery	0.00	0.00		
	3 months after surgery	0.16	0.57	1.00	—
	6 months after surgery	0.33	0.65	0.173	—
	9 months after surgery	0.33	0.65	0.173	—
	12 months after surgery	0.50	0.67	**0.038***	0.59
Trunk movement	Before surgery	4.33	0.99		
	3 months after surgery	5.17	0.84	**0.023***	0.66
	6 months after surgery	5.50	0.80	**0.010****	0.74
	9 months after surgery	5.75	0.62	**0.007****	0.78
	12 months after surgery	5.75	0.62	**0.007****	0.78
Sphincter control	Before surgery	0.00	0.00		
	3 months after surgery	1.42	1.44	**0.017***	0.69
	6 months after surgery	2.00	1.76	**0.011***	0.74
	9 months after surgery	2.25	1.71	**0.007****	0.77
	12 months after surgery	2.92	1.44	**0.003****	0.85
Bladder control	Before surgery	0.00	0.00		
	3 months after surgery	1.08	1.14	**0.011***	0.73
	6 months after surgery	1.25	1.14	**0.011***	0.73
	9 months after surgery	1.25	1.14	**0.011***	0.73
	12 months after surgery	1.67	0.89	**0.003****	0.86
Bowel control	Before surgery	0.00	0.00		
	3 months after surgery	0.33	0.65	0.102	-
	6 months after surgery	0.75	0.75	**0.014***	0.71
	9 months after surgery	1.00	0.85	**0.010****	0.75
	12 months after surgery	1.25	0.75	**0.004****	0.8
Muscular tension	Before surgery	1.33	0.99		
	3 months after surgery	1.92	0.52	**0.038***	0.60
	6 months after surgery	2.17	0.58	**0.008****	0.76
	9 months after surgery	2.25	0.62	**0.009****	0.75
	12 months after surgery	2.33	0.65	**0.006****	0.80
Pain	Before surgery	2.00	0.95		
	3 months after surgery	2.25	0.97	0.180	-
	6 months after surgery	2.42	0.90	0.059	-
	9 months after surgery	2.42	0.90	0.059	-
	12 months after surgery	2.58	0.79	**0.038***	0.60
Sexual function	Before surgery	1.11	0.78		
	3 months after surgery	1.56	0.73	**0.046***	0.58
	6 months after surgery	1.67	0.71	**0.025***	0.65
	9 months after surgery	1.67	0.71	**0.025***	0.65
	12 months after surgery	1.67	0.71	**0.025***	0.65

Significant *P* values are in **bold**.

pressure (ES, 0.62; $P = 0.002$) and bladder compliance (ES, 0.83; $P = 0.005$). However, at the end of the follow-up period, improvement in maximum cystometric capacity was not statistically significant ($P = 0.185$). See additional information in the Supplementary Appendix (Supplementary Tables S15 and S16, and Supplementary Figures S63–S65).

Neuroimaging studies

In the neuroimaging studies, 5 patients had spinal fixation devices that caused artefacts, being difficult to obtain a reliable analysis of lesion volume. However, they were not an impediment to study the area and extent of the SCI and plan for the cell number and volume to be injected into injured tissue. At the end of the follow-up, images did not suggest a worsening. In two cases we did not detect significant changes in the basal characteristics of the SCI, but in the remaining cases, we observed a reduction or disappearance of intramedullary hyperintense lesions, suggesting the replacement of a cyst or gliotic tissue with solid tissue, as a result of the cell therapy (Figure 4). Additional details and images are provided

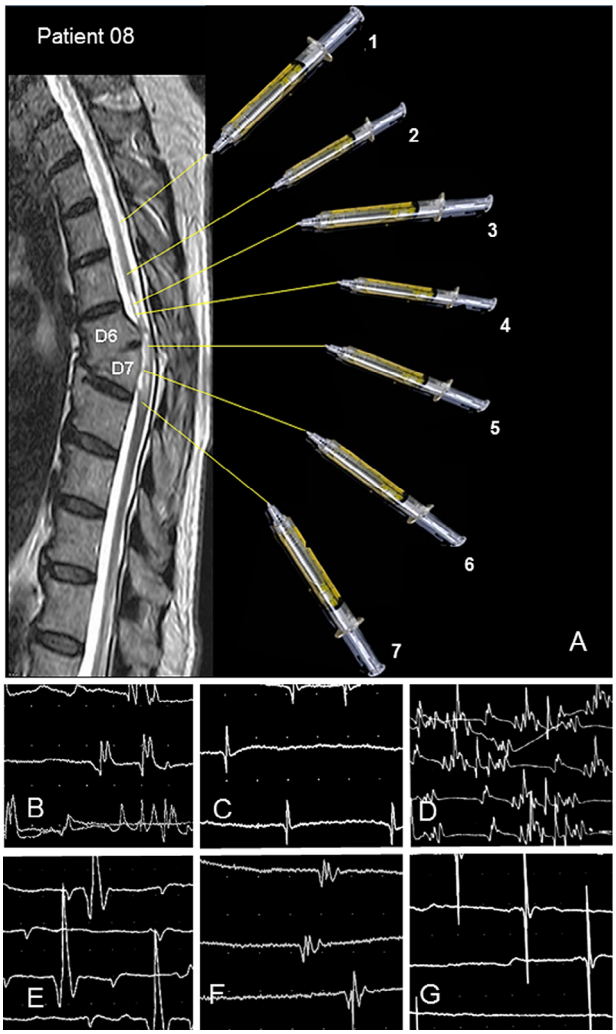

Figure 3. Patient 08. Complete paraplegia since 1996 (ASIA A). SCI level: D6–D7. (**A)** Surgical planning. Microinjections were performed in 7 different targets in the zones of the SCI and its edges. 30×10^6 MSCs were administered in targets 3–7. In target 2, 20×10^6 MSCs were administered. Furthermore, 30×10^6 MSCs were administered into the perilesional subarachnoid space (target 1). The total number of MSCs administered at surgery was 200×10^6. (**B–G)** EMG study 1 year after surgery showing voluntary muscle contraction with polyphasia in infralesional muscles (**B,** left rectus abdominus muscle at D12 level; **C,** left iliopsoas muscle; **D,** right quadriceps muscle; **E,** left medial gastrocnemius muscle; **F,** left tibialis anterior muscle; **G,** right bicep femoris muscle).

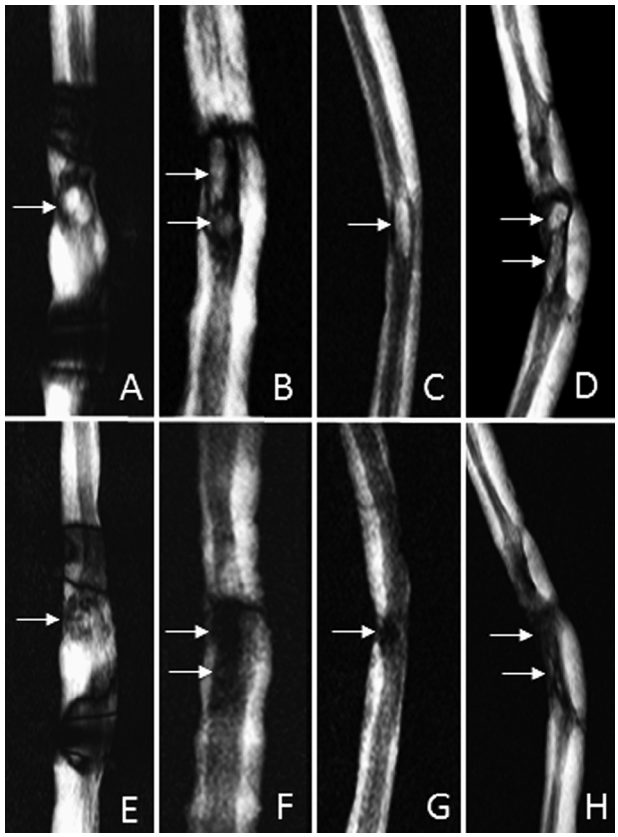

Figure 4. MR-myelography images in four patients in the study. (Above) Arrows show hyperintense lesions in the spinal cord (possible gliosis or cysts) prior to cell therapy. (Below) Arrows show the disappearance of these hyperintense lesions 12 months after cell therapy. (A and E, Patient 06; B and F, Patient 08; C and G, Patient 15; D and H, Patient 16). These patients received in the hyperintense zone between 70 and 150×10^6 cells (mean, 108×10^6; SD, 39×10^6) and all, except patient 16, had changed their classification to "incomplete" lesion, according to the ASIA scale.

in the Supplementary Appendix (Supplementary Figures S66–S70).

Discussion

In this clinical trial, as a result of the experience gained on animal models [10–14,16,36], we administered a cell therapy medicament consisting of autologous MSCs supported by autologous plasma to patients suffering complete and chronically established paraplegia.

The number of cells that we have directly administered into the spinal cord tissue during surgery ranged between 70×10^6 and 200×10^6 (with a local dose between 100×10^6 and 230×10^6 if we consider that during surgery another 30×10^6 cells were always administered in the perilesional subarachnoid space). This variability is due to the planning of treatment after the morphological study of the SCI. Given that we administered 100,000 cells/μL, our purpose was the administration in each intramedullary target of an acceptable number of cells, but trying to not produce additional tissue damage by volume.

Our primary outcome was safety, and assuming that patients with complete traumatic paraplegia may show some degree of improvement after the SCI [37], we only included patients with a long time since SCl.

Surprisingly, we found that all our patients, even those with the longest chronicity, experienced gradual improvement in clinical parameters without reaching

a plateau at the end of the follow-up period. The recovery of infralesional sensitivity and vegetative functions, such as bladder, bowel or sexual function, occurred soon after surgery, suggesting a possible effect through cytokines released by the transplanted cells, which activate preserved but non-functional circuits, rather than a mechanism of nerve pathway regeneration. Furthermore, MS in the ASIA scale showed significant improvement in some patients, a finding supported by neurophysiological studies.

In the present study, the patients showed improvement in most sections of the IANR-SCIFRS scale, with a clear parallelism between this improvement and that obtained from the ASIA scale. In addition, improvements in overall scores over ASIA, IANR-SCIFRS and NBD scales showed a positive correlation with the number of cells administered, suggesting that the beneficial effect of our cell therapy is dose-dependent. The high number of cells used in our trial may also explain the important and early improvement obtained in the manifestations of neurogenic bowel. Currently, MSCs can be tracked *in vivo* after labeling with superparamagnetic iron oxide nanoparticles [38], and it is obvious that the use of these techniques can offer in the future useful data to optimize doses and routes of administration.

Although our analysis shows significant improvements in FIM scale scores after treatment, in our opinion, scales evaluating ADL are not very useful for the assessment of chronic paraplegic patients because they had generally adapted to chronic dysfunction and were able to perform most activities of daily living without assistance.

Neuropathic pain causes significant disability in many paraplegic patients. In the present study, improvement in neuropathic pain was difficult to ascertain because only seven patients had significant neuropathic pain prior to treatment. However, we did observe a tendency for the pain to decrease, with statistically significant improvements in later stages of monitoring, in both the VAS and the IANR-SCIFRS scale. Early and progressive improvement in spasms and spasticity was also a clear effect of the treatment.

To obtain objective evidence for improvements of our patients, we performed neurophysiological and urodynamic studies. In the neurophysiological studies, all patients showed significant improvement during the follow-up period after cell therapy, with the appearance of previously abolished SSEPs or MEPs or with EMG evidence of voluntary muscle contractions in infralesional muscles. Given the progressive improvement in neurophysiological studies, we think that long-term monitoring of our patients is necessary.

The urodynamic findings support the improvement in bladder function observed in the clinical scales. In patients with complete and chronically established paraplegia, reduced bladder capacity and increased detrusor muscle pressure are common findings. Therefore, both increased bladder capacity (maximum cystometric capacity) and decreased detrusor pressure are urodynamic signs of clinical improvement. The ratio of maximum cystometric capacity and detrusor pressure determines bladder compliance, and, therefore, an increase in bladder compliance objectively reflects the improvements in bladder function after treatment.

In the present study, basal neuroimaging studies enabled us to develop a personalized treatment. Although the presence of surgical artefacts and the difficulty in obtaining strictly superimposed images on successive scans hindered the full assessment of possible changes after cell therapy, our treatment reduced hyperintense SCI lesions observed in MR-myelography images. In previous studies performed on our model of chronic paraplegic minipigs [24], we described similar neuroimaging findings when paraplegic minipigs received intralesional cell therapy with administration parameters similar to those in the present study. In these animals, histological studies showed that MRI modifications were due to a progressive solidification of intramedullary post-traumatic cavities, with presence of regenerative phenomena [13].

Conclusions

In conclusion, our cell therapy treatment is a safe procedure. In the present clinical trial, we reproduced in humans our previous experience with upper mammals, and the results showed improvement in all patients, in different aspects of the neurological disability associated with chronic complete paraplegia. The use of a high number of cells, the characteristics of our cell therapy medicament, which included a high concentration of cells in a small volume and the use of autologous plasma as excipient, together with a personalized plan to select spinal cord targets where the cells would be administered are crucial factors influencing our results.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jcyt.2016.05.003.