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Neuroprotective effects of human umbilical cord mesenchymal stromal cells in an immunocompetent animal model of Parkinson's disease

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ABSTRACT

Microglial activation in the substantia nigra (SN) is a ubiquitous feature in PD which could mediate toxic effects. Human mesenchymal stromal cells (hMSCs) possess immunomodulatory properties. We evaluated whether the transplantation of hMSCs obtained from umbilical cord had a neuroprotective effect in a not-immunosuppressed rat Parkinson's disease (PD) model. Rats receiving hMSCs in the SN displayed significant preservation in the number of dopaminergic neurons in the SN at 21 days after lesion and an improved performance in behavioral tests compared to control rats. However, no differences in any inflammatory parameter tested were found. These results suggest that grafted hMSCs exert neuroprotection but not neuromodulatory effects on degenerating dopaminergic neurons.

1. Introduction

Parkinson's disease (PD) is a neurological disorder mainly characterized by the progressive loss of neurons from the substantia nigra *pars compacta* (SNpc). This neuronal loss leads to dopamine deficiency in the striatum, which is responsible for characteristic motor symptoms such as akinesia, rigidity and tremor (Hirsch and Hunot, 2009). None of the current treatments repair this dopaminergic pathway or delay nigral cell loss.

A main feature of the patho-physiology of PD is the ubiquitous presence of microglial activation in the SNpc in animal models and PD patients. The SNpc is highly susceptible to inflammation, possesses the highest density of microglial cells and inflammation has been shown to elicit or exacerbate dopaminergic neuronal demise in the SNpc (Lawson et al., 1990; Pott Godoy et al., 2008; De Lella Ezcurra et al., 2010; Pott Godoy et al., 2010). Therefore, immunomodulatory strategies are being studied as possible novel therapies against PD.

Recent works suggest that grafting of mesenchymal stromal cells (hMSCs) derived from human bone marrow in the striatum could induce neuroprotective effects (Kim et al., 2009; Sadan et al., 2009; Blandini et al., 2010; De Lella Ezcurra et al., 2010; Wang et al., 2010; Sheikh et al., 2011). This neuroprotective effect was related to the secretion of neurotrophic factors such as glial cell line derived neurotrophic factor (GDNF) (Sadan et al., 2009; Blandini et al., 2010; Wang et al., 2010). In addition, hMSCs were reported to possess immunomodulatory properties *in vitro* (Ren et al., 2008) or using *in vivo* models of lipopolysaccharide (LPS) induced inflammation (Kim et al., 2009). The Wharton jelly from the umbilical cord is an easy accessible source for these cells, but hMSC of this origin has not been tested in PD models.

Therefore, hMSCs seem to be good candidates to exert neuroprotective actions to the SNpc either by trophic factor release and/or immunomodulatory properties. However, no previous work has addressed the neuroprotective potential of hMSCs derived from umbilical cord when injected in the main degenerating area in PD, the SNpc. In this report, we show for the first time that hMSCs from umbilical cord can have a transient neuroprotective effect on nigral cell loss when injected in the degenerating SNpc. This neuroprotection was associated with an alleviation of motor symptoms. No immunomodulatory effect by hMSCs was detected that could explain these beneficial effects. However, no major innate or adaptive immune response was observed when these human cells were injected in immunocompetent animals, confirming previous reports on host immunological ignorance towards these cells and/or their immunomodulatory properties. These observations set the basis for further exploration in animal models on the neuroprotective properties of a cell source of easy access with immunological characteristics that could favor its clinical application in PD patients.

2. Materials and methods

2.1. Isolation and maintenance of human MSCs

We obtained written informed consent from all persons who agreed that the umbilical cord donated could be used for research

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purposes. Umbilical cords from full-term delivery donors were processed and hMSCs were isolated from the Wharton jelly with enzymatic digestion (Sarugaser et al., 2005). Cells were placed in 10-cm dishes, cultivated in Minimum Essential Medium Alpha medium (alpha-MEM, Gibco-Invitrogen), containing 10% FBS, 50 μg/ml gentamicin (GIBCO-Invitrogen), 0.3 ug/ml amphotericin B (GIBCO-Invitrogen) and 1% penicillim/streptomycin, and incubated in a humidified incubator at 37 °C in 5% CO_2. Medium containing non-adherent cells was replaced every 3 days of culture. When the cells reached 70–80% confluence, they were trypsinized and subcultured. For multipotency testing culture cells were subjected to adipogenic, chondrogenic and osteogenic *in vitro* differentiation according to established protocols (Sarugaser et al., 2009). At passage 6, cells were trypsinized and suspended in medium without FBS to perform the transplantations.

2.2. Animals and injections

Adult male Wistar rats (2 to 3 month old) were housed in groups of four animals, under controlled temperature (22 °C ± 2 °C), artificially lit under a 12-h cycle period and with water and food *ad libitum*. All animal procedures were performed according to the rules and standards of National Institute of Health, USA. Animal experiments were approved by the Ethical Committee of the Institute Leloir Foundation.

2.3. Nigrostriatal lesion

Animals were anesthetized by 3% isofluorane inhalation and arbitrary divided into two groups. Rats were placed in a stereotaxic frame (Stoelting, Wood Dale, IL, USA); the 6-OHDA group ($n = 16–22$) received 4 μl of a 6-OHDA solution (a total of 65 μg in 0.02% ascorbic acid solution; Sigma Aldrich, St. Louis, MO, USA) into the left striatum (bregma, +1.0 mm; lateral, +3.0 mm; ventral, −4.5 mm) (Paxinos and Watson, 1986). The sham group received an equal volume of ascorbic acid solution (AA) ($n = 12$), at the same stereotaxic coordinates. Injections were performed at a flow rate of 0.5 μl/min, using a Hamilton 10-μl syringe with a 26-gauge needle, and kept in place for additional 2 min before removal.

2.4. Intrastriatal grafts

Seven days after the nigrostriatal lesion, the 6-OHDA and AA groups were further divided into two subgroups. Transplanted animals received 20,000 hMSCs, while control groups received an injection of medium without FBS. All animals were injected into the left SN (bregma, −5.3 mm; lateral, +2.0 mm; ventral, −7.0 mm). The following four experimental groups were obtained: 1) 6-OHDA/hMSCs: animals lesioned with 6-OHDA that received hMSCs in the SN ($n = 11$); 2) 6-OHDA/Medium: animals lesioned with 6-OHDA that received medium in the SN ($n = 8–11$); 3) AA/hMSCs: unlesioned animals that received hMSCs in the SN ($n = 7$); 4) AA/Medium: unlesioned animals that received medium in the SN ($n = 5–6$).

All intracerebral infusions were performed at 0.5 μl/min, using a Hamilton 10-μl syringe with a 26-gauge needle. To avoid reflux along the inoculation track, the needle was left in place for 8 min before being retracted. No immunosuppressive treatment was used. The animals were killed at 14 or 28 days after the transplant.

2.5. Histology

The animals were deeply anesthetized and transcardially perfused with heparinized saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.2). After dissecting the brains, they were placed in the same fixative overnight at 4 °C. Then, the tissues were cryoprotected by immersion in 30% sucrose, frozen in isopentane and serially sectioned in a cryostat (40 μm). The 40-μm sections were used either for cresyl violet staining or for free floating immunohistochemistry.

2.6. Immunohistochemistry

Free-floating sections were incubated in blocking buffer (1% donkey serum, 0.1% Triton in 0.1 M PB) and incubated overnight with primary antibodies. The antibodies used were anti-tyrosine hydroxylase (TH) for dopaminergic neurons (diluted 1:1000; Chemicon, Temecula, CA) and anti-glial fibrillary acidic protein (GFAP) (1:600; for astrocytes; Dako, Carpinteria, CA). Alternatively, we used the biotinylated lectin Griffonia simplicifolia (GSA-1B4, 1:50; Vector Laboratories, Burlingame, CA), for microglial cells (Kaur and Ling, 1991). For immunohistochemical identification of dopaminergic neurons, the sections were incubated with donkey anti-rabbit biotin conjugated antibody (Jackson, ImmunoResearch Laboratories Inc., West Grove, PA) followed by Vectastain standard ABC kit (Vector Laboratories, Burlingame, CA) and developed with 3,3′-diaminobenzidine (Sigma, Saint Louis, Missouri). For double-labeling immunohistochemistry, the sections were incubated with either indocarbocyanine Cy3 (Cy3) conjugated donkey anti-rabbit antibody (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA), cyanine Cy2 (Cy2) conjugated donkey anti-mouse antibody (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or Cy2 conjugated streptavidin (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Digital images were collected in a Zeiss LSM Meta laser scanning confocal microscope equipped with a krypton–argon laser.

2.7. Quantification of TH-positive cells and area

The number of TH-positive neurons was assessed by using an unbiased optical fractionator designed by the Stereo Investigator analysis software (MicrobrightField Inc., USA), combined with a Nikon Eclipse E600 microscope and a CX900 video camera (MicrobrightField Inc., USA). We analysed the three main sections of the SN every sixth 40 μm section where the injection site was included (identified in the Nissl staining). The SN *pars compacta* (SNpc) was delineated using a 2× objective and a systematic sampling of TH-positive neurons on the outlined areas were counted with a 100× oil-immersion objective (numeric aperture 1.30). Counts were taken at predetermined intervals ($x = 120$, $y = 120$), and a counting frame (50 × 50 μm) was superimposed on the live image of the tissue sections. Section thickness was determined every fifth measurement by focusing on the top of the section, zeroing the *z*-axis and focusing on the bottom of the section. The media thickness was 15 μm and the dissector height was set at 9 μm with a 3 μm top guard zone. The media Coefficient of Error (Gundersen) was 0.11.

Results were expressed as percentage of TH-positive cells in the ipsilateral SN with respect to the contralateral side.

The TH-positive area in the striatum of 6-OHDA/Medium or 6-OHDA/hMSCs groups was determined by using the Image J program with a set measurement of 270 pixels/mm. The TH + area in the injected side was defined as the area with more than 80% of the TH intensity present in the contralateral side of the same brain section. The results were expressed as the percentage of TH + area in the injected ipsilateral side compared to the contralateral side. The TH + area was measure in four sections every sixth section between 1.7 to −0.40 mm from bregma (Paxinos and Watson, 1986) in each animal of each group.

2.8. Quantification of activated microglia

For the quantitation of microglial activation we adopted the classification of microglial activation according to Kreutzberg (Kreutzberg, 1996) and as previously described (Depino et al., 2003; Ferrari et al., 2006; Pott Godoy et al., 2008; Pott Godoy et al., 2010).

Stage 1: Resting microglia. Rod shaped soma with fine and ramified processes.

Stage 2: Activated ramified microglia. Elongated shaped cell body with long and thicker processes.

Stage 3: Amoeboid microglia. Round shaped body with short, thick and stout processes.

Stage 4: Phagocytic cells. Round shaped cells with vacuolated cytoplasm, no processes can be observed at light microscopy level.

All these cellular types are GSA positive. Stages of microglia activation were identified by their morphology on GSA staining under 40× magnification and counted in every sixth 40-μm-thick serial section of the SNpc of each rat using a double-blind procedure.

2.9. Behavioral test

2.9.1. Cylinder test

Forelimb akinesia was assessed using the test previously described (Schallert and Jones, 1993; Pott Godoy et al., 2008; De Lella Ezcurra et al., 2010; Pott Godoy et al., 2010) (n = 6-11 per group). This test evaluates the use of the forelimb to support the body against the walls of a cylinder. The rats were put individually in an acrylic cylinder (20 cm × 30 cm). The number of wall contacts performed independently with the left and the right forepaw was counted.

2.9.2. Adjusting steps

This test was performed as previously described (Depino et al., 2003) for assessment of akinesia in the unilateral Parkinson model ($n = 6$–8 per group). Briefly, the experimenter held the rat with one hand fixing the hind limbs and slightly raising the hind part. The rat was moved slowly sideways by the experimenter from right–left direction in first place. The number of adjusting steps was counted for both paws in the backhand and the forehand direction of the movement. The sequence of testing was right (contralateral) paw forehand and backhand and then the left paw. The experimenter handled the rats at least three times before testing to familiarize the animal with the experimenter's grip. The last assay before surgery was considered as the pre-lesion stepping test.

2.10. Statistical analysis

Results are expressed as mean ± SEM of different animals in the different treatment groups. Statistically differences among treatments were determined using two-factor analysis of variance (ANOVA) and Bonferroni's multiple comparison test. For statistical evaluation of the cylinder test and the adjusting step test, the data were subject to two-factor analysis of variance (ANOVA) and Bonferroni's multiple comparison test or one-factor analysis of variance, Kruskal–Wallis test, respectively.

3. Results

3.1. Effect of hMSCs grafting in the SN of 6-OHDA lesioned animals

Umbilical cord hMSC was first characterized for the expression of specific markers by flow cytometry. hMSC was defined as CD 90 (+), CD 73 (+), CD 105 (+), CD 44 (+), CD 34 (−), HLA-DR (−), CD 14 (−) and CD 45 (−), and was capable to differentiate into adipogenic, chondrogenic and osteogenic linages (Supplementary Fig. 1).

The hMSCs were grafted into the ipsilateral SN of rats 7 days after the intrastriatal injection of 6-OHDA and the level of neurodegeneration was assessed at 21 or 35 days post 6-OHDA inoculation (Fig. 1A). In order to identify transplanted cells we used an anti-human CD105 antibody to stain the hMSCs. Surviving cells were detected at 21 and 35 days after lesion near the injection tract (Supplementary Fig. 2). At those time points, hMSCs were in direct contact with the dopaminergic cells bodies.

The extent of the neurotoxin-induced nigrostriatal damage at 21 days after 6-OHDA injection was significantly reduced in animals transplanted with hMSCs in the SN compared with controls (Fig. 1B and D). The grafting of hMSCs increased approximately 20% the number of dopaminergic cell bodies in the SNpc in the injected hemisphere compared to lesioned animals that received medium (Fig. 1D). When the effect of the hMSCs graft was evaluated 35 days after 6-OHDA injection the amelioration of the neurodegeneration was no longer observed (Fig. 1C and E). This result shows a transient neuroprotective effect of the hMSCs transplanted in the SN. Transplantation of hMSCs in vehicle-injected animals (AA/hMSCs) did not modify *per se* the number of TH-positive cells in the SNpc (Fig. 1B to E).

In addition, in order to test the impact of the transplant in the lesioned striatum we analysed the TH-positive area of the striatum in comparison with the contralateral side (Fig. 2A). This analysis was performed at 21 and 35 days after the 6-OHDA lesion. We found that there were no differences in the TH-positive area between 6-OHDA/hMSCs and 6-OHDA/Medium groups, even at 21 days when the neuroprotective effect was observed in the SN (Fig. 2B). These results showed that the neuroprotective effect of the transplant was restricted to the SN area where the hMSCs were injected. Transplantation of hMSCs in vehicle-injected animals (AA/hMSCs) did not modify the density of dopaminergic terminals in the striatum (Supplementary Fig. 3).

3.2. Inflammatory response after hMSCs transplantation

The inflammatory response was analysed at the time when neuroprotection was observed (21 days after the lesion), initially by Nissl staining. We found that inflammation was composed exclusively of monocytes/macrophages with no polymorphonuclear (neutrophils) cells and was restricted to the injection site in all the experimental groups (Fig. 3). Monocytes/macrophages could be observed within the vessels which showed no vasodilatation. No edema or necrotic tissue can be observed in any of the experimental groups. As we previously described (Depino et al., 2003) no infiltrating inflammatory cells (lymphocytes) were seen in the SNpc after 6-OHDA administration.

Microglial activation at 21 days after 6-OHDA injection was detected by GSA-lectin staining in the SN ipsilateral to the intrastriatal neurotoxin injection. GSA-positive cells with stage 2, 3 and 4 of activation were observed in the SNpc (Fig. 4A). In a previous work we observed that the amount of activated microglia after striatal 6-OHDA injection started to increase at day 6, with an increment at 12 days and a tendency to reduction 28 days after lesion (Depino et al., 2003). In agreement with this previous report our results showed that the amount of activated microglia in the SNpc at 21 days after the 6-OHDA lesion was scarce (Fig. 4A and B). In addition, there were no significant differences between the 6-OHDA/Medium and 6-OHDA/hMSCs groups (Fig. 4A and B). The numbers of the lectin-positive cells were restricted to a total of approximately 35 or 28 of type 2–3, and only 3 or 2 of type 4 GSA-positive microglial cells in the 6-OHDA/Medium or 6-OHDA/hMSCs groups along the whole SNpc, respectively (Fig. 4B). Stage 4 of activation was detected mainly near the injection site. Alternatively, we used MHCII (class II major histocompatibility complex) to stain activated microglial cells. Using this marker we only observed MHCII-positive cells in the injection tract and a scarce amount of positive cells in the SNpc ipsilateral to the lesion (Supplementary Fig. 4). In addition, no differences in the number

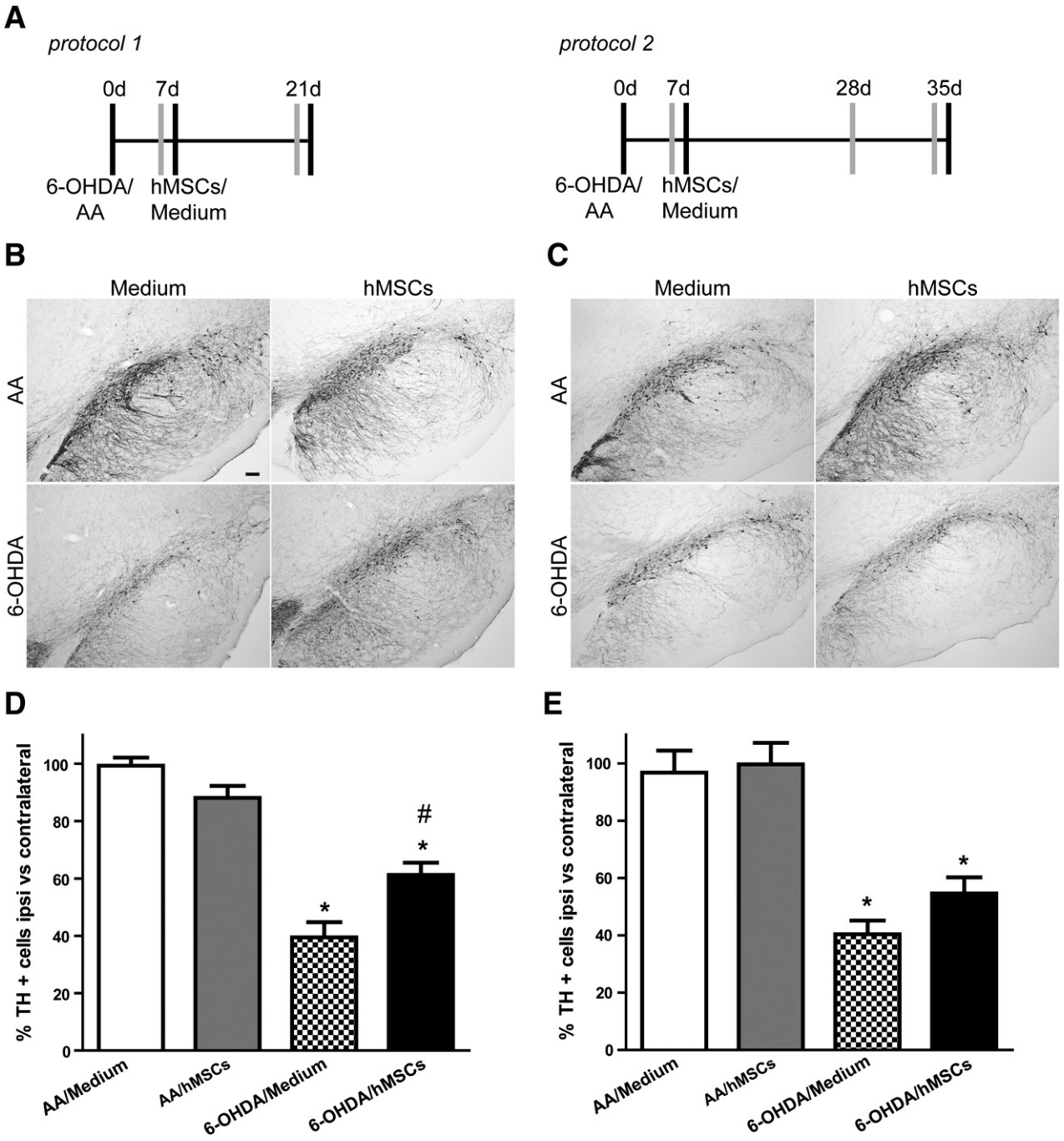

Fig. 1. Transient neuroprotective effect of transplanted hMSCs in the SNpc. Experimental schedules are shown in (A). Protocol 1 and 2 lasted for 21 or 35 days, respectively. Grey bars represent the behavior testing time points. Representative images for the immunohistochemical detection of TH + cells in the ipsilateral SN at 21 (B) or 35 (C) days after 6-OHDA injection for each experimental groups (6-OHDA or vehicle (AA)) injected and transplanted (hMSC) or not transplanted (Medium). Scale bar: 0.1 mm. The results for the immunohistochemical staining of TH + cells were expressed as the ratio between the TH + cells in the ipsilateral side related to the contralateral side for animals sacrificed 21 (D) or 35 (E) days after the lesion. Values are expressed as mean ± SEM for the different treatment groups. Statistically differences among treatments were determined using two-way analysis of variance test followed by Bonferroni's multiple comparison test. $p<0.001$, *, statistical difference between 6-OHDA and AA injected animals; $p<0.01$, #, statistical difference between 6-OHDA/Medium and 6-OHDA/hMSCs groups.

of GFAP-positive astrocytes were detected in the SNpc regardless of the treatment and time point studied (Fig. 4C). Moreover, a similar response was observed in control groups (AA/Medium, AA/hMSCs) (Supplementary Fig. 5), which indicate that the scarce response observed is mainly due to the Hamilton injection in the SN.

3.3. Behavioral tests

The cylinder test was performed to assess forelimb akinesia as it has been described previously (Schallert and Jones, 1993). As expected, the injection of 6-OHDA decreased the number of wall contacts with the right forepaw in 6-OHDA injected groups (Fig. 5A). A decrease in the percentage of right paw placement was already observed at 7 days post lesion, although there was no statistical difference between the groups. At 28 and 35 days after the dopaminergic lesion, the performance of the 6-OHDA/Medium and 6-OHDA/hMSCs groups was statistically different from the AA/Medium control group (Fig. 5A). A slight tendency of motor improvement in the 6-OHDA/hMSCs group at both time points could be observed by this test (Fig. 5A).

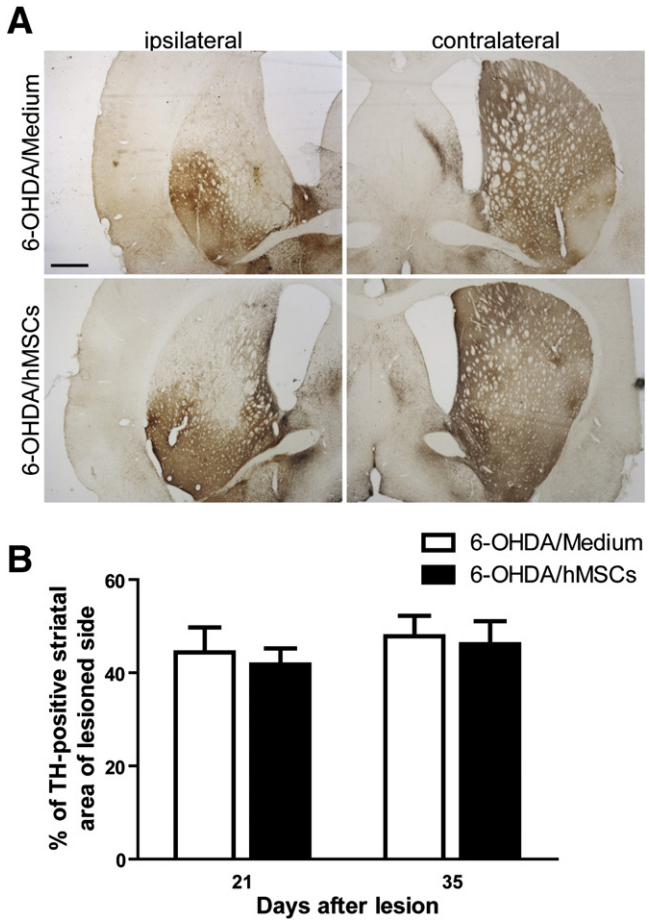

Fig. 2. Immunohistochemical analysis of the TH-positive area in the striatum. The effect in the striatum of the SN-graft was evaluated in 6-OHDA groups after 21 or 35 days after 6-OHDA injection. Representative images are shown in (A). Results were expressed as the percentage of TH-positive striatal area of lesioned side in comparison with contralateral side (B). No statistical differences were observed between these groups. Scale bar: 1 mm.

As expected, the adjusting step test of animals injected with medium after 6-OHDA showed a significant impairment in the performance of the right (contralateral) paw in the forehand direction at 28 and 35 days after lesion compared with the left paw or the same paw of AA/medium or AA/hMSCs groups (Fig. 5B and data not shown). Also, the rats injected with AA and medium or hMSCs (AA/medium, AA/hMSCs) showed no statistically significant differences in the number of steps with both paws at any time point studied (Fig. 5B). Interestingly, in the 6-OHDA/Medium group the motor impairment was already significant at 28 days after lesion, whereas the 6-OHDA/hMSCs showed that the impairment in the adjusting steps did not take place until 35 days after neurotoxin injection, showing a delay of the start of movement impairment in the animals grafted with hMSCs. This test shows a functional correlation with the transient neuroprotective effect observed on dopaminergic cells bodies 21 days after lesion (compare Figs. 1D and E, and 5B).

Overall these results demonstrate that the neuroprotection achieved with this protocol account for a minor but significant behavioral improvement. Therefore, these data are encouraging to further analyse conditions that will improve neuroprotective effects of hMSCs in PD models.

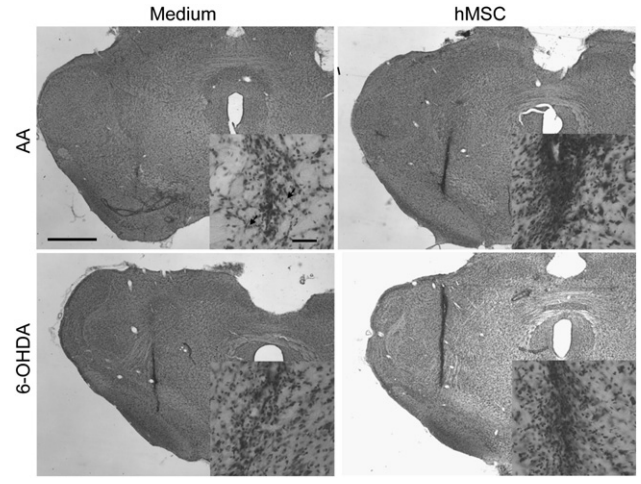

Fig. 3. Inflammatory response after hMSCs transplant. To evaluate the inflammatory response towards the transplanted cells, 40 micron brain sections every 0.24 mm were stained with cresyl violet. Representative ipsilateral SN sections are shown. Insets show higher magnification of the injection site. Inflammatory infiltrate can be observed at the injection tract and is mostly composed of macrophages (inset, arrow). Scale bars: 1 mm, inset 0.05 mm.

4. Discussion

In the current study, we investigated the neuroprotective effect of undifferentiated hMSCs obtained from the umbilical cord in a rat PD model without immunosuppression. We have transplanted these cells or control medium directly into the degenerating SN and assessed changes in neurodegeneration, inflammation and motor behavior. We have observed that a mild and transient neuroprotection and improved motor behavior were achieved with this treatment. Surprisingly, no adaptive immune response or major signs of inflammation as expected from the injection of a xenograft in the SN were observed. To the best of our knowledge, this is the first report that explores the effects of an intranigral transplant of umbilical cord hMSCs into the degenerating SN. This is the first proof of concept that human MSC grafted in the SN could be neuroprotective in the 6-OHDA model.

We evaluated the neuroprotection at two time points, 14 and 28 days post-transplant. Compared to previous works where the cell transplant was performed a few hours or less post 6-OHDA inoculation (Sadan et al., 2009; Wang et al., 2010), we have performed the transplant at a time point when neurodegeneration has already started in the SNpc. The results showed that the hMSCs were able to protect the dopaminergic cell bodies from the on-going degeneration at 21 days after transplant. This neuroprotective effect increased the survival of the SNpc neurons in approximately 20%. The analysis of the remnant dopaminergic neurons 28 days after transplant showed that the neuroprotective effect was no longer present, suggesting that it was a transient effect.

Although the neuroprotection was not evident in the striatum, the behavioral test showed that there was a minor, but significant, improvement in the motor performance of the treated animals. This effect correlates with the neuroprotection achieved at day 21 post 6-OHDA by the administration of hMSCs in the SNpc. The fact that a few hMSCs were found in direct contact with the SNpc cell bodies at the time points analysed makes our results encouraging for the evaluation of different transplantation approaches in order to obtain a greater number of transplanted cells in the proximity of SNpc dopaminergic neurons. We speculate that a higher number of hMSCs near the SNpc neurons could result in an increased motor improvement. PD patients start showing clinical signs of the disease when more than one third of nigral neurons and 70–80% of striatal dopamine are lost (Bohnen et al., 2006; Greffard et al., 2006). In addition, compensatory mechanisms prevent neuronal demise to translate into clinical symptoms (Nandhagopal et al., 2011). Therefore, minor changes in dopaminergic cell survival or striatal dopamine levels may have overt consequences in improving clinical symptoms. It is tempting to speculate that the transient neuroprotective effect observed at the cellular and behavioral level in this

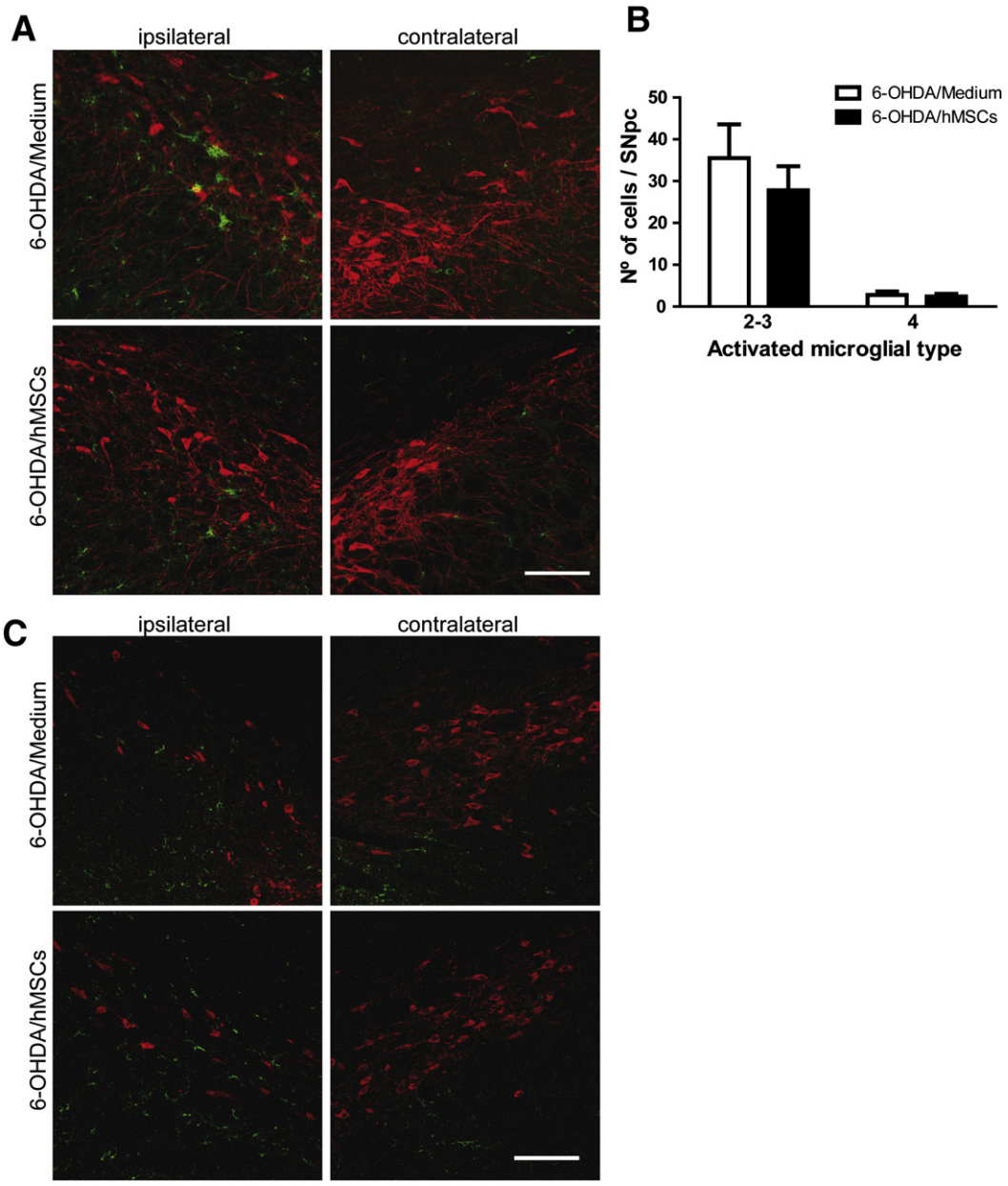

Fig. 4. Effect of hMSCs transplant in glial cell activation. Microglial activation was evaluated by GSA (green). The area of the SNpc was determined by TH (red) immunostaining (A). The degree of microglial cell activation was determined by counting the number of cells GSA + with morphology type 2–3 or 4 all along the ipsilateral SNpc (B). There was no statistical difference between the number of activated microglial cells in the 6-OHDA/Medium and 6-OHDA/hMSCs groups. Scarce GFAP + cells were observed in the SNpc in both 6-OHDA groups (C). Scale bar: 0.1 mm.

animal model may increase the probability for compensatory mechanisms to be expressed and/or complement the traditional pharmacological treatment to compensate dopamine deficiency. Either way, hMSCs transplantation in the SN may result into an increased time of reduced symptoms or symptoms-free intervals in PD patients.

Previous works had pointed out the immunomodulatory capacity of MSCs (Ren et al., 2008; Kim et al., 2009; Sheikh et al., 2011) and it was previously reported that there is an activation of microglial cells during nigral neurodegeneration and a functional effect of inflammation on dopaminergic cell demise (Lawson et al., 1990; Depino et al., 2003; Whitton, 2007; Pott Godoy et al., 2008; Hirsch and Hunot, 2009; De Lella Ezcurra et al., 2010; Pott Godoy et al., 2010). Therefore, one of the goals of this work was to evaluate the immunomodulatory effect of the hMSCs in this model. In order to evaluate the effect of the xenograft on the inflammatory component, no immunosuppressive treatment was used. The inflammatory response against the transplant was only evident in the injection site and was mainly composed of macrophages and microglia. The analysis of microglial activation showed that there were no statistical differences in the 6-OHDA animals receiving hMSCs or medium. At a first glance, this result suggests that immunomodulation of the neurodegenerative processes by the hMSCs might not be responsible for the neuroprotection observed. Interestingly however, there were no differences observed in microglial activation in vehicle-injected animals receiving hMSC or medium. This result, in the absence of an immunosuppressive treatment, suggests an active immunomodulatory capacity of these cells or immunological ignorance by the host cells. A deeper analysis of inflammation at additional time points and a thorough study on the functional contribution of the molecules secreted by hMSC are needed to truthfully dismiss immunomodulatory actions of hMSCs in the neuroprotective effect observed.

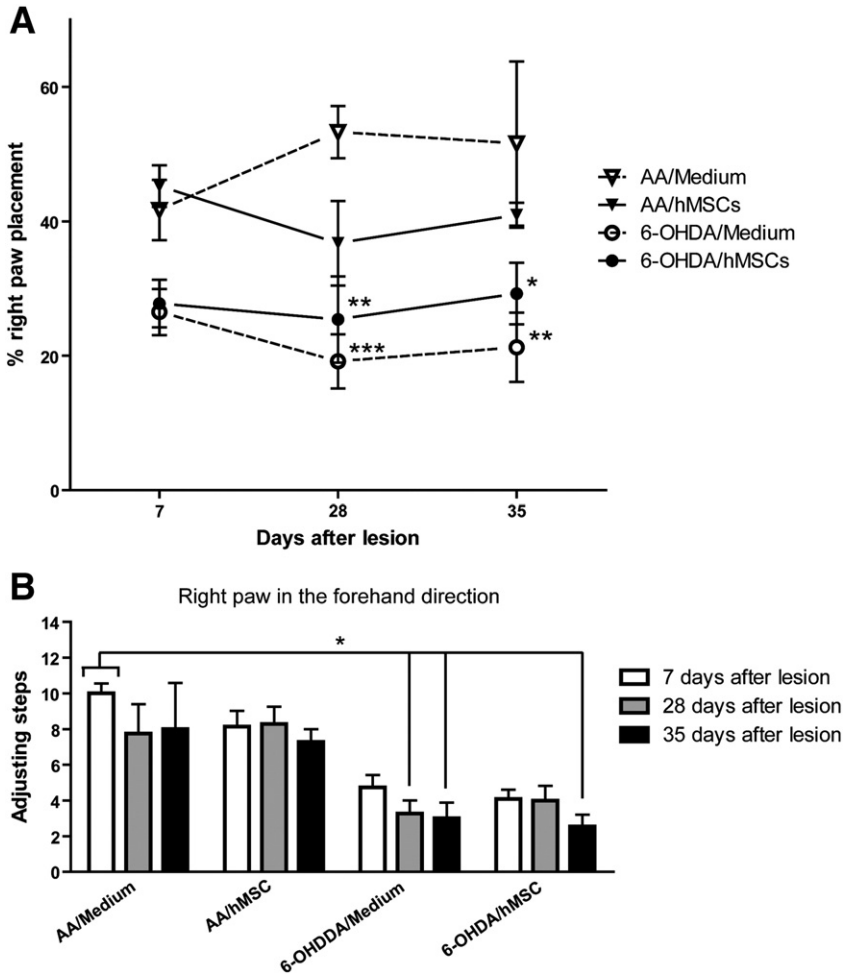

Fig. 5. Behavioral tests. Results for cylinder tests are expressed as the percentage of right forepaw placement of total touches for each experimental group and for each time point (A). For statistical evaluation the data were subject to a two-factor analysis of variance and Bonferroni's multiple comparison tests. All groups were compared to AA/Medium group and the level of statistical significance was set at $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Results for adjusting step test are expressed as the number of adjusting steps for the right paw in the forehand direction for each experimental group at 7, 28 or 35 days after the neurotoxin injection (B). The statistical differences were assessed in comparison to the values obtained for control group AA/Medium at 7 days after lesion. For statistical evaluation the data were subject to a one-factor analysis of variance, Kruskal–Wallis test. The level of statistical significance was set at $^{*}p<0.05$.

Other mechanism by which MSC could exert neuroprotective effects is the secretion of trophic factors capable of increasing the survival of the dopaminergic neurons. In this regard several groups have demonstrated that MSCs could secrete GDNF, brain derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), stromal cell derived factor 1 alpha (SDF-1α) and nerve growth factor (NGF) (Koh et al., 2008; Sadan et al., 2009; Blandini et al., 2010; Nicaise et al., 2010; Wang et al., 2010). Moreover, the secretion of these neurotrophic factors was related to neuroprotective effects in a PD model (Sadan et al., 2009; Blandini et al., 2010; Wang et al., 2010), an ischemic stroke model (Koh et al., 2008) and a ALS model (Nicaise et al., 2010). A recent study has pointed out that GDNF increased survival of DA neurons by increasing the expression of Pitx3 and BDNF and that the final BDNF secretion by the neurons is responsible for the neuroprotection (Peng et al., 2011). Because an immunomodulatory effect was not overtly observed we favor the possibility that trophic factor's secretion by the hMSCs might be key contributors for the neuroprotection observed in our model.

The striatum is the traditional clinical site for transplantation studies aiming at restoring the loss of dopaminergic innervations (Lindvall and Kokaia, 2010). When the aim is neuroprotection of nigral bodies, intrastriatal transplantation needs to elicit its effect via de axonal terminals to the nigral cell bodies. Neuroprotective strategies have been studied by gene transfer of different trophic factors, especially GDNF, in the SN and/or the striatum in PD models (Choi-Lundberg et al., 1997; Connor and Dragunow, 1998; Kozlowski et al., 2000). In the studies where GDNF was administered after neurodegeneration started in the SNpc, intranigral administration showed a better neuroprotective effect (Kozlowski et al., 2000). On the other hand, the neuroprotective effects of injecting MSC in the striatum of an MPTP PD disease model were recently described (Pereira et al., 2011). Our results are in agreement with this previous report and warrant further studies where both possible sites of intervention (SN and striatum) could be investigated simultaneously in a similar animal model. This study shows that the injection of hMSCs in the degenerating SN can have a neuroprotective effect and elicit an increased motor performance. Although the beneficial effects were mild and transient, it provides a proof of principle that human MSC could be injected into the rat SN without a major and toxic inflammatory reaction and opens up the possibility to improve this new therapeutic strategy in animal models.

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