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Modeling chronic cervical spinal cord injury in aged rats for cell therapy studies

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ABSTRACT

With an expanding elderly population, an increasing number of older adults will experience spinal cord injury (SCI) and might be candidates for cell-based therapies, yet there is a paucity of research in this age group. The objective of the present study was to analyze how aged rats tolerate behavioral testing, surgical procedures, post-operative complications, intra-spinal cell transplantation and immunosuppression, and to examine the effectiveness of human iPSC-derived Neural Progenitor Cells (IMR90-hiPSC-NPCs) in a model of SCI. We performed behavioral tests in rats before and after inducing cervical hemi-contusions at C4 level with a fourth-generation Ohio State University Injury Device. Four weeks later, we injected IMR90-hiPSC-NPCs in animals that were immunosuppressed by daily cyclosporine injection. Four weeks after injection we analyzed locomotor behavior and mortality, and histologically assessed the survival of transplanted human NPCs. As rats aged, their success at completing behavioral tests decreased. In addition, we observed high mortality rates during behavioral training (41.2%), after cervical injury (63.2%) and after cell injection (50%). Histological analysis revealed that injected cells survived and remained at and around the grafted site and did not cause tumors. No locomotor improvement was observed in animals four weeks after IMR90-hiPSC-NPC transplantation. Our results show that elderly rats are highly vulnerable to interventions, and thus large groups of animals must be initially established to study the potential efficacy of cell-based therapies in age-related chronic myelopathies.

1. Introduction

Spinal cord injury (SCI) is a devastating condition most commonly caused by trauma, which has a life-long impact on patients and their families and high social and economic costs for public and private healthcare systems. The global incidence of SCI is increasing in the elderly population [1,2]. Additionally, myelopathies, neurologic deficits that commonly develop into SCI, typically caused by compression, increase with age [3]. Non-traumatic SCI caused by vascular ischemia or neoplasia is also more prevalent due to an increase in life expectancy [4]. Indeed, cervical spondylotic myelopathy is most common in adults over 55 years of age [5], with hospitalizations estimated at 4.04/100,000 person-years, and greater surgical rates [6].

Both, traumatic and non-traumatic SCI, cause focal cervical damage and, despite the differential timing, they can lead to demyelination and alpha-motoneuron and axonal damage, ultimately resulting in upper limb impairment [7].

Presently, the approved therapies for clinical use in injured patients aim to decompress the spinal cord and stabilize the patient's condition, but do not have regenerative potential. Cell-based therapies are emerging as promising treatments for SCI based on successful studies in animal models [8–10]. Nonetheless,

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the translation of such studies for human SCI treatment has so far been disappointing [11].

Induced pluripotent stem cells (iPSCs) can differentiate to neural progenitor cells (NPCs) and, subsequently, produce neuronal cells, offering an alternative for SCI treatment. Several pre-clinical studies involving therapies based on iPSC-derived neural cells have shown encouraging results, with therapeutic benefits including synaptic integration into the neuronal circuitry and locomotor recovery [12–15]. Accordingly, there is great interest in the use of iPSCs for cell therapy [16] particularly for the treatment of SCI [17]. While several studies have evaluated the use of other stem cell types, such as mesenchymal stromal cells (MSCs) [18] with trophic competence, NPCs would seem to offer a better approach, as they exert paracrine effects and also may serve as a replacement of some neural populations [19]. A previous analysis on SCI in aged rats concluded that age is a factor for functional recovery, as it is associated with a greater degree of axonal damage and demyelinization [20]. This would be consistent with the observation of higher microglial activation, oxidative stress and expression of inflammation-associated genes in older rats than in younger ones after SCI [21–23]. Furthermore, axonal repair and locomotor improvement might be related not only to these adverse conditions, but also to the limited innate capacity of neurons to respond to them [24]. Overall, these factors create an unfavorable environment in the injured spinal cord for transplanted cells to thrive.

While the majority of pre-clinical studies on SCI have used animal models with thoracic lesions, some studies have focused on cervical SCI [25–28]. These studies are remarkable given that cervical lesions are more frequent than thoracic ones and the symptoms are much more severe. Chronicity is defined by the development of the glial scar after contusion injury. In rats, astrocytes cluster around the lesion 1–2 weeks post-injury (sub-acute phase); the scar matures at 2–3 weeks post-injury and, by 8 weeks, the lesion is considered chronic [29,30]. In the present study, we elected to use an early chronic SCI model for cell transplantation, at 4 weeks post-injury (PI) [31].

The European Medicines Agency has stated the need to produce more faithful animal models in pre-clinical studies to reproduce SCI and its treatment [32,33]. Given the increased occurrence of SCI in elderly populations and their diminished capacity to recover, the establishment of a valid model that mimics SCI in the elderly is of great importance.

In the present work, we differentiated human iPSCs into NPCs and subsequently transplanted them into an early chronic cervical SCI model of aged rats. Our aim was to examine whether aged rats can tolerate extensive behavioral training and testing, surgical procedures, intra-spinal cell transplantation and immunosuppression. We also sought to examine the effectiveness and safety of human iPSC-derived NPCs for SCI in this setting.

2. Materials and methods

2.1. Cell culture

The NPCs used in this study were donated by The Institute for Stem Cell and Regenerative Medicine, University of Washington, Seattle, WA (USA), and were differentiated from iPSCs generated from human fetal lung fibroblasts (IMR90) as described [34] (Supplementary Fig. 1). Briefly, iPSCs were mechanically picked and cultured in suspension in TeSR2 medium (StemCell Technologies, Vancouver, Canada) containing 10 μM Rock inhibitor (Y-27632; Tocris Bioscience, Bristol, UK) for embryoid body (EB) formation. On day 4, the medium was changed to neural differentiation medium (NDM) containing DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, USA), penicillin/streptomycin (Sigma-Aldrich, Madrid, Spain), 1% N2 supplement (Thermo Fisher Scientific), 2 μg/mL heparin (Millipore, Darmstadt, Germany), 0.1 mM non-essential amino acids (Sigma-Aldrich) and 20 ng/mL bFGF (Miltenyi Biotec, Bergisch Gladbach, Germany). After 3 days in culture, EBs were seeded onto Matrigel (Corning, Corning, NY, USA) coated plates, and 0.1 μM retinoic acid (Sigma Aldrich) was added to the culture medium at day 10. At day 15, neuronal rosettes were mechanically isolated and seeded into plates coated with 20 μg/mL poly-L-ornithine (Sigma-Aldrich) and 20 μg/mL laminin from human placenta (Sigma-Aldrich) in NDM supplemented with 2% B27 (Thermo Fisher Scientific) and 20 ng/mL EGF (PeproTech, London, UK) for NPC growth. Cells were expanded either in coated plates or in suspension as neurospheres and were used for injection at passage 6–7. The study was approved by the Andalusian Ethical Committee of Research with Biological Samples of an Embryonic Origin and Similar Cells (RC/001/2013).

2.2. Expression arrays

We extracted RNA with the RNeasy® Mini kit (Qiagen, Hilden, Germany). RNA sample quantification and expression array analysis were performed at the Genomics Unit in CABIMER, Sevilla, Spain, as previously described [35]. The microarray expression dataset is publicly available at the GEO repository under the identifier GSE124361 (https://www.ncbi.nlm.nih.gov/geo/).

2.3. Behavioral tests

We trained 36 female Long–Evans rats (Charles River, Wilmington, MA, USA) for behavioral tests from 8 weeks to 19 months of age. Only female rats were used because of their smaller size and less aggressive behavior. The training consisted of a skilled Forelimb Reaching Task (FRT) [36], where the animals extend their paw through a slot to grasp (and eat) a treat. Animals were trained progressively in 10-minute sessions, and after approximately three months they consistently reached the food with a success rate of 70%. The trial was considered successful when the animal took the treat with the correct paw and placed it in its mouth without dropping it. Rats also undertook the Irvine, Beatties and Bresnahan (IBB) test. Briefly, we videotaped the rats eating two types of cereal––round and doughnut--shaped inside a transparent cylinder. We analyzed the recordings in slow motion to assess fine control of the forelimb and digits [37,38]. We also evaluated the forelimb preference during vertical exploration of the cylinder with the limb-use asymmetry test (LUAT) [39] to determine the degree of dysfunction from the hemi-contusion. For this test, we counted the number of wall contacts with the right, left or both paws at the same time, when the animals lift over their posterior paws. Sessions lasted until the animals made 20 wall contacts. All animals had a mean (±standard deviation, SD) score of 0.48 ± 0.11 prior to injury, indicating no preference for the use of the left or right paw. Scores lower than 0.5 would indicate preference for the unaffected paw. The animals were tested the week prior to injury to obtain the initial scores, and once per week thereafter (PI) in order to assess their state and potential locomotor recovery.

2.4. Sample size justification

Our previous experience indicated that training a minimum of 8–10 animals per group would be necessary to achieve a power of 90% for detecting a 5% difference in forelimb function results. We considered the following additional factors for calculating the initial number of animals needed: pre-surgical training dropout (not all the animals are able to learn the task); demanding surgical procedures; losses due to anesthesia or possible surgical or trans-

planting errors; and natural causes due to aging. Therefore, we used 18 animals per group.

2.5. Laminectomy and spinal cord injury surgeries

Cervical hemi-contusion injury and laminectomy were performed on 20-month-old female Long-Evans rats as described [26]. Briefly, rats were anesthetized with an injection of 80 mg/kg ketamine and 15 mg/kg xylazine, and a laminectomy of the spinous process ipsilateral to the animal's predominant paw (determined at FRT test) at C4 level was performed. We then induced a contusion on the animal's spinal cord using a fourth-generation Ohio State University Injury Device, lowering the magnetically controlled probe to the spinal cord, lateral to the midline. The spinal cord was displaced to induce the injury during 14 ms (peak force = 0.60 ± 0.10 N; average force = 0.03 ± 0.13 N; average displacement = 0.53 ± 0.16 mm) producing a severe hemicontusion. Prior to closing the surgical zone, the exposed spinal cord was covered with a small piece of GELFOAM® (Pfizer, New York, NY, USA) to prevent adhesion formation.

The animals received special care measures one-week PI or post-transplant (PT), including cleaning urine residues, administering analgesia and controlling body weight. If the animals showed signs of dehydration, they received a subcutaneous injection of Ringer's solution. In addition, softened chow was placed at the bottom of the cage to help them feed. Antibiotics were administered if the rats showed signs of infection.

All procedures involving animal use were carried out under the Animal Welfare Regulations and were approved by the Animal Welfare Commission of the "Hospital Virgen del Rocío/ IBiS" and "Consejería de Agricultura" of Andalucía (2013PI/025).

2.6. Cell transplants

Rats were divided into two groups at 4 weeks PI: the experimental group received a cell transplant and was immunosuppressed by daily injection of cyclosporine (10 mg/kg: Sandimmune, Novartis, Basilea, Sweden) starting from the day of transplantation; the control group was not immunosuppressed and did not receive cells or any other surgical procedure after the injury. Cell transplants were performed as described [26]. Briefly, under anesthesia, the injury site was re-exposed and the animals were placed in a spinal frame. A pulled glass capillary (OD 70–100 μm) was positioned on the lateral border of the gray matter and stereotaxically lowered to a depth of 1.0 mm, targeting the gray-white border of the dorsolateral funiculus. Rats received two human IMR90-hiPSC-NPC grafts, rostral and caudal to the contusion site, each containing 200,000 viable cells (determined by trypan blue dye exclusion), in a maximum volume of 8 μl of vehicle, which consisted of Hanks Balanced Salt Solution (Thermo Fisher Scientific) containing 1 mM glucose (Grifols, Barcelona, Spain), and 0.5 mM EDTA (Thermo Fisher Scientific). Cells were treated with 0.4 U/μL DNAse (Thermo Fisher Scientific) for 10 min before transplantation to prevent cell clumping.

2.7. Tissue processing

At 4 weeks PT, the animals were perfused transcardially with 4% paraformaldehyde in PBS (Santa Cruz Biotechnology, Dallas, TX, USA) under deep anesthesia. Spinal cords were extracted, post-fixed overnight and cryoprotected. The tissue was embedded in OCT medium (Sakura Finetek, Alphen aan den Rijn, The Netherlands), frozen and then 20-μm coronal sections were cut on a Leica CM 1950 cryostat (Wetzlar, Germany) and stored at −80 °C.

2.8. Immunostaining

We permeabilized the tissue with 0.4% Triton (Millipore) and blocked with 5% BSA (Millipore) in 0.4% Triton. Primary antibodies were incubated at 4 °C for 72 h (see Table 1). Species-specific secondary antibodies were incubated at room temperature for 2 h.

With the aim of characterizing the injured area in terms of inflammation, we performed triple staining for glial fibrillary acidic protein (GFAP), CD68 (ED-1) and neurofilament (NF) markers. To determine whether the grafted cells differentiated within the spinal cord, we stained for human nuclei (HuNu) (which enabled us to distinguish between rat [host] and human [grafted] cells) together with Sox1, neuron-specific class III β-tubulin (Tuj1), GFAP and oligodendrocyte transcription factor 2 (Olig2). Cells expressing HuNu were counted in sections spanning the graft area, and the total number and cell survival was calculated using the Abercrombie correction [40]. Co-expression of Tuj1, GFAP and Olig2 was also quantitatively assessed and expressed as a percentage of total HuNu-positive cells.

2.9. Statistical analysis

Differences between groups for FRT success rates were compared with repeated measures ANOVA and the Newman-Keuls post hoc test. Animal mortality was analyzed by Kaplan-Meier survival analysis. All tests were carried out with GraphPad 8.4.3 (GraphPad, La Jolla, CA, USA).

3. Results

3.1. Rats show reduced performance in behavioral tests along their lifespan

Female rats (n = 36) were trained for behavioral tests from 8 weeks of age. 2 of them were taken out of the study because they did not learn to perform the tasks. As shown in Fig. 1, the FRT success rate along the training period decreased significantly from 9 months of age onwards, and this was particularly evident from 12 months of age.

For the IBB test, the animals obtained the maximum score, showing normal features of forelimb use: joint position, object support, digit movement and grasping technique.

3.2. Rats show high mortality rates during the study and related to the procedures

Almost half of all the rats (41.2%) developed tumors and required euthanasia during the training stage (Table 2) (Supplementary Fig. 2). Histological analysis revealed that the lesions were

Table 1
Primary antibodies.

Primary antibody	Marker of	Dilution	Source
CD68 (ED-1)	Microglia/macrophages	1:50	Millipore
GFAP	Astrocytes	1:2000	Millipore
HuNu	Human nuclei	1:300	Millipore
NF	Neurofilaments	1:1000	Biolegend (San Diego, CA)
Olig2	Oligodendrocytes	1:100	Millipore
Sox1	Neural Stem/ progenitor cells	1:100	R&D Systems (Minneapolis, MN)
Tuj1	Early-stage neurons	1:1000	Biolegend

Abbreviations: Cluster of differentiation 68 (CD68), glial fibrillary acidic protein (GFAP), human nuclei (HuNu), neurofilament (NF), oligodendrocyte transcription factor 2 (Olig2), Sox1, Neuron-specific Class III β-tubulin (Tuj1) markers.

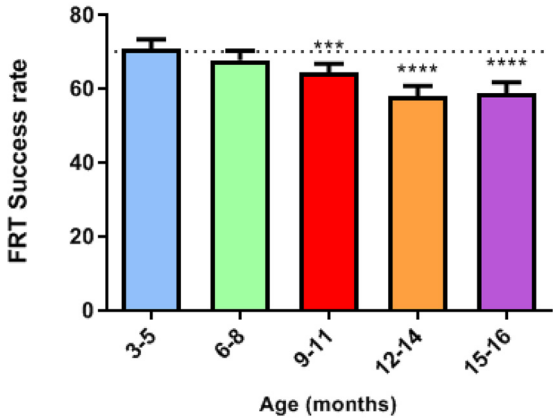

Fig. 1. Forelimb Reaching Task (FRT) success rate along lifespan. Plots show monthly success rate (mean ± SEM). The dotted line represents 70% success rate, set as the average optimum rate after a 3-month training period. Only those animals that could perform the FRT test with an optimum initial rate and that reached at least 16 months of age were considered for data analysis (n = 20). Repeated measures ANOVA test and Newman-Keuls multiple comparisons test show significant decrease in FRT success rates over time (*** $p \leq 0.001$; **** $p \leq 0.0001$).

mammary gland tumors (data not shown). Mortality rates of 63.2% and 50% were observed PI and PT, respectively (Table 2). Survival curve analysis of the animals along the training and experimental stages is shown in Fig. 2A.

Analysis of body weight at one week PI showed that rats experienced an average loss of 15.7% of their initial body weight (Fig. 2B), which they never recovered.

3.3. Transplanted IMR90-hiPSC-NPCs survive for one month in the spinal cord of aged animals

Five human iPSC lines and one embryonic stem cell line were initially differentiated to NPCs using an EB-based protocol (Supplementary Fig. 1) and were characterized by transcriptomic analysis (Fig. 3). The IMR90-hiPSC-NPC line was selected for transplantation based on the robust expression of neural differentiation and caudalization genes including HoxA5 (Fig. 3). IMR90-hiPSC-NPCs were expanded until passage 7 and were then injected into injured animals after confirmation of a normal karyotype (Supplementary Fig. 3).

Animals were sacrificed at four weeks PT. We characterized the spinal cord lesion site by immunostaining for neurofilament (NF), GFAP and ED-1. As shown in Fig. 4A–D, the side ipsilateral to the contusion appeared smaller suggesting a loss of tissue volume 8 weeks after the lesion. Axonal disruption was evident in the decrease of NF immunoreactivity inside the injured area (Fig. 4A, E). GFAP staining revealed the presence of reactive astrocytes surrounding the zone, especially in the ventral area (Fig. 4B, F). Within the injury site a few cells stained positive for ED-1 (microglia and macrophages), (Fig. 4G, H).

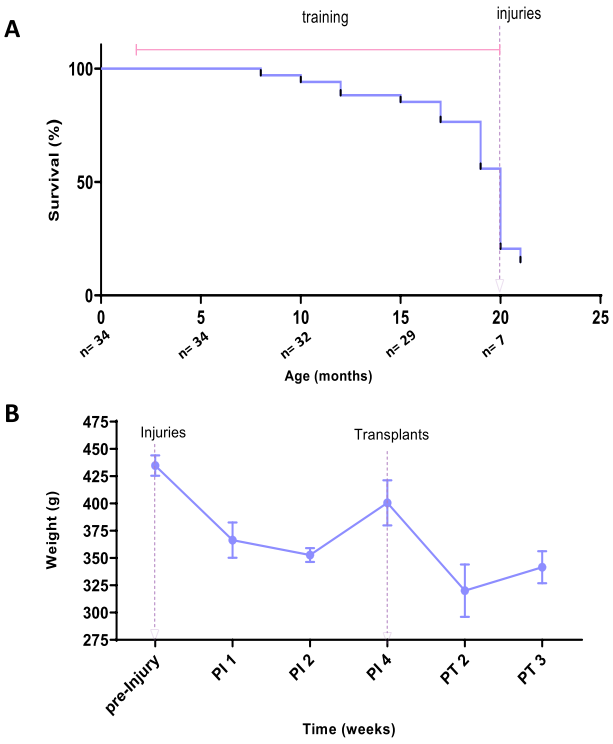

Fig. 2. Survival curve and rat body weights at different stages of the experiment. A Kaplan-Meier survival analysis was performed to examine the overall survival in the experiment (initial n = 34). Gradual loss of animals during the training period reflects deaths due to tumor appearance. The two sharp drops correspond to the mortality related to surgical procedures (contusion injury and transplantation). B Evolution of weight loss after the injury. Graph shows mean body weights ± SEM (initial n = 20; final n = 5).

IMR90-hiPSC-NPCs could be detected in the tissue at 4 weeks PT and no signs of tumor development or adverse reactions were noted. The implanted cells were positive for human nuclei (HuNu), Olig2, Tuj1 and GFAP markers (Fig. 5), and negative for Sox1 (data not shown). We quantified the cell survival and differentiation rates in the animals that survived 4 weeks PT (n = 2). In animal 1, the survival rate was 2.2% and differentiation rates were: 8.6% Tuj1, 61.6% Olig2 and 22.2% GFAP over HuNu; in animal 2, the survival rate was 6.7% and differentiation rates were: 11.2% Tuj1, 53.0% Olig2 and 18.3% GFAP over HuNu. No further statistical analysis was done due to the low number of surviving animals.

Table 2
Mortality rate. Number of animals, casualties, and mortality rate at each phase of the experiment. Despite the initial number of animals was 36, only 34 were considered, since 2 of them were taken out of the study because they did not learn to perform the tasks.

		N. of rats		N. of casualties	Mortality rate (%)
		Start	End		
Training time		34	20	14	41.2
Injury		19	7	12	63.2
	Dead during intervention			1	5.3
	Dead within 24 h after intervention			2	10.5
	Dead within 2–10 days			9	47.4
Cell injection		4	2	2	50.0
	Dead during intervention			0	0.0
	Dead within 24 h after intervention			0	0.0
	Dead within 2–10 days			2	50.0
Total process		34	5	29	85.3

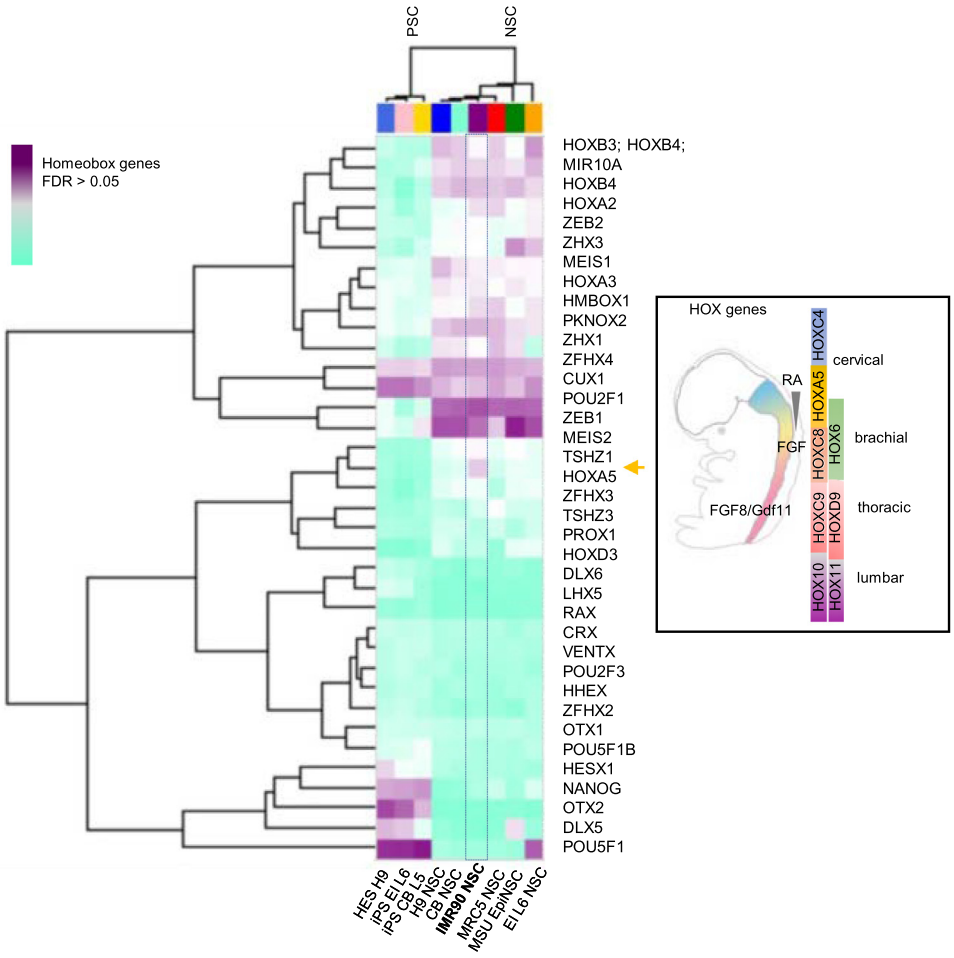

Fig. 3. Neural progenitor cell characterization by genome wide microarray profiling. IMR90-human iPSC-derived neural progenitor Cells (IMR90-hiPSC-NPCs) showed expression of neural differentiation and caudalization genes like HoxA5 and were therefore chosen for transplantation. Inset: schematic depicting the expression of *HOX* genes in human development.

No animals showed locomotor improvement in behavioral tests PI or PT in the treatment or control groups. The animals were unable to perform the FRT test from the moment they were injured (Fig. 6A). Regarding the LUAT, most of the animals did not explore the cylinder PI or PT and scored 0 at this task (Fig. 6B). Finally, the IBB scores fell dramatically PI and improved slightly in the following weeks in both control and treatment groups. Behavioral scores did not improve after cell transplantation (Fig. 6C, D).

4. Discussion

Given the increased incidence of SCI due to traumatic and non-traumatic lesions, the establishment of a valid model that mimics SCI in the elderly is of great importance. Our results show that pre-clinical animal model studies of cervical SCI involving aged rats require a large number of animals due to their intrinsic age-related frailty, which makes them more vulnerable to lesions and interventions. Behavioral assessment scoring progressively worsened in aged rats, and there was a high level of attrition during the extended study period due to unrelated causes such as tumor formation. Furthermore, aged animals experienced high mortality rates during surgical interventions. Nevertheless, the transplanted cells survived in the spinal cord of aged animals with no signs of tumor development or adverse reactions, although no locomotor improvement was observed.

The decrease in success rates during training is likely attributable to an age-related loss of capacity and interest in tasks, which is in agreement with the impairment in learning and performing executive functions experienced by older people.

The mortality rate during the training period was high given that––within the same group of animals––no rats died or were euthanized before 6 months of age. The main cause of mortality in our population was the presence of mammary gland tumors, which has previously been described in rats as they age. Indeed, Esfandiari *et al.* found that Long-Evans rats developed tumors at 18–26 months of age [41]. The difference between this study and ours was that our animals were younger when they developed tumors (8–20 months old) and the Esfandiari *et al.* study had a higher mortality (85%). Therefore, our considerations for calculating the initial sample size for the study were affected by the fact that 41.2% of the rats were euthanized due to tumor formation at an earlier age than that reported in the literature. It has been demonstrated that a relationship exists between high-fat and high-calorie diets and mammary carcinogenesis in rodents [42,43]. It is possible that earlier tumor formation in our study may be related to diet since treats were offered to the animals during the behavioral tests, increasing both the daily calorie and fat intake. Tumor development is a burden to consider in long-term studies with elderly animals.

At the end of the training stage, the rats had an average weight of 424 g, which posed an additional limitation. Magnetic resonance

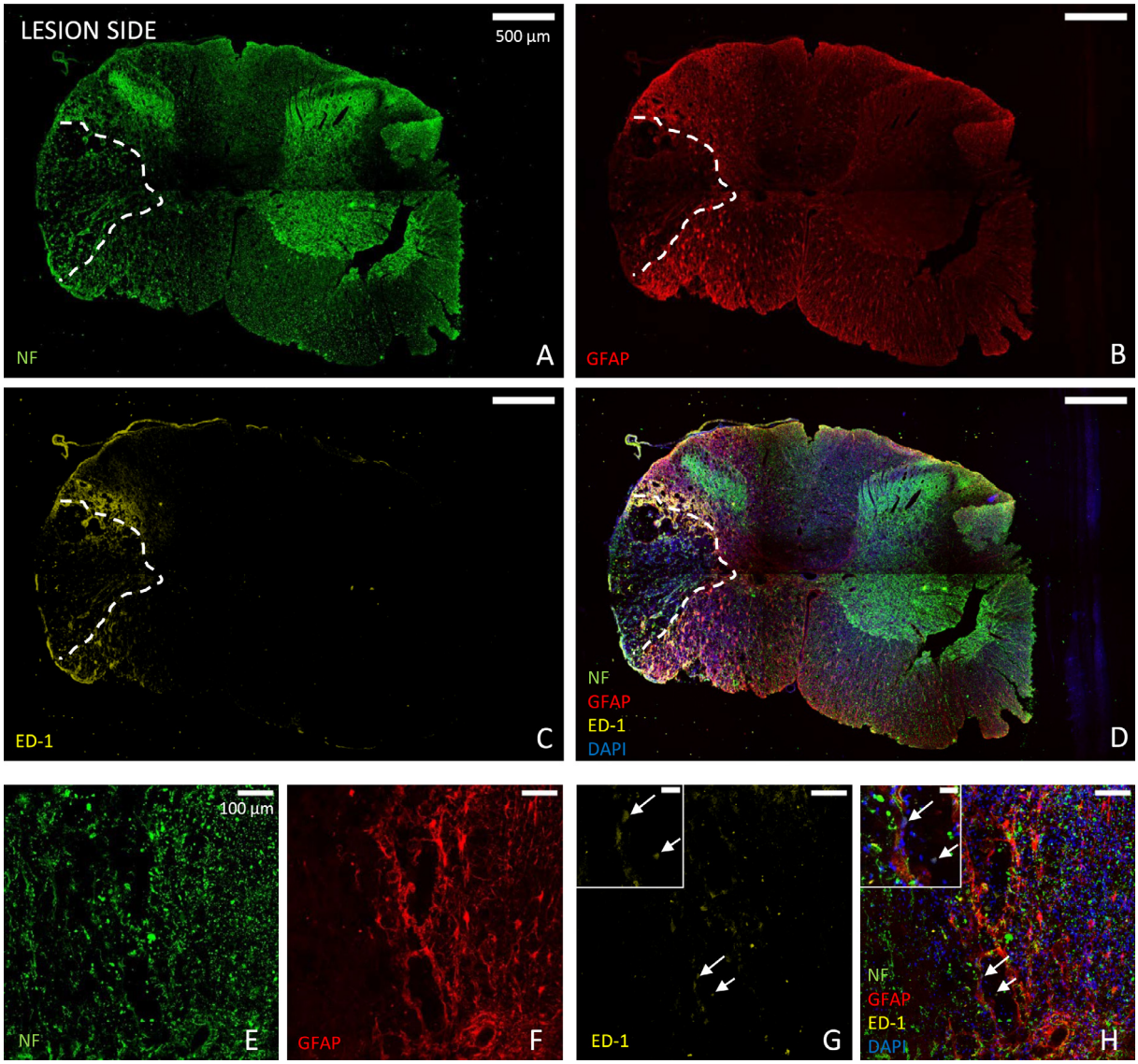

Fig. 4. Histological analysis of the spinal cord at injury level 8 weeks after the contusion (4 weeks after transplantation). (A–D). Characterization of the injured area (dashed line). The ipsilateral side of the spinal cord appears smaller suggesting loss of volume. (A) Loss of Neurofilament (NF) staining at the injury site illustrates axonal loss and disruption. (B) Glial fibrillary acidic protein (GFAP) staining shows reactive gliosis, delimiting the injury area (dashed lines). (C) CD68 (ED-1) staining reveals scattered macrophage/microglia cells located at the injury site. (E–H) NF, GFAP, ED-1 positive cells within and around the injury site observed at higher magnification at a different level. Arrows show ED-1 positive cells.

imaging was not feasible as our available magnet is designed to hold smaller animals.

The high mortality observed after SCI agrees with other studies involving aged rats, which showed that age affects mortality rates after thoracic SCI [20,44]. For example, Siegenthaler *et al.* observed that the mortality rate was much higher in aged rats (50%) than in young rats (8.3%) [20]. Our results show an even higher mortality rate (63.2%) in aged rats, which can be attributed to the cervical lesion, the severity of the injury, and the fact that our animals were slightly older than in the aforementioned study. Siegenthaler et al. also assessed animals for weight changes and observed that aged groups lost weight, agreeing with our results showing marked weight loss. Muscle atrophy after SCI may have contributed to this [45].

In our study, 50% of the animals died PT, likely illustrating their general weakened state. Nonetheless, the transplanted cells remained in the tissue and differentiated into the three neural lineages. Also, no tumor formation was observed in the tissue after cell transplantation, suggesting that IMR90-hiPSC-NPC transplantation is safe.

Histological analysis revealed that the grafted cells located at the contralateral side of the spinal cord, indicating that the anatomical landmark used to determine the injection site (lamina media) was displaced after the contusion. This might be due to the cell loss at the lesion site and the reduction in tissue volume, which can ultimately cause the midline to shift. These observations have been reported in a similar study in young adult rats [26]. Moreover, morphological age-related differences are known to occur at the cervical level in rats [46]. For future investigations, the precise location of lesions within the spinal cord must be examined preferably using imaging techniques [47] before cell implantation. A study on aged and young rats carrying thoracic contusions also found volume-associated differences between the two groups, describing a greater size lesion area and, therefore, more extensive cavitation and less tissue sparing caudal to the epicenter in aged animals [22]. The same study found differences in cell death and

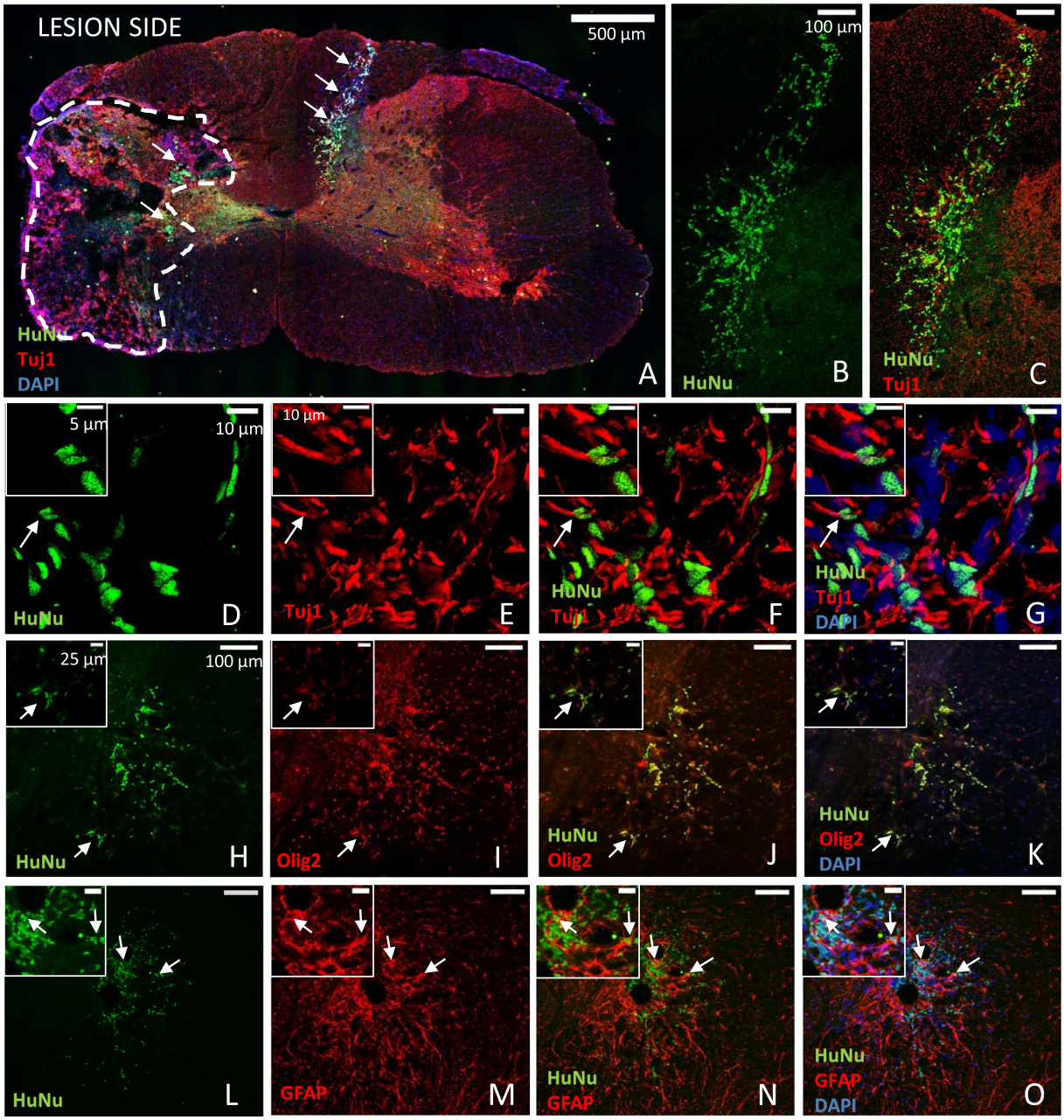

Fig. 5. Histological analysis locates IMR90-human iPSC-derived neural progenitor cells (IMR90-hiPSC-NPCs). (A) Human nuclei (HuNu)-positive cells mark the transplanted IMR90-hiPSC-NPCs (arrows) in the host tissue 4 weeks after the transplant. Loss of volume is seen at the lesion area. (B–G) Colocalization of grafted cells with neuron-specific class III β-tubulin (Tuj-1). Some grafted cells were Oligodendrocyte transcription factor 2 (Olig2)-positive cells (H–K) or Glial fibrillary acidic protein (GFAP)-positive cells (L–O). Arrows show HuNu positive cells for each marker; scale bars are stated in the panels.

inflammation when comparing aged and young rats at an acute phase after injury. This pro-inflammatory and apoptotic niche could affect the role of stem cells on the modulation of inflammation/scarring and the provision of support to the damaged neural cells [48].

Our results are in accord with recent published data on the evolution of SCI in elderly patients, where mortality can be up to 7-times higher than in younger peers [49]. Loss of tissue volume also occurs in patients, and the spinal cord can show enormous variations after traumatic SCI.

Despite the presence of differentiated cells in the injured tissue, animals did not show locomotor recovery. These results have also been observed in similar studies involving younger rats with early chronic SCI [26], suggesting that mobility is difficult to recover in chronic SCI with treatments involving stem cell therapies alone.

Recently, Nishi et al. [28] published a similar approach to ours in terms of modeling cervical unilateral SCI in young and aged mice, using two different mouse strains to characterize this type of injury. To our knowledge, however, ours is the first study on cell therapy-based treatment of severe, early chronic SCI in a population of aged rats. Given the increased global numbers of older people experiencing SCI and myelopathies, our model has great relevance. Cervical spondylotic myelopathy presents with a different physiopathology to what we have modeled in this study in terms of the compression exerted on the spinal cord, which is continuous for spondylosis. However, the lesion produced with the

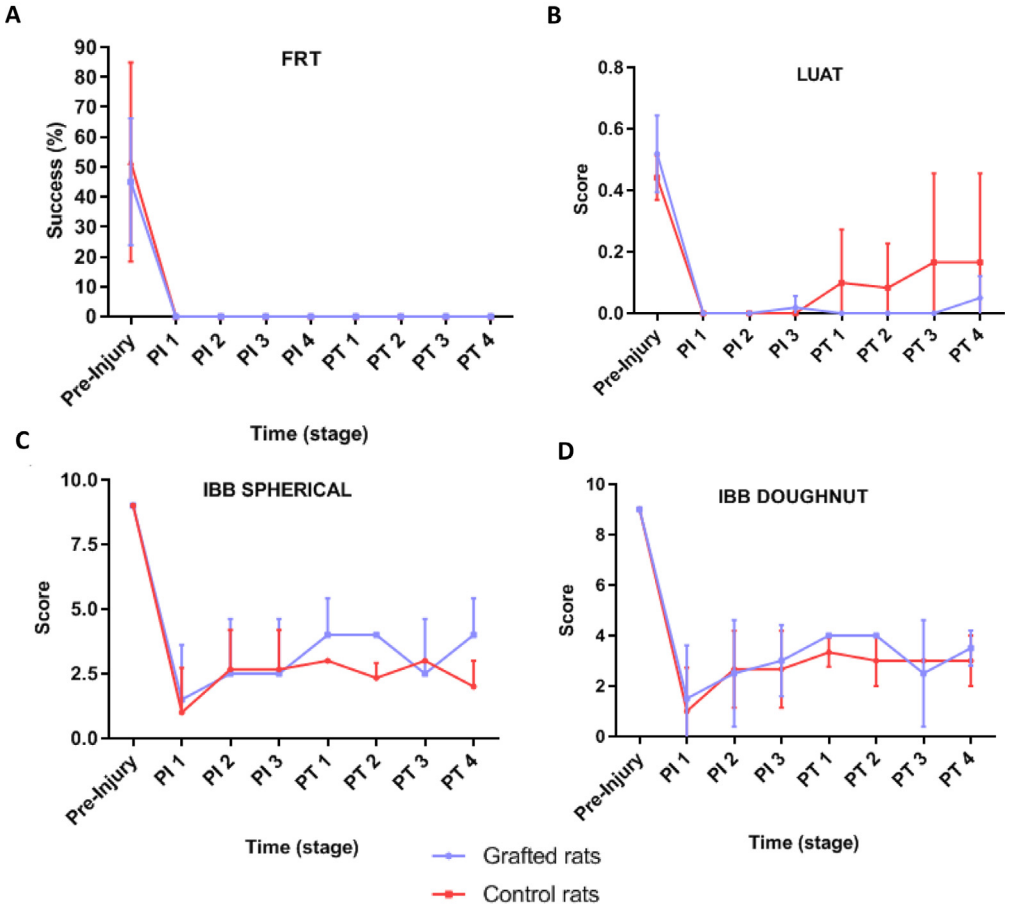

Fig. 6. Locomotor recovery assessment. (A) Forelimb Reaching Task (FRT). The test showed no difference in performance between control and grafted groups. (B) Limb Use Asymmetry Test (LUAT). Most animals did not explore the cylinder and scored 0 at this test. (C, D) Irvine, Beatties and Bresnahan (IBB) test. The scores drastically decreased post-injury and slightly increased in the following weeks in both groups. Graphs show mean scores. Control rats, n = 3; injected rats, n = 2.

fourth-generation Ohio State Injury Device is very reproducible and has been validated in several studies [26,50,51]. It can thus represent the chronic final lesion after long-term compression. Still, larger pre-clinical studies of cervical SCI considering both male and female rodents and with additional sham and immunosuppressant control groups are required to establish the potential efficacy of cell-based therapies in age-related chronic myelopathy. Indeed, the major limitation of the present work is the small sample size at the end of the experiment; however, because there are few data published on aged animal models our preliminary findings might help guide future works.

5. Conclusion

Larger pre-clinical studies of cervical SCI are required to establish the potential efficacy of cell-based therapies in age related conditions given the high vulnerability of aged rats.

Ethical statement

This study was approved by the Andalusian Ethical Committee of Research with Biological Samples of an Embryonic Origin and Similar Cells (RC/001/2013).

All procedures involving animal use were carried out under the Animal Welfare Regulations and approved by the Animal Welfare Commission of the "Hospital Virgen del Rocío/ IBiS" and "Consejería de Agricultura" of Andalucía (2013PI/025).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jocn.2021.09.042.

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