

Nasal Administration of Stem Cells: A Promising Novel Route to Treat Neonatal Ischemic Brain Damage

CINDY T.J. VAN VELTHOVEN, ANNEMIEKE KAVELAARS, FRANK VAN BEL, AND COBI J. HEIJNEN

Laboratory for Neuroimmunology and Developmental Origins of Disease [C.T.J.V., A.K., C.J.H.], Department of Neonatology [C.T.J.V., F.B.], University Medical Center Utrecht, 3584 EA Utrecht, The Netherlands

ABSTRACT: Mesenchymal stem cell (MSC) transplantation is a promising therapy to regenerate the brain after an ischemic event. We investigated the possibility to use the nasal route as a noninvasive method to repair the neonatal damaged brain. Nine-day-old mice underwent cerebral hypoxia-ischemia (HI), and MSCs were transplanted intranasally 10 d after HI. At 28 d after HI, MSCs were still present in the affected hemisphere but had not differentiated into cerebral cell types. Intranasal MSC treatment significantly improved sensorimotor function in the cylinder rearing test at 21 and 28 d after HI. Furthermore, intranasal MSC treatment decreased gray and white matter area loss when determined 28 d after HI by 34 and 37%, respectively. MSC cultured *in vitro* with brain extracts obtained 10 d after HI, responded to the ischemic brain by up-regulation of several growth factors, including fibroblast growth factor 2 and nerve growth factor in comparison with brain extracts of sham-operated controls. In conclusion, MSC can reliably be delivered to the brain via the nasal route to induce functional recovery and a reduction in brain lesion size. We propose that MSC function by stimulating endogenous cerebral repair by adapting their secretion profile to the ischemic brain leading to up-regulation of repair promoting factors. (*Pediatr Res* 68: 419–422, 2010)

Neonatal hypoxia-ischemia (HI) causes severe brain damage and remains a major cause of neonatal morbidity and mortality (1). At present, treatment options for neonatal HI brain damage are very limited (2). Recent clinical trials have shown that hypothermia (cooling of the brain by 2–4°C) has modest effects on outcome (3,4).

The therapeutic potential of mesenchymal stem cells (MSCs) has been studied in several rodent models of ischemic brain injury (5–7). The data show that MSC transplantation improves functional outcome and can also restore brain structure (7–9). Recently, we and others have shown that transplantation of bone marrow-derived MSC markedly improves functional outcome after neonatal HI in rodents (7,9–11). Furthermore, we have shown that intracranial MSC transplantation after neonatal HI enhanced neurogenesis and oligodendrogenesis and reduced lesion volume (7). It has been suggested that MSC promotes repair of injured tissue by replacement of damaged cells. However, MSC also secrete growth and differentiation factors that could stimulate endogenous repair processes (12–14).

The intracranial route of administration is frequently used in rodent studies, but for clinical application, a less invasive route is preferred. Although *i.v.* MSC delivery can be effective, the loss of cells in peripheral organs before they reach the brain is an important possible drawback. Existing evidence indicates that after intranasal delivery, MSCs are capable of crossing the cribiform plate and migrate throughout the brain using the rostral migratory stream (15). The goal of this study was to determine whether intranasal delivery of MSC can be used as the route of entry to treat neonatal hypoxic-ischemic brain damage.

MATERIALS AND METHODS

MSC. Bone marrow from femur and tibia of 6- to 8-wk-old C57Bl/6-Tg (UBC-GFP) 30Scha/J mice (Jackson Laboratories, Bar Harbor, ME) was cultured in DMEM/15% fetal bovine serum as described (7). Cells were negative for myeloid and hematopoietic cell lineage specific antigens and positive for Sca-1, CD90, CD29, CD44, and MHC class I (7). Before intranasal application, cells were labeled with PKH-26 according to manufacturers' instructions (Sigma-Aldrich Chemical Co., Steinheim, Germany).

Animals. The animal committee of Academic Biomedical Center Utrecht approved all experiments. At postnatal d 9 (p9), C57Bl/6 mice pups of both sexes underwent HI by permanent right common carotid artery occlusion under isoflurane anesthesia [3% induction and 1% maintenance in O₂:N₂O (1:1)] followed by exposure of 10% oxygen in nitrogen for 45 min (16). This procedure induced a lesion involving hippocampus, neocortex, and striatum. Pups from at least three different litters were used in each experimental group, both genders were equally distributed among experimental groups, and data were obtained in at least two independent experiments. All analyses were performed in a blinded set up. Mortality (~10%) only occurred immediately after HI.

At 10 d after HI, MSC or vehicle was delivered intranasally in awake animals. Thirty minutes before MSC or vehicle administration, two doses of 3 μ L hyaluronidase (total 100 U; Sigma-Aldrich Chemical Co.) in PBS were applied to each nostril and spontaneously inhaled (15). Subsequently, a total of 5 \times 10⁵ MSC in 12 μ L PBS or vehicle were administered as two doses of 3 μ L applied to each nostril. Animals were killed at 28 d after HI and perfused with 4% paraformaldehyde in PBS. Brains were embedded in paraffin or cryoprotected in 20% sucrose and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Zoeterwoude, The Netherlands).

Functional outcome. The cylinder rearing test was used to assess forelimb use asymmetry at 10, 21, and 28 d after HI. The weight-bearing forepaw(s) to contact the wall during a full rear was recorded as left (impaired), right (nonimpaired), or both. Paw preference was calculated as [(nonimpaired – impaired)/(nonimpaired + impaired + both)] \times 100% (7,17).

Histology. Coronal paraffin sections (8 μ m) were incubated with mouse anti-microtubule-associated protein (MAP2; Sigma-Aldrich Chemical Co.) or mouse anti-myelin basic protein (MBP; Sternberger Monoclonals, Lutherville, MD), and binding was visualized with a Vectastain ABC kit (Vector

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Correspondence: Cobi J. Heijnen, Ph.D., University Medical Center Utrecht, Room KC03.068.0, Lundlaan 6, 3584 EA Utrecht, The Netherlands; e-mail: C.Heijnen@umcutrecht.nl

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Abbreviations: BDNF, brain-derived neurotrophic factor; FGF, fibroblast growth factor; HI, hypoxia-ischemia; MAP2, microtubule-associated protein-2; MBP, myelin basic protein; MSC, mesenchymal stem cell; NGF, nerve growth factor; NT3, neurotrophin-3; SDF, stromal cell-derived factor

Table 1. Primers used for quantitative real-time RT-PCR

Symbol	RefSeq	Forward (5'→3')	Reverse (5'→3')
β -actin	NM_007393	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT
BDNF	NM_007540	CACATTACCTTCCAGCATCTGTGG	ACCATAGTAAGGAAAAGGATGGTCAT
CXCR4	NM_009911	CATAATCTCTTATTCCGAGGAG	CTACCACCATTTCAGGCTTTG
FGF2	NM_008006	GCGAGAAGAGCGACCCACAC	GAAGCCAGCAGCCGTCATC
GAPDH	NM_008084	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG
IL1 β	NM_008361	TCTATACCTGTCTGTGTAATG	GCTTGTGCTCTGCTTGTG
IL6	NM_031168	CCGCTATGAAGTTCCTCTCTGC	AGTGGTATCCTCTGTGAAGTCTCC
NGF	NM_013609	ACGGGCAGCATGGTGGAG	TGTAGAACAACATGGACATTACCG
NT3	NM_008742	CCCTGGAAATAGTCACACGGATG	ATGCTGTTGCCCTGGATGCC
SDF1	NM_021704	CGGTGTCTCTGTGCTGCC	CTCTGGCGATGTGGCTCTC
TGF β 1	NM_011577	GTGACAGCAAAGATAACAAAC	CTGAAGCAATAGTTGGTATCC

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Laboratories, Burlingame). Brain damage was analyzed at a location equivalent to -1.58 mm from bregma in adult mice by outlining both hemispheres on full section images using ImageJ software (National Institutes of Health, Bethesda, MD; <http://rsb.info.nih.gov/ij/>, 1997–2007). Ipsilateral MAP2 area loss was calculated as follows: $[1 - (\text{area ipsilateral MAP2 staining}/\text{area contralateral MAP2 staining})] \times 100\%$. Similarly, MBP area loss was determined (16).

To determine whether transplanted MSC differentiated into cerebral cell types, frozen sections ($10 \mu\text{m}$) were incubated with mouse anti-NeuN, mouse anti-S100 β (Chemicon, Temecula, CA) or rabbit anti-Iba1 (Wako, Richmond, VA) followed by AlexaFluor-488-anti-IgG (Molecular Probes, Eugene, OR).

MSC gene expression profile after culture with ischemic brain extracts. To assess whether MSC can sense and respond to signals provided by the ischemic brain, MSC gene expression was performed after culturing MSC *in vitro* with extracts from the brain from sham animals and animals after HI (13). Brains were collected from HI or sham-operated animals at 10 d after surgery. A standardized section of the ischemic hemisphere, centered at the HI lesion (bregma $-2 \sim 0$ mm), was obtained by dissection on ice. Brain sections were homogenized at 150 mg/mL in knockout DMEM (Invitrogen) and clarified by centrifugation for 10 min at $10,000 \times g$ at 4°C . Protein concentration was determined using a protein assay (BioRad, Hercules, CA) with BSA as standard and stored at -80°C until use. Approximately, 1×10^6 MSC per well were seeded in six-well plates in complete MSC medium. After 24 h, culture medium was replaced with knockout DMEM containing brain extract (final concentration 1 mg protein/mL) from HI + vehicle-treated or HI + MSC-treated ischemic brain extract ($n = 4$ per condition). After 72 h in culture, total RNA was isolated from the MSC using the RNeasy mini kit (Qiagen, Venlo, The Netherlands) and transcribed to cDNA using oligo (dT), dNTP mix, first-strand buffer, DTT, RnaseOUT, and Superscript III (Invitrogen). Gene expression changes in several growth factors (Table 1) were measured using quantitative RT-PCR with SYBR green mastermix on the BioRad iQ5. Data were normalized using multiple housekeeping genes and analyzed by comparing $2^{-[\Delta\Delta\text{Ct}]}$ of the normalized data. Fold changes were calculated relative to MSC cultured in brain extracts from sham-operated animals and killed at 10 d after HI.

Statistical analysis. Data are presented as mean \pm SEM and analyzed by two-way analysis of variance with Bonferroni posttests, and $p \leq 0.05$ was considered significant.

RESULTS

MSC in the ischemic brain after nasal application. Mice underwent HI on p9, and PKH 26-labeled MSC were administered intranasally at d 10 after HI. At d 28 after HI, which is 18 d after intranasal administration, MSC could still be detected in the brain (Fig. 1). Nasally applied MSC were present in several layers of the olfactory bulb but predominantly in the glomerular layer of the olfactory bulb of both hemispheres. At the level of the subventricular zone, few MSCs were detected in both hemispheres. In the severely damaged ipsilateral hippocampus, many cells were present, whereas no MSCs were detected in the contralateral hippocampus.

Sensorimotor function and brain damage. Exposure to HI induced a preference for the use of the unimpaired forepaw in

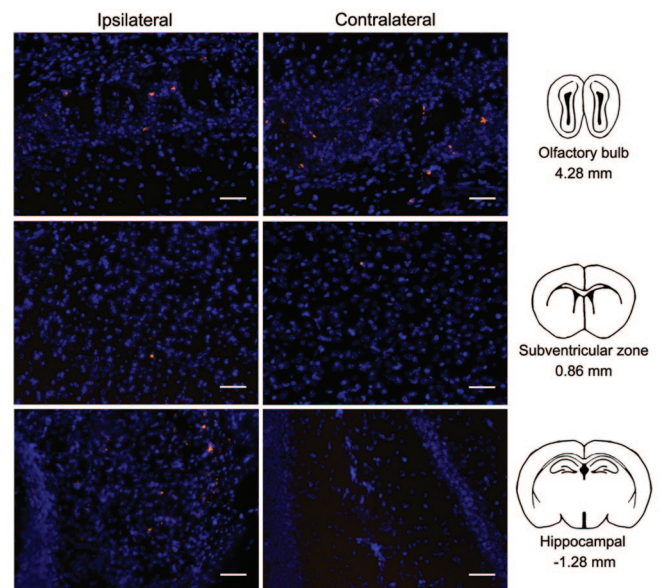


Figure 1. MSC location in brain after intranasal administration. Brain sections 18 d after intranasal administration of MSC. Sections were stained with DAPI (blue) to visualize all nuclei. MSCs (red) were found in both the ipsi- and contralateral olfactory bulb. Few cells could be found in both hemispheres at the level of the subventricular zone. In the contralateral hippocampus, no MSC could be found. However, in the ipsilateral hippocampus, which is damaged, many MSCs are present. Scale bar represents $50 \mu\text{m}$.

the cylinder rearing test when performed at 10, 21, and 28 d after HI. After intranasal application of MSC, forepaw preference was significantly reduced, indicating restoration of sensorimotor function (Fig. 2A). Intranasal administration of MSC at 10 d after HI also significantly reduced neuronal ($34 \pm 4\%$ reduction of MAP2 loss; $p < 0.05$) and white matter ($37 \pm 8\%$ reduction of MBP loss; $p = 0.05$) loss at 28 d after HI (Fig. 2B and C). Vehicle treatment, *i.e.* hyaluronidase followed by saline, had no effect on sensorimotor function and lesion size.

Gene expression of MSC after culture with brain extracts. Transplantation of only 5×10^5 MSC resulted in a robust reduction in lesion volume. However, analysis of expression of NeuN, S100, or Iba-1 showed that cells of transplant origin had not differentiated into mature neurons, astrocytes, or microglia (data not shown). Therefore, we hypothesized that MSC may respond to signals in the ischemic brain by adapting

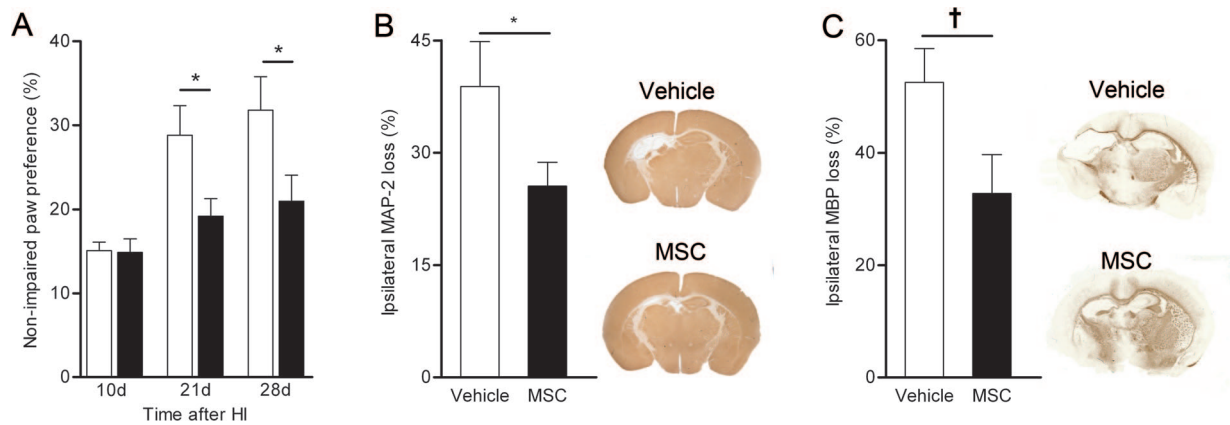


Figure 2. Sensorimotor function and brain damage after intranasal transplantation of MSC. Preference for use of the impaired paw was determined as a measure of sensorimotor function (A). Quantification of MAP2+ (B) and MBP+ (C) area loss expressed as ratio ipsi- or contralateral area after intranasal administration of MSC at 10 d after HI. *White bars*: vehicle treated animals ($n = 8$); *black bars*: MSC-treated animals ($n = 12$). Data represent mean \pm SEM ($*p < 0.05$; $^{\dagger}p = 0.05$).

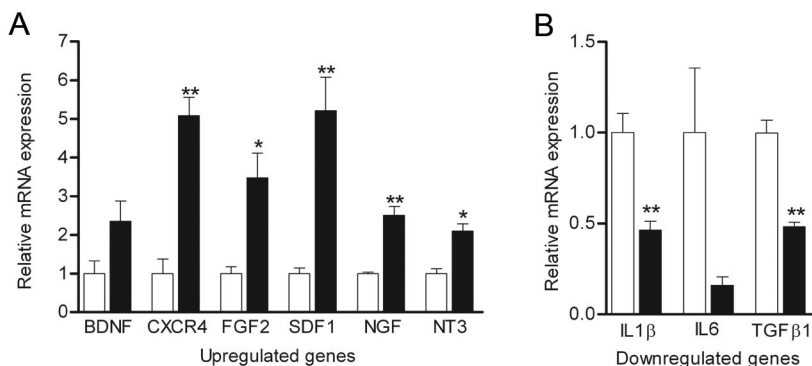


Figure 3. Changes in mRNA levels growth factors in MSC cultured with brain extracts. MSCs were cultured for 72 h with brain extracts obtained from sham-operated mice (*white bars*) or HI mice (*black bars*; $n = 3$ per group). Culture of MSC with HI brain extracts induced increased mRNA expression of CXCR4, FGF2, SDF-1, NGF, and NT3 (A) and down-regulation of IL1 β and TGF β 1 (B) as determined by quantitative real-time RT-PCR. Data represent mean \pm SEM ($*p < 0.05$, $**p < 0.001$).

their expression of growth factors to stimulate endogenous repair processes. To test this hypothesis, MSCs were cultured in conditioned medium containing brain extracts from sham-operated animals or HI animals obtained at 10 d after HI. After culturing the MSC with the brain extracts for 72 h *in vitro*, mRNA expression of growth and differentiation factors was analyzed by qPCR. In response to HI brain extracts compared with sham brain extracts, MSC significantly up-regulated expression of chemokine (C-X-C motif) receptor 4 (CXCR4), fibroblast growth factor 2, nerve growth factor, neurotrophin-3, and stromal cell-derived factor (SDF)-1, whereas expression of IL1 β and transforming growth factor beta 1 (TGF β 1) by MSC was down-regulated (Fig. 3). mRNA expression of brain-derived neurotrophic factor and interleukin (IL)-6 was not changed by MSC cultured with HI brain extracts.

DISCUSSION

This study shows for the first time that nasally applied MSCs improve functional outcome and reduce gray and white matter loss in a model of neonatal HI brain damage. These findings indicate that the nasal route is an efficient route for stem cell transplantation after brain injury in the neonate.

Improved outcome after MSC treatment has been attributed to the inhibition of inflammatory processes that could contribute to damage, the differentiation of transplanted MSC into

new neurons, and the stimulation of endogenous repair processes. In this study, MSC were applied via the nasal route at 10 d after HI. At this time point after HI, maximal reduction in MAP2 and MBP staining has already been obtained. Therefore, we anticipate that inhibition of injurious processes or prevention of damage are not the major mechanisms underlying the marked improvement in functional outcome and reduction in lesion size, but that MSC transplantation at this time point rather leads to regeneration of gray and white matter.

At d 28 after HI, which is 18 d after bilateral intranasal administration, MSCs were still present in the brain. However, it is hard to imagine that the relatively low number of transplanted MSC will exclusively be responsible for the profound reduction in lesion size that we observed. Moreover, no differentiation of transplanted MSC into a mature cerebral cell type was observed. This is in line with previous reports showing very limited differentiation of MSC into cerebral cell types after transplantation directly into the ischemic brain (9,12,18). Therefore, we propose that the major mechanism underlying the improved outcome after nasal MSC treatment is enhancement of endogenous repair mechanisms. This notion is in line with results from our earlier studies in which we showed that intracranial administration of MSC stimulated proliferation and/or survival of newly formed cells of host origin (12). Moreover, we showed that intracranial MSC

transplantation stimulated differentiation of new cells into neurons and oligodendrocytes (12).

It is of particular interest that at the level of the hippocampus, MSCs were predominantly found in the ipsilateral and lesioned hemisphere after intranasal delivery. These findings indicate that on intranasal administration, MSCs migrate to the lesion site not only from the ipsi- but also from the contralateral hemisphere. This migration is probably effected by chemokines secreted at the site of injury such as SDF-1. SDF-1 is up-regulated after HI brain injury near the lesion site and is expressed for at least 14 d after induction of the injury (19). Furthermore, we show here that MSCs increase the expression of CXCR4 and the receptor for SDF-1, as a response to the ischemic environment in the brain.

Directed migration of nasally applied MSC to the lesion site may have the advantage that MSCs are allowed to adapt better to the milieu in the brain then, *e.g.* after intracranial administration leading to better MSC survival. This notion is supported by our finding that MSC applied intracranially, directly adjacent to the lesion site, could not be detected anymore in the brain at d 28 after HI, which is 18 d after administration (7).

As described above, we propose that the reduction of lesion size and improved functional outcome induced by intranasal or intracranial administration of MSC after HI are mainly mediated by stimulation of endogenous repair. We show here that MSCs express a number of growth and differentiation factors that could contribute to stimulation of repair. Moreover, MSC cultured with HI brain extracts obtained at 10 d after HI change their gene expression of several secreted factors that are known to regulate cell proliferation and differentiation and to improve outcome after brain injury (20–24). Although it is not possible to define the exact contribution of each of these factors to repair processes, we can conclude that a trophic milieu, including brain-derived neurotrophic factor, fibroblast growth factor 2, neurotrophin, and nerve growth factor, is provided by MSC when in contact with signals provided by the damaged brain. It is also of interest that the proinflammatory cytokines such as IL-1 and IL-6 are down-regulated by MSC after interaction with the ischemic brain, which may also contribute to controlling proper repair.

The setbacks that come with *i.v.* or intraarterial transplantation of MSC, such as potential loss of cells in the periphery and systemic immunosuppression, are avoided by nasal application. We demonstrate here that MSCs applied via the nasal route have therapeutic effects for repairing the neonatal brain after a hypoxic-ischemic insult. Therefore, we propose that this route of administration provides an effective alternative allowing frequent MSC treatments with minimal burden for the patient.

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