

Mesenchymal stem cells transplantation attenuates experimentally induced brain injury after neonatal hypoxia by different two routes of administrations

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Abstract: The neonatal hypoxic–ischemic encephalopathy (HIE) is an important cause of neurological morbidity and mortality in neonates. Cell therapy is considered a promising method for treating severe neurological disorders such as this one. Stem cells have the capacity for self-renewal and differentiation into certain cell lineages. The present study was aimed to find out the most beneficial route of bone marrow-derived mesenchymal stem cells (BMSCs) administration for the attenuation of experimentally induced HIE in neonatal rats. Sixty neonatal rats were divided randomly into four groups. Group 1: control group. Group 2: rats were exposed to bilateral ligation of cephalic arteries. Group 3: rats were exposed to bilateral ligation of cephalic arteries and then underwent intravenous (IV) BMSC injection. Group 4: rats were exposed to bilateral ligation of cephalic arteries and then underwent intracerebroventricular (ICV) BMSC injection. The animals were evaluated by (a) neurobehavioral tests; (b) histopathology, i.e., histological and immunohistochemical studies; and (3) gene expression studies. The BMSC treated groups (3 and 4) showed improvement in neurobehavioral tests, histopathological studies, and gene expression, as compared to non-injected lesioned rats (Group 2) with better improvement in Group 4 (ICV injections) than in Group 3 (IV injections).

Introduction

A neonatal stroke occurs in ~1:4000 live human births and the associated hypoxic–ischemic encephalopathy (HIE) is a major cause of morbidity and mortality during the prenatal and perinatal periods (Kirton and deVeber, 2009). Between 20 and 30% of affected infants will die during the neonatal period, whereas < 25% of the survivors will exhibit long-term motor or mental disabilities (Wei *et al.*, 2009).

HIE is caused by a partial or complete reduction of cerebral blood flow and is the most common brain injury in full-term newborns during the perinatal period (Fang *et al.*, 2013). HIE may be followed by permanent neurological deficits such as cerebral palsy, epilepsy, memory deficiency, hypothermia, and even death (Quattrocchi *et al.*, 2016). Neonates with similar degrees of ischemic damage may differ in the extent of brain injury, ranging from none to near total brain injury

(Harteman *et al.*, 2013). There is no standard therapy for HIE, however many potential therapies that may prevent injury progression and enhance repair of the suffered injury are under investigation (Fang *et al.*, 2012).

Mesenchymal stem cells (MSCs) have the capacity of renewal and differentiation into a variety of tissue lineages such as osteoblasts, chondrocytes, and adipocytes (Du *et al.*, 2016). MSCs can derive from various tissues, like bone marrow, adipose tissue, and umbilical cord (Wang *et al.*, 2016). MSCs have a low immunogenicity, anti-apoptotic, anti-fibrotic and anti-inflammatory effects through the secretion of bioactive trophic factors that make them suitable for cell therapy and regenerative medicine (Dhoke *et al.*, 2016). MSC therapies provide a good chance for treating diseases of the brain, which have a limited regenerative capacity (Nguyen *et al.*, 2014). The potential therapeutic effect of stem cells is critically dependent on the number of stem cells retained at the site of injury (Marquez-Curtis *et al.*, 2015).

The present study was conducted to test the most beneficial route for the administration of bone marrow-derived mesenchymal stem cells (BMSCs) to ameliorate

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several neurobehavioral and molecular parameters in a rat model of experimental HIE.

Material and Methods

Experimental animals

Sixty healthy 7-day-old male albino rats weighing about 20 g were kept with their mothers, maintained in an air-conditioned animal house under specific-pathogen-free conditions. Rats were subjected to a normal light/dark cycle and allowed unlimited access to chow and water. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (NIH publication No. 85-23, revised 2011). All protocols were approved by the Institutional Review Board for Animal Experiments of the Faculty of Medicine, Benha University, and the Animal Committee of the Faculty of Medicine, Cairo University.

Induction of hypoxic brain injury

Hypoxic brain injury was induced by ligating the bilateral cephalic arteries. The animals were anesthetized by intraperitoneal injection of 4% chloral hydrate (0.5 ml / 100 mg body weight). A 0.5 cm incision was made at the ventral side of the neck through which the cephalic arteries were isolated and ligated. The incision was then closed with stitches. Afterward, the animals were placed in an incubation chamber (37°C) for 70 min during which their body temperature and behavior returned to normal, before being placed in a container aerated with 8% O₂ and 92% N₂, for 1.5 h at 37°C (Zhang *et al.*, 2013).

Preparation and administration of BMSCs

Cells were prepared in the Biochemistry Department, Faculty of Medicine, Cairo University. Bone marrow cells were flushed from tibia and fibula of rats' bones with phosphate-buffered saline (PBS) containing 2 ml EDTA. Over 15 ml Ficoll-Paque (Gibco-Invitrogen, Grand Island, NY), 35 ml of the diluted sample was carefully layered, centrifuged for 35 min at 400 g and the upper layer was aspirated leaving undisturbed the mononuclear cell (MNC) layer at the interphase. The MNC layer was aspirated, washed twice in PBS containing 2 ml EDTA and centrifuged for 10 min at 200 g at 10°C. The cell pellet was resuspended in a final volume of 300 µl of buffer. Isolated BMSCs were cultured on 25 ml culture flasks in minimal essential medium (MEM) supplemented with 15% fetal bovine serum (FBS) and incubated for 2 h at 37°C and 5% CO₂. Adherent BMSCs were cultured in MEM supplemented with 30% FBS, 0.5% penicillin, streptomycin, at 37°C in 5% CO₂ in air (Abdel Aziz *et al.*, 2011). All cultures were examined using an inverted microscope (Leica DM IL LED) with a camera (Leica DFC295; Leica Microsystems CMS GmbH, Ernst-Leitz-Straße 17-37, Wetzlar, D-35578, Germany).

The cells were administered either into the tail vein (IV injections) or into the cerebral ventricle (ICV injections).

Experimental groups

Rats were divided into four groups: (1) Group 1 (control group), 20 rats were divided in turn into four equal

subgroups, which were either (1a) intact, (1b) sham-operated, (1c) injected with 0.5 ml of PBS into the tail vein, or (1d) injected with 5 µl of PBS into the cerebral ventricle; (2) Group 2 (hypoxic brain injury group), 20 rats that underwent bilateral ligation of cephalic arteries; (3) Group 3 (hypoxic brain injury + IV BMSC injection), 10 rats that underwent bilateral ligation of cephalic arteries and that were injected with 3 × 10⁶ BMSCs suspended in 0.5 ml PBS into the tail vein, 24 h thereafter (Attia *et al.*, 2015); (4) Group 4 (hypoxic brain injury + ICV BMSC injection), 10 rats that underwent bilateral ligation of cephalic arteries and that were slowly injected ICV with 3 × 10⁶ BMSCs suspended in 0.5 ml PBS, 24 h thereafter (Sato *et al.*, 2008).

Neurobehavioral evaluation

Two neurobehavioral tests, the geotactic reflex and the righting reflex (Zhang *et al.*, 2013) were performed 3, 7, 14, and 28 days after induction of hypoxic ischemic brain injury. The time (in seconds) needed to complete the righting reflex and the geotactic reflexes were recorded.

Histological and immunohistochemical studies

All rats were sacrificed 28 days after induction of hypoxic-ischemic brain injury. The fasted rats were anesthetized with ether and sacrificed by means of cervical dislocation. Cerebral cortex tissue was excised out and posterior parietal lobe samples were fixed in 10% buffered formaline, dehydrated and embedded in paraffin. Sections (4-6 µm thick) were mounted on glass slides and stained with hematoxylin and eosin (Bancroft and Layton, 2013). Other sections were mounted on positively charged slides for immunohistochemical staining (Jackson and Blythe, 2013) using (1) anti-nestin antibodies at 1:200 dilution, (2) monoclonal antibody against glial fibrillary acidic protein (GFAP) at 1:400 dilution, and (3) anti-neurofilament 200 (NF) antibody at 1:80 dilution (Sigma-Aldrich, St Louis, Missouri, USA). Antigen retrieval was performed in all cases by steam heating the slides in a 1 mmol/l solution of EDTA (pH 8.0) for 30 min. After blocking endogenous biotin, staining was performed using an automated immunostainer followed by using a streptavidin-biotin detection system (Dako, Glostrup, Denmark). Sections were counterstained with hematoxylin. As a negative control, the primary antibody was replaced with PBS.

Morphometric study

The mean percent area of nestin, GFAP and NF immunorexpression were quantified in five images from five non-overlapping fields of each rat of each group using Image-Pro Plus version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA).

Real-time quantitative PCR (RT-qPCR)

Samples of brain tissue were obtained 28 days after operations and homogenized. Total RNA was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany) and further analyzed for quantity and quality with a dual beam spectrophotometer (Beckman Coulter, Fullerton, California, USA). For quantitative expression of vascular endothelial growth factor-receptor 2 (VEGF-R2) and endothelial nitric oxide synthase (eNOS) genes, the following procedure was followed: (1)

200 ng of total RNA from each sample were used for cDNA synthesis by reverse transcription using High capacity cDNA Reverse Transcriptase kit (Applied Biosystems Inc., Foster City, CA, USA); (2) cDNA was subsequently amplified with the Sybr Green One-Step PCR Master Kit in a 48-well plate (Applied Biosystems Inc., Foster City, CA, USA) (10 min at 95°C for enzyme activation followed by 40 cycles of 15 s

at 95°C, 20 s at 55°C and 30 s at 72°C for the amplification step). Changes in the expression of each target gene were normalized relative to the mean critical threshold (CT) values of GAPDH housekeeping gene by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). We used 1 μ of both primers specific for each target gene. Tab. 1 shows the specific primer sequences used for each gene.

TABLE 1

Sequences of the specific primers used

Target gene	Primer sequence: 5' - 3'	Gene bank accession number
VEGF-R2	Forward: CTATTCTGTCAGCACTTTGG Reverse: CAGACTTTGGTTCTCCAACCT	NM_031054.2
eNOS	Forward: AAATGTGGGTGTACACAGGC Reverse: TTCACCCGGTTGTGGAAACT	NM_031055.1
GAPDH	Forward: CACCCTGTTGCTGTAGCCATATTC Reverse: GACATCAAGAAGGTGGTGAAGCAG	NG028301.2

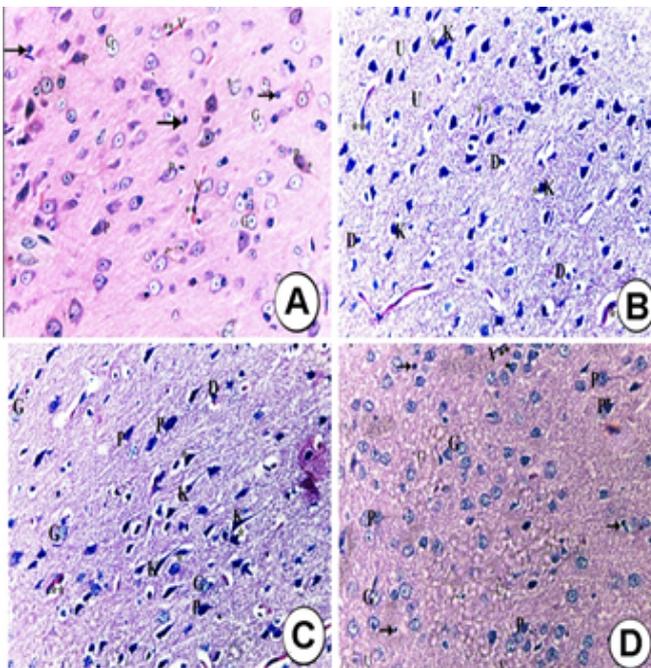


FIGURE 1. (A) Cerebral cortex of a Group 1 rat (control) showing pyramidal cells (P) with their typically large nuclei, granule cells (G), neuroglia cells (arrow) and a blood vessel (V). Narrow perineural (*) and perivascular spaces (**), and no obvious neuropil vacuolations were observed. (B) Cerebral cortex of a Group 2 rat (hypoxic brain injury) showing many degenerated (D) and shrunken (K) pyramidal cells. Excess vacuoles (U) in neuropils and wide perineural (*) and perivascular spaces (**) were also observed. (C) Cerebral cortex of a Group 3 rat (hypoxic brain injury, plus IV injection of BMSCs) showing few apparently normal pyramidal cells (P) and excess granule cells (G). Few pyramidal cells appeared shrunken (K) with slightly wide perineural spaces (*), together with excess vacuoles in neuropils (U) and narrow perivascular spaces (**) were also observed. (D) Cerebral cortex of a Group 4 rat (hypoxic brain injury, plus ICV injection of BMSCs) showing many apparently normal pyramidal cells (P) and excess granule cells (G). Few pyramidal cells appeared shrunken (K) with slightly wide perineural spaces (*). Also, vacuoles (U) in neuropils and narrow perivascular spaces (**) were observed. Hematoxylin and eosin, 400x.

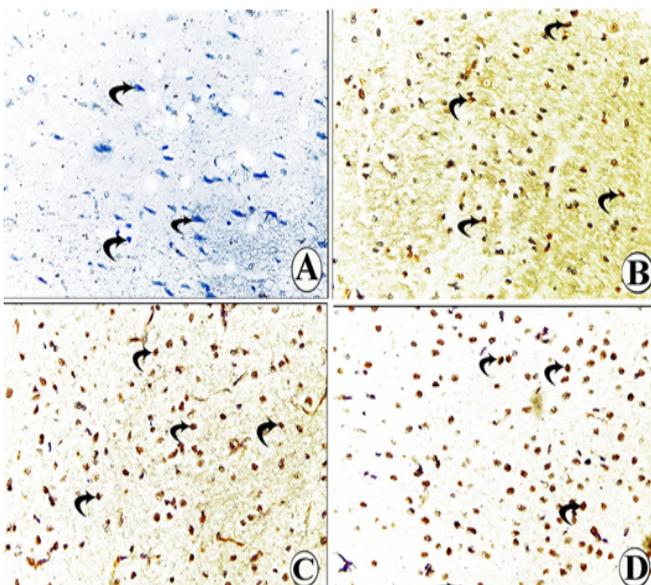


FIGURE 2. Nestin immuno-expression in the cerebral cortex, 400x. (A) Group 1 (control group) showing negative immuno-expression (curved arrows). (B) Group 2 (hypoxic brain injury) showing mild immunoexpression (curved arrows). (C) Group 3 ((hypoxic brain injury, plus IV injection of BMSCs) showing moderate immunoexpression (curved arrows). (D) Group 4 (hypoxic brain injury, plus ICV injection of BMSCs) showing marked immuno-expression (curved arrows).

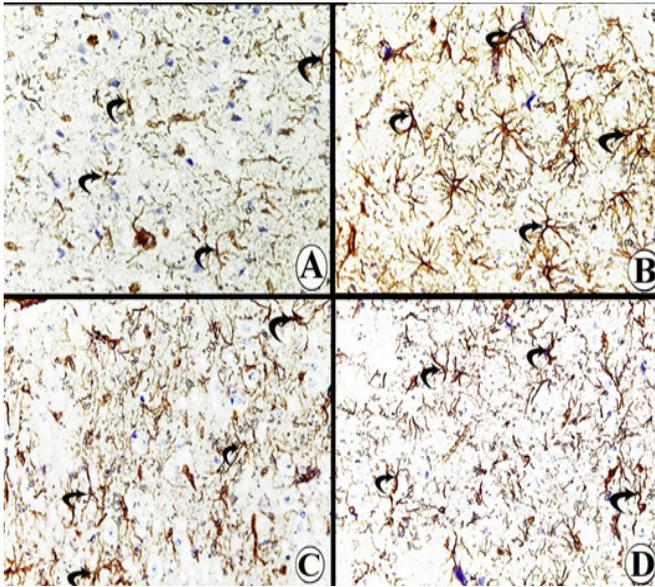


FIGURE 3. GFAP immuno-expression in the cerebral cortex, 400x. (A) Group 1 (control group) showing mild immuno-expression (curved arrows). (B) Group 2 (hypoxic brain injury) showing marked immuno-expression (curved arrows). (C) Group 3 ((hypoxic brain injury, plus IV injection of BMSCs) showing mild immuno-expression (curved arrows). (D) Group 4 (hypoxic brain injury, plus ICV injection of BMSCs) showing marked immuno-expression (curved arrows).

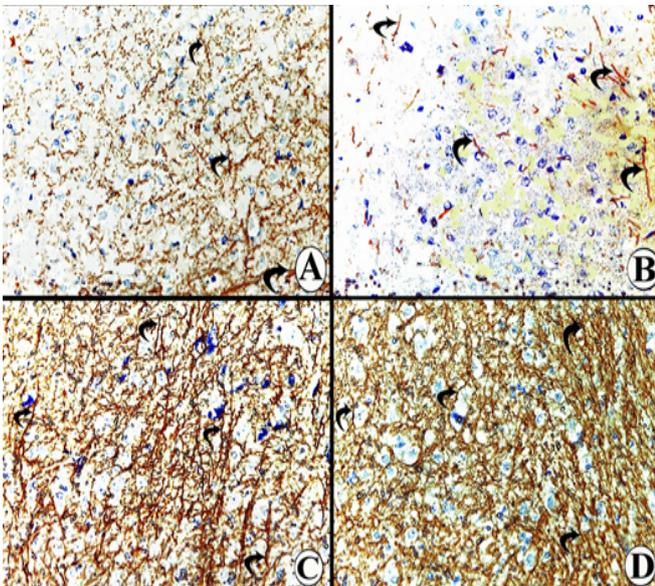


FIGURE 4. NF immuno-expression in the cerebral cortex, 400x. (A) Group 1 (control group) showing marked immuno-expression (curved arrows). (B) Group 2 (hypoxic brain injury) showing mild immuno-expression (curved arrows). (C) Group 3 (hypoxic brain injury, plus IV injection of BMSCs) showing moderate immuno-expression (curved arrows). (D) Group 4 (hypoxic brain injury, plus ICV injection of BMSCs) showing marked immuno-expression (curved arrows).

TABLE 2
Righting reflex responses in seconds, at days 3, 7, 14, and 28. M = mean, SD = standard deviation

Day	Group 1			Group 2			Group 3			Group 4		
	M	SD	Signvs.									
3	1.9	0.1	2, 3, 4	6.53	0.5	1	6.0	0.7	1	5.9	0.7	1
7	2.1	0.1	2, 3, 4	6.19	0.3	1, 4	6.0	0.1	1, 4	5.5	0.4	1, 2, 3
14	2.1	0.1	2, 3, 4	5.58	0.3	1, 3, 4	4.8	0.7	1, 2, 4	4.1	0.3	1, 2, 3
28	2.0	0.2	2, 3	4.77	0.4	1, 3, 4	3.2	0.3	1, 2, 4	2.3	0.3	2, 3

Group 1, control

Group 2, injured

Group 3, injured plus IV BMSC injection

Group 4, injured plus ICV BMSC injection

Sign vs = statistically significant differences vs. the indicated groups (Post Hoc LSD test)

TABLE 3
Geotactic reflex responses in seconds, at days 3, 7, 14, and 28. M = mean, SD = standard deviation

Day	Group 1			Group 2			Group 3			Group 4		
	M	SD	Sign vs.									
3	5.2	0.5	2, 3, 4	15.7	0.7	1	14.8	0.5	1	14.8	1.3	1
7	6.3	0.5	2, 3, 4	15.4	0.5	1, 4	15.1	0.5	1	14.6	0.5	1, 2
14	5.8	0.6	2, 3, 4	10.8	0.8	1, 3, 4	9.1	0.8	1, 2, 4	8.2	0.5	1, 2, 3
28	5.2	0.2	2, 3	10.1	0.4	1, 3, 4	6.3	0.4	1, 2, 4	5.6	0.8	2, 3

Group 1, control

Group 2, injured

Group 3, injured plus IV BMSC injection

Group 4, injured plus ICV BMSC injection

Sign vs = statistically significant differences vs. the indicated groups (Post Hoc LSD test).

TABLE 4
Nestin immunoeexpression in Groups 1, 2, 3 and 4

	Group 1	Group 2	Group 3	Group 4
Mean % area	0	1.43	3.37	4.51
SD	0	0.07	0.43	0.58
Sign vs.	2, 3, 4	1, 3, 4	1, 2, 4	1, 2, 3

Group 1, control

Group 2, injured

Group 3, injured plus IV BMSC injection

Group 4, injured plus ICV BMSC injection

Sign vs = statistically significant differences vs. the indicated groups (Post Hoc LSD test).

TABLE 5
GFAP immunoeexpression in Groups 1, 2, 3 and 4

	Group 1	Group 2	Group 3	Group 4
Mean % area	3.45	7.19	4.65	4.14
SD	0.31	0.73	0.58	0.53
Sign vs.	2, 3, 4	1, 3, 4	1, 2	1, 2

Group 1, control

Group 2, injured

Group 3, injured plus IV BMSC injection

Group 4, injured plus ICV BMSC injection

Sign vs = statistically significant differences vs. the indicated groups (Post Hoc LSD test).

TABLE 6
Neurofilaments immunoeexpression in Groups 1, 2, 3 and 4

	Group 1	Group 2	Group 3	Group 4
Mean % area	10.5%	1.6%	15.3%	22.0%
SD	0.98	0.48	1.15	1.29
Sign vs.	2, 3, 4	1, 3, 4	1, 2, 4	1, 2, 3

Group 1, control

Group 2, injured

Group 3, injured plus IV BMSC injection

Group 4, injured plus ICV BMSC injection

Sign vs = statistically significant differences vs. the indicated groups (Post Hoc Scheffé's test).

TABLE 7
VEGF-R2 and eNOS gene expression (mean \pm SD)

	VEGFR2 gene expression	eNOS gene expression
Group 1	0.276 \pm 0.07 a	0.750 \pm 0.06 a
Group 2	0.563 \pm 0.09 b	0.795 \pm 0.045 b
Group 3	1.180 \pm 0.08 c	1.560 \pm 0.44 c
Group 4	1.830 \pm 0.03 d	2.100 \pm 0.03 d

Group 1, control

Group 2, injured

Group 3, injured plus IV BMSC injection

Group 4, injured plus ICV BMSC injection

Different letters indicate significant differences between means (Scheffé's test).

Statistical analysis

All the data collected from the experiments were recorded and analyzed using IBM SPSS Statistics software for Windows, Version 20 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by Post Hoc LSD test were used to compare differences between groups of neurobehavioral tests, morphometric results and quantitative gene expression. In each test, the data was expressed as the mean value (M) \pm standard deviation (SD) and differences were considered to be significant at $p < 0.01$.

Results

Neurobehavioral tests

The data are presented in Tabs. 2 and 3. As expected, hypoxic brain injury (Group 2) provoked significant delays for the completion of both neurobehavioral tests. Although there was a tendency to recover with time in Group 2 animals, both tests were still significantly above control levels after 28 days ($p < 0.01$). The IV injection of BMSCs helped some degree of recovery, but a significant difference with the injured group occurred only after 14 and 28 days. However, the ICV injection of BMSCs was associated to a recovery of the response time, which was already significant after day 7 (as compared to the injured group) and was significantly larger than that associated to IV injection, both after 14 and 28 days.

Histopathological findings

All rats were sacrificed 28 days after the operations, when brain samples were processed for histopathological examinations. All subgroups of the control Group 1 (subgroups 1a, 1b, 1c, and 1d) showed no apparent histological alteration of the cerebral cortex, which showed pyramidal cells with large vesicular nuclei and prominent nucleoli, granule cells, neuroglia cells, and blood vessels (Fig. 1A). Group 2 (hypoxic brain injury) showed degenerating pyramidal cells, many of which appeared shrunken, with a dark cytoplasm and small pyknotic nuclei. Excess vacuoles in neuropils, and wide perineural and

perivascular spaces were observed (Fig. 1B). Group 3 (hypoxic brain injury + IV-injected BMSCs group) showed signs of recovery, with some apparently normal pyramidal cells, even though many of them appeared shrunken and showed wide perineural spaces. There was also an excess number of granule cells. Also, there were abundant neuropil vacuoles (Fig. 1C). Group 4 (hypoxic brain injury + ICV-injected BMSCs group) showed a clear recovery from injury, with many apparently normal pyramidal cells and granule cells. However, some pyramidal cells still appeared shrunken, with slightly wide perineural spaces. Excess neuropil vacuoles and narrow perivascular spaces were also noticed (Fig. 1D).

Immunohistochemistry and morphometry

Immunohistochemical detection and morphometry was made 28 days after the operations, for (1) nestin, (2) glial fibrillary acidic protein (GFAP), and (3) neurofilament protein (NF). Since no apparent differences between the subgroups of Group 1, they were pooled for presentation.

Nestin is a class VI intermediate filament protein that is expressed in adult neural progenitor cells and the results are shown in Figs. 2A-D. GFAP is a marker of astrocytes and the results are shown in Fig. 3A-D. NF is expressed in neuronal somata and projections, and the results are shown in Fig. 4A-D.

The changes in the mean % area and standard deviation (SD) of nestin, GFAP and NF immuno-expression are shown in Tabs. 4-6. Nestin- or GFAP-positive areas increased significantly after the hypoxic brain injury (Group 2) and increased even further after any of the treatments (Groups 3 and 4). NF positive area dramatically decreased after the injury but recovered above control levels after any of the treatments. However, rats after ICV treatment (Group 4) showed significantly higher levels than those after IV treatment (Group 3).

Quantitative gene expression

Changes in the expression of VEGF-R2 and eNOS genes are shown in Tab. 7. The expression of both genes increased

slightly but significantly after ischemic brain injury, increased notably after IV injection of BMSCs, and even further after ICV injection.

Discussion

HIE, as a result of asphyxia at term, remains a major cause of neurological disabilities. It occurs in ~20 of 1000 full-term human deliveries. Between 20 and 50% of asphyxiated newborns exhibiting HIE die during the newborn period. Of the survivors, up to 25% have permanent neuropsychological handicaps in the form of cerebral palsy, with or without associated intellectual disability, or epilepsy (Chicha *et al.*, 2014; Gonzales-Portillo *et al.*, 2014).

Neural stem cells have been proposed as a useful treatment approach for the diseases of the central nervous system, and has been used in animal models of neonatal HIE. However, their lack of accessibility and the inhomogeneity in neuronal differentiation of various neurospheres limit their utility (Fan *et al.*, 2006).

MSCs, or particularly BMSCs, also offer a potential source of therapies, as shown in several rodent models and clinical trials of human diseases, such as Parkinson's disease, Huntington's disease, myocardial infarction, and stroke (Oh *et al.*, 2012).

In the current paper, experimental HIE was induced by bilateral ligation of cephalic arteries (Group 1) in 7-day-old rats, i.e. when the peak of brain growth occurs. It may thus be equivalent to the gestational term in humans (Bennet *et al.*, 2012). Twenty-eight days later, there were severe histological alterations in cerebral tissues, in the form of necrosis and cellular degeneration, with mild immuno-expression of nestin and NF while the immunoexpression of GFAP was increased. Also, the gene expression of VEGF-R2 and eNOS were increased.

The administration of BMSCs, either IV or ICV, to injured rats (Groups 3 and 4 of the current study) was followed by amelioration of all parameters studied (neurobehavioral tests, histopathological studies, and genes expression) as compared to the hypoxic brain-injured rats (Group 2). Although the IV transplantation of BMSCs was successful to ameliorate the effects of brain injury, the results of the ICV administration were even better. In fact, BMSCs would reach the lesioned sites in higher concentrations after ICV administration, while those injected IV would be diluted in the systemic circulation and many of them may be retained in the spleen and lung, and would never recirculate.

In principle, the MSC-mediated functional recovery after ischemic brain injury may occur through (a) cell replacement, and (b) a paracrine effect (Gonzales-Portillo *et al.*, 2014). For cell replacement, MSCs are capable of migration to the injury site, differentiation into specific lineages, help in the repair of brain tissue through a possible replacement of oligodendrocytes and neurons, and may also help in the modulation of the host inflammatory response. The paracrine effects include enhancement of the regenerative processes of parenchymal cells, and revascularization by newly-formed blood vessels (Gonzales-Portillo *et al.*, 2014). The repairing process may also be enhanced by a significant dampening of inflammatory pathways (Mitsialis and Kourembanas, 2016).

Moreover, it is still an open question how MSCs, which only reside for a short period in the brain, can induce long-term recovery. Future research will have to show whether transplanted MSCs redirect or stimulate the autologous stem cell pool, which could lead to sustained functional and anatomical regeneration.

Declarations

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Disclosure of conflict of interest

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Authors' contributions

NE, DS, A SH, designed and planned the study. NE and A SH performed the experiments. NE collected the data, and DS, E A E and E A analyzed the data. NE was a major contributor in writing the manuscript. NE amended the manuscript. All authors read and approved the final manuscript.

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