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Intralesional administration of allogeneic bone marrow stromal cells reduces functional deficits after intracerebral hemorrhage

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Summary. When a severe neurological lesion occurs as a consequence of intracerebral bleeding, no effective treatment for improving the outcome is currently available. In the present study, intracerebral hemorrhage (ICH) was induced by stereotactic injection of 0.5 U of collagenase type IV in the striatum of adult Wistar rats, and three days later, intralesional administration of $2x10^6$ allogeneic bone marrow stromal cells (BMSC) in saline (n:10), or saline only (n:10), was performed. In the following 30 days, functional outcome was evaluated in each animal by rotarod and the modified neurological severity score (mNSS) test. Progressive and functional improvement was observed in BMSC-transplanted rats compared with controls, together with morphological images suggesting that intracerebral administration of BMSC increases endogenous neurogenesis at the level of subventricular zone (SVZ). These findings suggest that local administration of allogeneic BMSC could be useful to reduce the neurological deficits caused by intracerebral hemorrhage.

Key words: Bone marrow stromal cells, Intracerebral hemorrhage, Endogenous neurogenesis, Cell therapy

Introduction

Intracerebral hemorrhage (ICH) is one of the most devastating forms of stroke and the third cause of death in developed countries. 38% of patients survive the first year, but ICH leads to severe neurological deficits, and currently no effective treatment for improving the outcome is available (Ferro, 2006; Andres et al., 2008). In the last years, cell therapy using adult stem cells offers new strategies for the treatment of neurological diseases, and some experimental studies suggested that local or systemic administration of bone marrow-derived stem cells can reduce the neurological deficits caused by intracerebral bleeding (Andres et al., 2008). Nevertheless, nowadays it is difficult to find arguments favouring the superiority of bone marrow stromal cells (BMSC) or bone marrow containing hematopoietic stem cells, when the repair of injured central nervous tissue is needed (Vaquero and Zurita, 2009). On the other hand, we must still obtain better knowledge of the mechanisms by which adult stem cells carry out their beneficial effects. Keeping in mind the advantage of the low antigenicity of BMSC, the results obtained after the use of these cells for spinal cord repair (Zurita and Vaquero, 2004, 2006; Zurita et al., 2008), and the absence of previous studies showing the potential utility of BMSC in ICH, we studied here whether intracerebral administration of allogeneic stromal cells, obtained from bone marrow, can restore neurological functions previously suppressed by intracerebral bleeding.

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Material and methods

In the present study, care of the animals complied with that stipulated by the principles of Animal Laboratory Care and the Guide for the Care and Uses of Laboratory Animals issued by the American National Society for medical research.

Experimental model

Female adult Wistar rats weighing 200 to 250 g were subjected to an experimental model of intracranial hemorrhage (ICH). The animals were anesthetized with

Sevorane 3% using a face mask with oxygen flow at 3 l/min. After subcutaneous injection of Meloxicam (2 mg/kg) and Morphine (2.5 mg/kg), the animals were placed in a stereotactic frame. Craniotomy was performed adjacent to bregma and intrastriatal hemorrhage was induced by administration of bacterial collagenase type IV (Sigma-Aldrich, Madrid, Spain). Using a microinjector pump (mod 310 Stoelting Co., Wood Dale, IL,USA), 2 μl of saline containig 0.5 U of collagenase were injected into the striatum over a period of 5 minutes (coordenates: 0.04 mm posterior, 3.5 mm lateral, 6 mm ventral). At this moment, the animals were randomly divided into two groups. Three days later, ten animals (treated group) were subjected to a stereotactic injection of 10 μl of saline containing $2x10^6$ BMSC, into the injured zone. In the other ten animals (control group) 10 μml of saline without BMSC were administered. Additionally, five adult female rats without any lesion type were studied to compare the functional results obtained in our experimental groups and to study the subventricular zone (SVZ).

BMSC isolation and characterization

Bone marrow stromal cells were obtained from adult male donor Wistar rats. Using a 1-ml syringe and a 21-gauge needle, fresh whole bone marrow was harvested aseptically from tibias and femurs. Both ends of the bones were cut and the marrow was extruded with 5 ml of alpha-MEM medium (Gibco BRL Co. Ltd, USA). Bone marrow was mechanically dissociated to obtain a homogeneous cell suspension. The cell suspension was filtered through a 70-mm mesh nylon strainer and placed in a 75-cm^2 flask for tissue culture with 12 ml alpha-MEM medium containing 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 25 ng/ml amphotericin B. The cells were incubated at 37°C in 5% CO_2 for three days. At this time, non-adherent cells were removed by replacing the medium. The culture medium was replaced 3 times per week. After the cultures reached confluency, they were lifted by incubation in a solution containing 0.25% trypsin and 1 mM EDTA for 5 min at 37°C. Flow cytometry experiments were performed on a flow cytometer (Cytomic FC 500-MPL, Beckman Coulter Inc., Fullerton, CA, USA) for the characterization of BMSC. Antibodies used were unconjugated CD31 (Becton Dickinson, Franklin Lakes, NJ, USA), PE-labeled CD45 (Becton Dickinson, Franklin Lakes, NJ, USA), FITC-labeled CD105 (Southern Biotech, Birmingham, Alabama, USA) and APC-labeled CD133 (Miltenyl Biotec, Bergisch Gladbach, Germany). BMSC were strongly positive for CD105 and negative for CD31, CD45 and CD133. The BMSC obtained, which showed viability higher than 95%, were injected into the lesion zone. In five animals, previous to intracerebral administration, BMSC were labeled with 5-bromo-2'-deoxiuridine (BrdU). For BrdU-labeling, BMSC were cultured with 10 μM BrdU (Sigma-Aldrich, Madrid, Spain) for 48 h. They were rinsed three times with alfa-MEM without serum and the culture was rinsed with 0.25% trypsin and 1 mM EDTA for 5 min at 37°C.

Behavioral tests

Rotarod test was used to evaluate motor coordination, and the modified neurological severity scores. The mNSS test previously described (Li et al., 2001) was used in order to measure the sensitive and motor deficits. In the Rotarod test the rats were placed on the rotarod cylinder, and the time the animals remained on the Rotarod was measured. The speed was slowly increased from 4 to 40 rpm within a period of 1 minute. The mNSS test is a composite of motor, sensory, balance and reflex tests, and neurological function was graded on a scale of 0 to 18. A score of 0 is associated wth normal neurological function, and a score of 18 represents the maximal functional deficit. In the severity scores of injury, 1 point is awarded for a specific abnormal behavior or for the lack of a tested reflex; thus, the higher score is the most severe injury.

The animals were training in both behavioral tests for 10 days before ICH, and basal data are recorded as the mean of the last two days of training. After transplantation, the animals were evaluated twice per week, for four weeks, and data for each group were recorded as mean ± standard deviation (SD). Kolmogorov-Smirnow test was performed to study whether the data were normally distributed, and we used a repeated measures ANOVA test for a comparison of the mean scores within each group, at the different time points of follow-up. The statistical analysis was performed by means of the InStat statistical system (v 1.01, GraphPad Software Inc., San Diego, CA), with $p<0.05$ considered as statistically significant.

Histological studies

One month after BMSC administration, the rats were sacrificed for histological studies. For sacrifice, the animals were anesthetized with 8% sevoflurane in a continuous oxygen flow of 3 l/min, and perfused transcardially with 20 ml heparinized saline followed by 60 ml of 4% paraformaldehyde in 0.1M PBS (pH 7.4). The brains were then dissected, post-fixed in 4% formalin for 1-2 days at room temperature, and for each rat, a block containing the zone of the ICH was processed for paraffin sectioning. A series of 5-μm thick sections were cut with a microtome through each block and mounted on glass slides for histological observations after hematoxylin-eosin staining. For immunohisto-chemical studies, adjacent slides were placed in a boiled citrate buffer (pH 6) in a microwave oven (650-720 w). After rinsing in PBS, the sections were exposed to 3% H_2O_2 for 30 min to quench endogenous peroxidase activity. Before incubation of primary antibodies, non-specific binding was blocked for one hour with 3% normal serum from the species in which the secondary

antibody was raised. Primary antibodies used were directed against: BrdU monoclonal antibody (1:100, Chemicon International, Inc. Temecula, CA.), nestin monoclonal antibody (1:100, Chemicon International Inc., Temecula, CA, USA), Ki-67 monoclonal antibody (1:200 Master Diagnostica) and doublecortin policlonal antibody (1:300, Santa Cruz Biothecnology). The sections were incubated with secondary antibodies conjugated to biotin, 1:200 (Vector Inc, CA, USA). Subsequently, the sections were washed in PBS, and incubated with avidin-biotin-horseradish peroxidase complex (Vector Inc, CA, USA). 3,3'-diaminobenzidine (DAB) was used as a chromogen. Control slices, lacking primary or secondary antibodies, were analyzed with each series. The sections were studied using light microscopy.

For identification of male donor cells, in situ hybridization studies were performed. For this, 5-μm thick sections were dewaxed and rehydrated with xilene and graded ethanol and subsequently digested with proteinase K (30 μg /ml) for 15 minutes at 37°C. We used a biotinilated-DNA probe for sequence specificity for murine Sry gene, the sex-determining region of Y-chromosome. Hybridization was performed in a hybridization mixture consisting of 50% deionized formamide, 2% salmon test DNA, 10% dextran sulphate, 10% 50X Denhardt's solution and 400 ng biotinilated-labeled probe at 50°C overnight. For conventional inmunohistochemistry to visualize the biotinilated-labeled probe, a mouse anti-biotin mouse monoclonal antibody (1:100, Jackson ImmunoResearch Laboratories, Inc., Baltimore Pike, USA), secondary antibodies anti-mouse biotin-conjugated antibody (5 μg/ml, Vector Laboratories Inc, Burlingame, CA, USA) and incubated with avidin–biotin–horseradish peroxidase complex (Vector Inc, CA, USA) were used. DAB was used as a chromogen to visualize under conventional microscopy. For double stain, the secondary antibody used was Rhodamine(TRITC)-conjugated anti-mouse antibody (1:200, Jackson ImmunoResearch Laboratories, Inc., Baltimore Pike, USA). The primary antibodies used after washing in PBS were anti-NeuN monoclonal antibody (1:500 Chemicon International, Inc. Temecula, CA.) and anti-Glial Fibrillary Acidic Protein (PGFA) (1 μl/ml, Lab Vision Corporation). The secondary antibody used was CyTM 2-conjugated anti-mouse antibody (1:200, Jackson ImmunoResearch Laboratories, Inc., Baltimore Pike, USA). After washing in PBS the sections were incubated in Dapi and mounted in Glicerol medium. The slices were visualized by fluorescence microscopy.

In order to analyze a possible increase in the proliferative activity following BMSC transplantation, ten sections corresponding to the lesion zone were selected at random from each animal and the number of Ki-67-positive cells, at least in ten different microscopical fields, at 400x, was recorded from each histological section. These recordings were made by image analysis morphometry (Optimas, 6.2 software package, Optimas Corporation, Bothell, WA) using a macro application, and conducted by two investigators trained in morphometric determinations, with no knowledge of the experimental group from which each sample had been obtained. The means recorded by these investigators were regarded as final values. For each experimental group the total number of immunopositive cells was averaged and values were expressed as count means ± standard deviations. We used the unpaired Student's t test, for a comparison of the number of immunopositive cells in the two experimental groups.

Results

In our present study, all the animals showed significant neurological dysfunction after ICH, measured with rotarod and mNSS tests, but a clear improvement was recorded in the course of follow-up in the group of animals that received intralesional BMSC.

In rotarod test, the basal mean scores (± standard deviation) were 34.05±1.87, 33.21±2.19 and 33,70±2.06 for BMSC-transplanted group, control group and non-injured group, respectively. The scores in rotarod test decreased significantly until 24.45±1.65 and 25.41±3.77 (BMSC-transplanted group and control group respectively) one week after ICH. Nevertheless, at the third week after treatment, the mean score in the rotarod test improved significantly in the BMSC-transplanted group compared with controls and compared to the previous time points ($p<0.05$). Significant modifications were not observed in the control group throughout the follow-up. The performances were homogeneous in non-injured animals (Fig. 1). In the mNSS analysis, all the animals showed a clear dysfunction after ICH (scores of 5.31±0.55 and 5.38±0.5, for BMSCs-transplanted group and control group respectively) except the intact group which showed 0 performances throughout the full study.

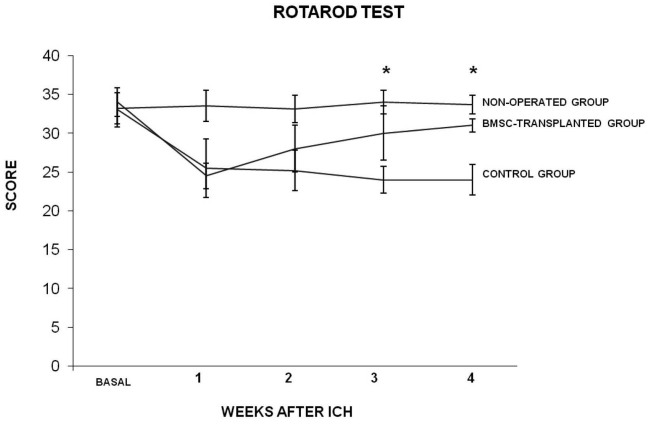

Fig. 1. Rotarod test. BMSC-transplanted animals started to show a significantly higher rotarod score at third week ($p<0.05$) compared with saline-treated animals. We could not see improvement in the saline-treated group. Non-injured animals showed a homogeneous behavior throughout the test.

However, at the third week after treatment, the BMSCs-transplanted group showed significantly better scores that the control group ($p<0.05$). This improvement was progressive in the course of follow-up, without significant improvement in the control group (Fig. 2).

Morphological study identified the zone of lesion in all the animals, as a central cavity near to the ventricle, surrounded by a scar wall and partially filled by macrophages. The size of the lesion was variable, but it was not significantly different when the two experimental groups were compared. At level of SVZ a clear increase in the number of cells was observed in the animals that received BMSC, compared with the animals that received saline only. In the animals that received BrdU-labeled-BMSC, a variable number of BrdU-positive cells were localized like isolated cells in the lesion surroundings and next to the SVZ, and some cells were identified as male-donor cells by means of the in situ hybridization study. Some of these cells showed GFAP or NeuN expression (Fig. 3). The presence of a large number of immunostained cells showing nestin and doublecortin expression was a constant finding in SVZ of the animals belonging to the BMSC-transplanted group, and ocasionally, nestin and doublecortin positive cells were identified in clusters of undifferentiated cells near the lesion zone. However, in the animals of the control group the presence of cells expressing nestin and doublecortin was lower than the BMSC-transplanted group (Figs. 4, 5). Only exceptionally some immunostained cells showing Ki-67, nestin or doublercortin expression, were observed in SVZ in the non-injured animals. Furthermore, in the group of animals that received BMSC, nestin and doublecortin-positive cells were occasionally identified inside the injured tissue.

When the proliferative activity of cells in SVZ was compared in both experimental groups, a clear difference could be observed, with an obvious increase in the BMSC-transplanted rats. Occasionally, clusters of undifferentiated cells could be seen in the surroundings of the lesion zone, in apparent continuity with proliferative cells that extended from SVZ. With regard to animals of the transplanted-BMSC group, a great number of cells showing Ki-67 expression could be seen, a finding that could not be observed in the animals of the control group (Fig. 5). In the transplanted-BMSC group, the mean (± SD) of SVZ-cells expressing Ki-67 was 94.7±6.4 per microscopical field, at 400x, while in the control group, the mean of Ki-67-positive cells was 29.4±3.6 (Fig. 6). Furthermore, in the non-injured animals, only some Ki-67-positive cell could be identified.

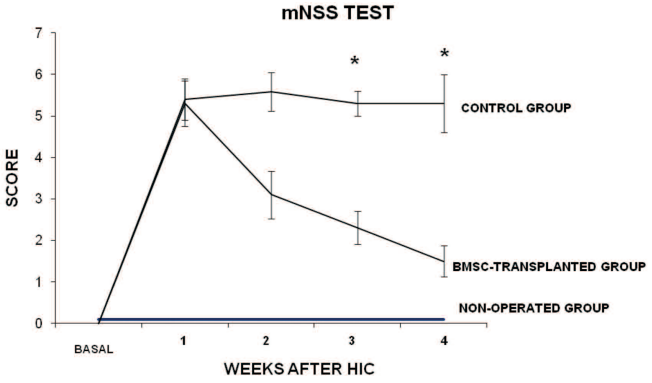

Fig. 2. mNSS test. A clear behavioral improvement is demonstrated in BMSC-transplanted animals ($p<0.05$). In saline-treated animals, no significant functional recovery was seen. The non-injured animals showed 0 performances during the full study.

Discussion

It is well known that ICH is one of the most devastating forms of cerebrovascular disease, causing neurological sequels which are generally irreversible. Therefore, it is necessary to develop novel therapeutic strategies to improve the outcome of patients suffering ICH. Based on previous experimental studies suggesting the utility of cell therapy in this disease (Andres et al., 2008), we designed an experimental strategy of cell therapy that could be applied to patients. It is well known that in the course of the first days after a hemorrhagic stroke, surgical evacuation of the intracerebral hematoma can be considered if clinical deterioration occurs in a previously stable patient. In these circumstances, local administration of previously cryopreserved allogeneic BMSC would be possible. The low antigenicity of BMSC (Le Blanc and Ringden, 2005) offers an additional advantage, making immunosupression unnecessary and facilitating the use of allogeneic BMSC. On the other hand, none of the previous reported studies evaluating cell therapy for ICH used the intracerebral administration of only bone marrow-derived stromal cells (Seyfried et al., 2006; Zhang et al., 2006; Andres et al., 2008). Furthermore, most of these previous studies used a vascular way for administration of progenitor cells (Nan et al., 2005; Seyfried et al., 2006; Zhang et al., 2006). Although this way may be valid in the acute phase after injury, where there is a disruption of the blood-brain barrier, in a previous study we demonstrated the superiority of local administration so that enough cells can reach the injured tissue (Vaquero et al., 2006).

On the other hand, keeping in mind that previous studies demonstrated that allogeneic BMSC can survive and differentiate toward neurons and glial cells after their local administration in the injured nervous tissue (Zurita et al., 2006, 2008; Vaquero and Zurita, 2009) the main purpose of our present study was to verify whether the intracerebral administration of allogeneic BMSC, in the first days after ICH, improves functional outcome.

Our present study showed that after ICH is induced, significant and maintained neurological dysfunction appears. Nevertheless, a few days after allogeneic BMSC were administered, functional recovery begins.

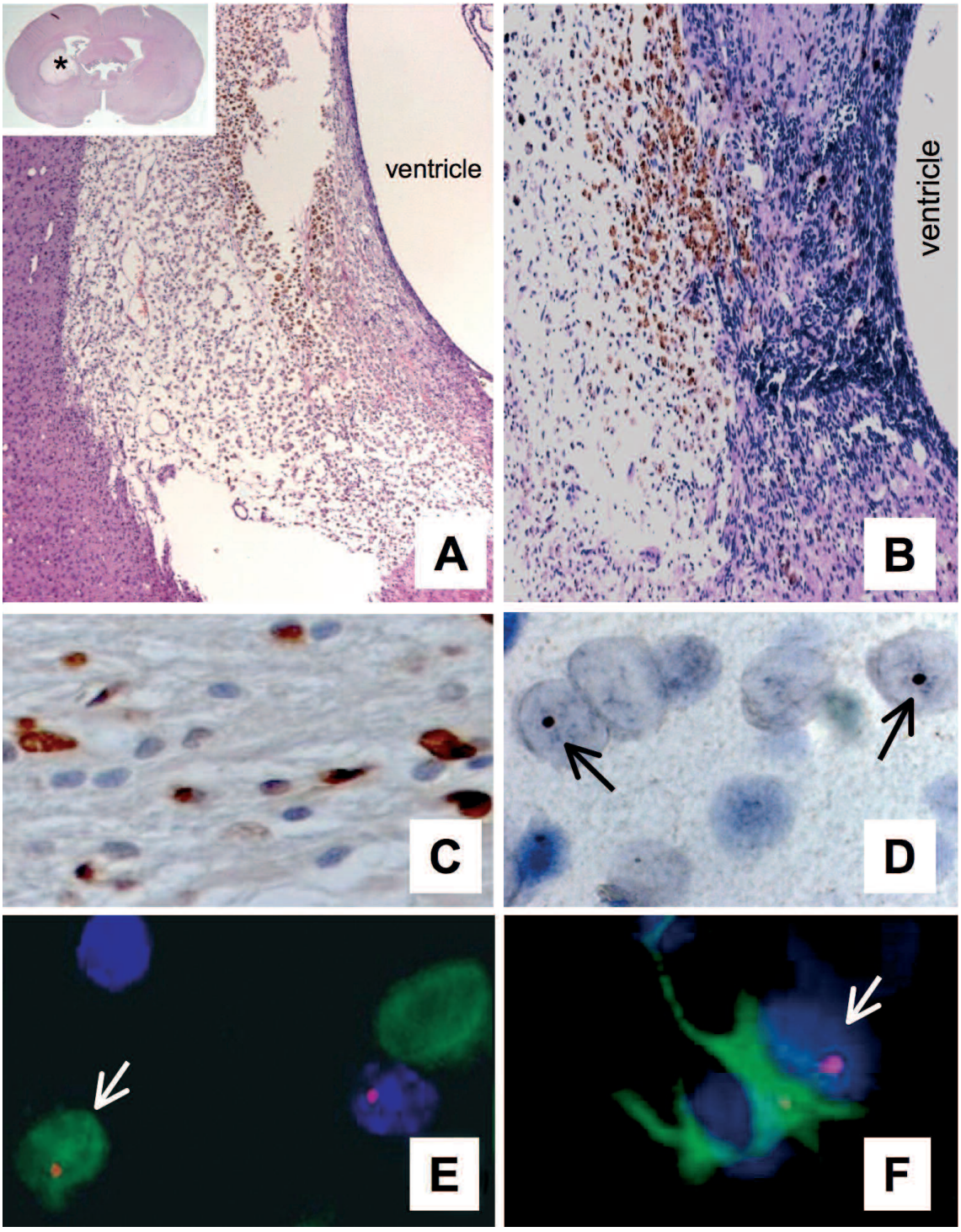

Fig. 3. Morphological findings in the animals of the series. **A.** Rat belonging to the control group. Histological aspect of the lesion zone, four weeks after intracerebral hemorrhage (H.E. stain). Inset shows a macroscopical view of the residual lesion (asterisk). **B.** Rat belonging to the BMSC-trasplanted group. Increased cellularity in the subventricular zone (SVZ) can be seen (H.E. stain). **C.** Rat belonging to the BMSC-trasplanted group. BrdU-immunostained cells can be seen in the vicinity of the lesion. **D.** Rat belonging to the BMSC-trasplanted group. Arrows show the presence of Y-chromosome in male donor cells (In situ hybridization technique). **E, F.** Double immunostain of rats belonging to the BMSC-transplanted group. In **E**, the arrow shows NeuN (green) and Y-chromosome (red) positivity in a male donor cell (In situ hybridization technique). In **F**, The arrow shows GFAP (green) and Y chromosome (red) positivity in a male donor cell (In situ hybridization technique). A, x 40; B, x 100; C, x 400; D-F, x 1000.

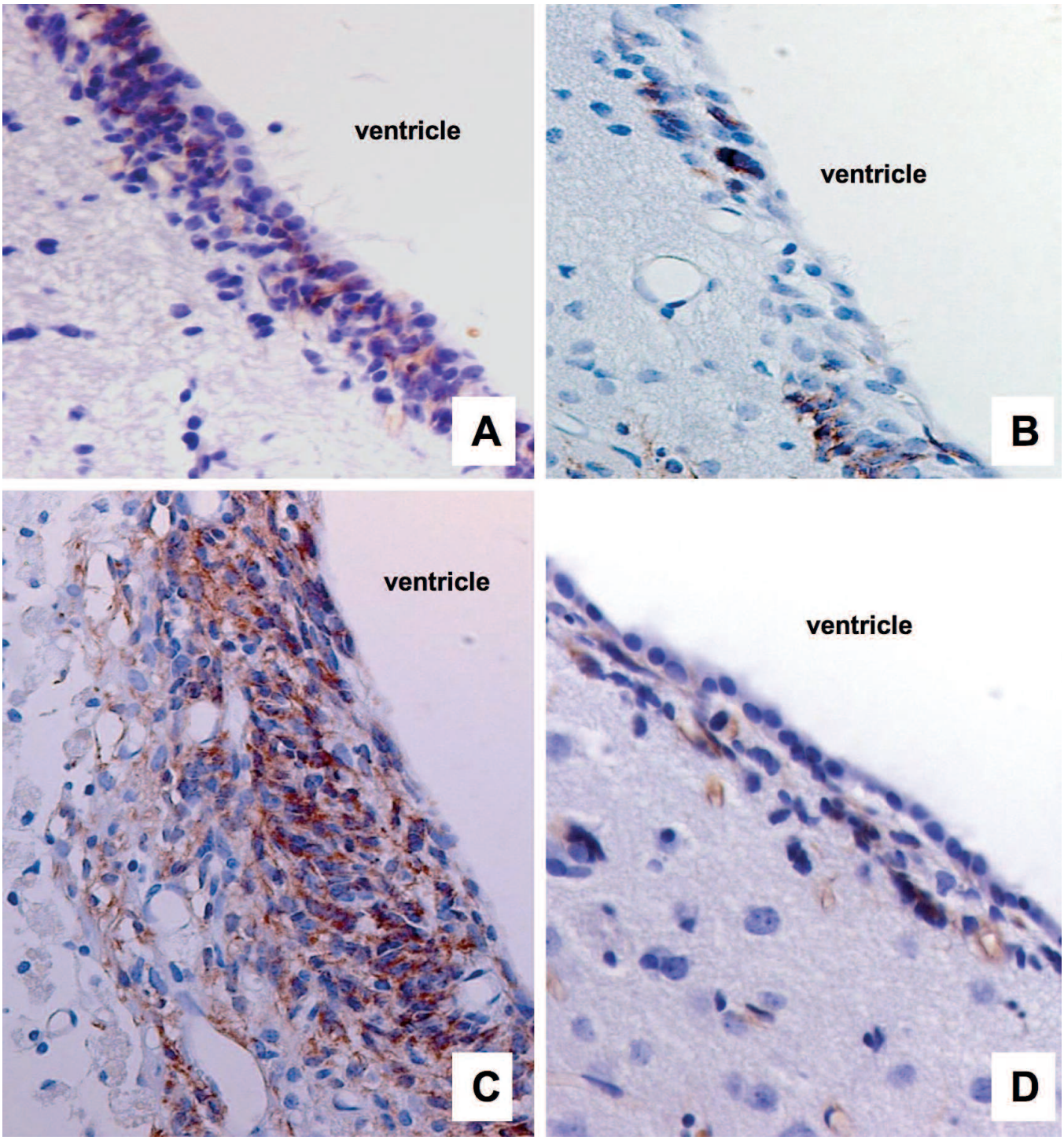

Fig. 4. Immunohistochemical findings in the animals of the series. **A and C.** Rats belonging to the BMSC-transplanted group. Cells in the SVZ expressing nestin **(A)** and doublecortin **(C)** can be seen. **B and D.** Rats belonging to the control group. Immunostain for demonstration of nestin **(B)** and doublecortin **(D)**. Increased cellularity can be seen in the SVZ of BMSC-transplanted rats, with strong expression of nestin and doublecortin, compared to the control group. x 200.

This recovery is clearly significant compared with controls at the third week after treatment and not plateaued by the end point of the follow-up. These results agree with the limited number of previous studies of stem cell-based therapy for ICH. In most available reports, the first signs of beneficial effects on behavioral

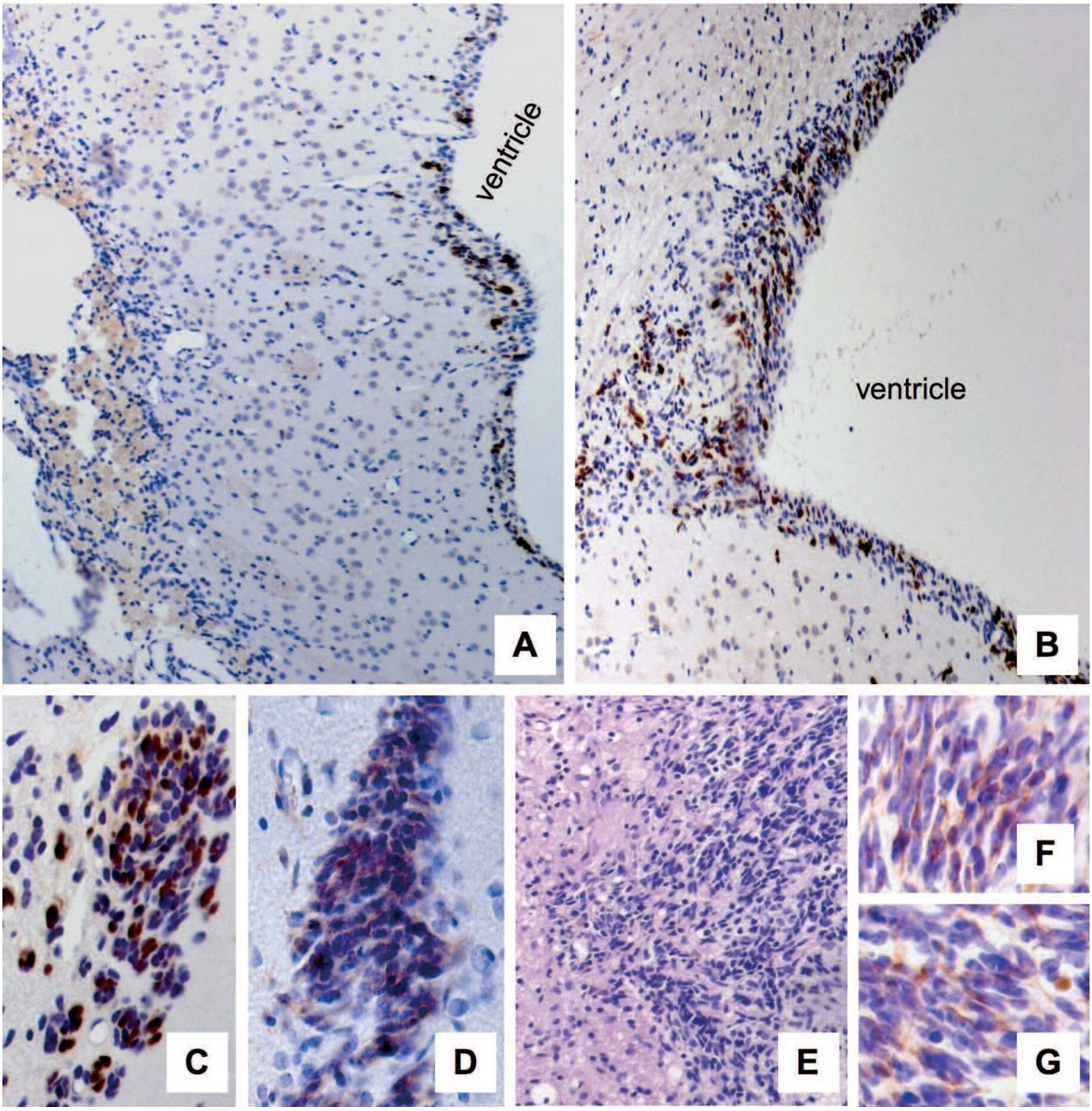

Fig. 5. A. Rat belonging to the control group. Cells expressing Ki-67 can be seen in the SVZ. **B.** Rat belonging to the BMSC-trasplanted group. A large number of Ki-67-positive cells, localized in SVZ, can be seen. **C.** Rat belonging to the BMSC-trasplanted group. A cluster of undifferentiated cells expressing Ki-67, can be seen in the vicinity of the lesion zone. **D.** Rat belonging to the BMSC-trasplanted group. A cluster of undifferentiated cells expressing doublecortin, can be seen in the vicinity of the lesion zone. **E.** Rat belonging to the control group. Undifferentiated cells, near the lesion zone and in continuity with SVZ can be seen, but without Ki-67 expression. **F and G.** Undifferentiated cells, near the lesion zone can be seen, expressing nestin **(F)** and doublecortin **(G)**. A, B, x 40; C-E, x 100; F, G, x 200.

recovery were seen between 2-4 weeks after transplantation, with some reports showing significant effects after only 1-7 days (Andres et al., 2008). In this study, some male-donor bone marrow stem cells were localized in the lesion surroundings expressing GFAP and NeuN, and these findings suggest that BMSC-transplantated differentiated into astroglial cells and neurons respectively. It is obvious that at this time, cell integration, transdifferentiation and establishment of functional connections is not enough reason for absolute improvement of the functional deficits. Moreover, when cell therapy has been used in stroke, the number of transplanted cells found in the brain of recipients does not necessarily correlate with the degree of functional improvement (Borlongan et al., 2004; Nan et al., 2005). These findings suggest that the beneficial effects of stem cell therapy should be mediated partially also by other mechanisms than direct graft-host interactions (Zurita et al., 2006, 2008; Vaquero and Zurita, 2009). Our dates show that four weeks after intracerebral administration of BrdU-labeled BMSC, they are localized in the lesion zone and in their surroundings, Although it is possible that at this time point immunostain can mark some macrophagic cells with incorporation of BrdU, the identification of cells showing Y-chromosome allow us to confirm the intracerebral survival of male donor cells. Nevertheless, the number of donor cells at the end of the follow-up suggests that BMSC suffer apoptotic death or autophagia in the following weeks after the transplant. Therefore, it is logical to suppose that in an immediate way after the transplant, and although a considerable number of transplanted cells can die in the following weeks, the presence of BMSC unchains a series of biological phenomena in the host tissue that allow a quick and progressive functional recovery (Shen et al., 2007; Vaquero and Zurita, 2009). It is well known that BMSC can secrete numerous trophic factors, including brain-derived neurotrophic factor (BDNF) nerve growth factor (NGF) and vascular endothelial growth factor (VEGF), which could be involved in the neurological recovery of the animals through a direct effect, or by exercising a neuroprotector effect (Andres et al., 2008; Seung-Wan et al., 2008). Therefore, the possibility that BMSC promote endogenous repair due to the production of neural restorative factors should be considered. In our present study we found an important proliferating activity in cells of the SVZ, which is significantly bigger in the animals belonging to the BMSC-transplanted group. Also, we found Ki-67-positive undifferentiated cells that extend from SVZ toward the lesion area, while in the case of the animals belonging to the control group Ki-67 expression in these cells is practically nonexistent.

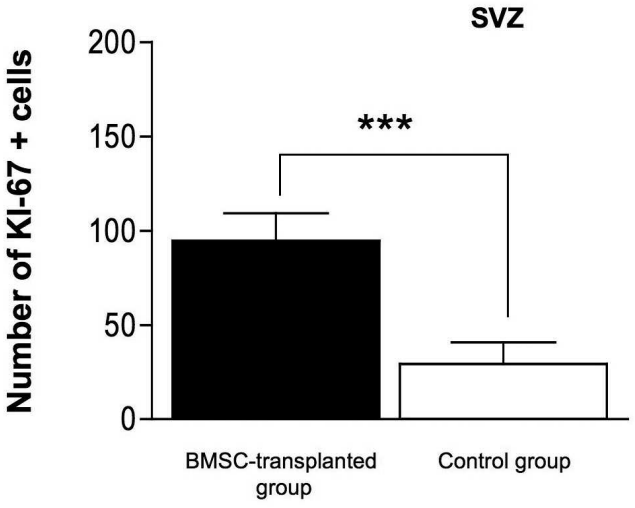

Fig. 6. Graph bars, showing the different Ki-67 expression in the SVZ, when the two experimental groups were compared. BMSC-treated rats showed a clear increase in cellularity and proliferative activity ($p<0.0001$).

On the other hand, when the group of BMSC-transplanted animals was studied, we found strong nestin-positivity in cells of the SVZ and occasionally in cells that were localized in the zone of damaged tissue or in their surroundings. These cells coexisted with some cell populations which presented doublecortin inmunopositivity. Keeping in mind that nestin is considered as an immunohistochemical marker for neural stem cells (Lendahl et al., 1990) these findings suggest that the intracerebral administration of BMSC promotes regeneration mechanisms in the injured cerebral tissue. Furthemore, doublecortin protein is not yet well defined, but it is believed to play a critical role in neuroblast migration (Brown et al., 2003). In this study we found high expression of nestin and doublecortin proteins in cells belonging to SVZ, indicating that BMSC transplantation activates the differentiation of neural stem cells, not only to glial cells, but also to neuroblasts. These newborn cells not only migrate to the cerebral cortex where they were found 1 month after transplantation, but also migrate toward the lesion area. These data suggest that BMSC-transplantation enhances endogenous neurogenesis and neural differentiation, and these newborn cells might contribute some compensation for the loss of neuronal function caused by hemorrhagic stroke.

In the last years, growing evidence exists that cells of SVZ proliferate after traumatic or ischemic brain injury, and that neural stem cells of this region move toward the injured tissue (Rice et al., 2003; Romanko et al., 2004; Salman et al., 2004; Richardson et al., 2007; Urrea et al., 2007; Seung-Wan et al., 2008). Activation of endogenous neurogenesis seems to exist also after ICH (Tang et al., 2004; Masuda et al., 2007) but at present, we do not know how the intracerebral administration of BMSC can modify the pattern of cerebral endogenous repair following HIC.

Our present study agrees with previous experimental reports showing a synergic function of BMSC transplantation after HIC. After allogeneic BMSC-transplantation, donor cells can survive in the brain

tissue, showing morphological evidence of transdifferentiation to neurons and astroglial cells. Furthemore, BMSC-transplantation enhances endogenous neurogenesis that usually exists after intracerebral hemorrhage. Both mechanisms can contribute to compensate the loss of neural tissue and of neurological function. On the other hand, the observation that after this type of cell therapy quick and progressive neurological recovery can be obtained suggests that the beneficial effect of BMSC can be attributed mainly to a stimulation of neuronal plasticity. Although our results should be extrapolated to the human disease with caution, it is obvious that the use of allogeneic BMSC offers great promise for developing novel and efficacious strategies in patients suffering ICH.

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