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Authors

Larpthaveesarp, Amara
Pathipati, Praneeti
Ostrin, Samuel
et al.

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Enhanced Mesenchymal Stromal Cells or Erythropoietin Provide Long-Term Functional Benefit After Neonatal Stroke

Amara Larpthaveesarp, BS; Praneeti Pathipati, PhD; Samuel Ostrin, BS; Anthony Rajah, BS; Donna Ferriero, MD; Fernando F. Gonzalez, MD

BACKGROUND AND PURPOSE: Perinatal stroke is a common cause of life-long neurobehavioral compromise. Mesenchymal stromal cells (MSCs) and EPO (erythropoietin) have each demonstrated short-term benefit with delayed administration after stroke, and combination therapy may provide the most benefit. The purpose of this study is to determine the long-term histological and functional efficacy of enhanced, intranasal stem cell therapy (MSC preexposed to EPO) compared with standard MSC or multidose systemic EPO.

METHODS: Transient middle cerebral artery occlusion or sham surgery was performed in postnatal day (P) 10 Sprague-Dawley rats, who were treated with single-dose intranasal MSC, MSC preexposed to EPO (MSC/EPO), multidose systemic EPO (EPO3; 1000 u/kg per dose×3 every 72 hours), or cell-conditioned media on P13 (day 3 [P13–P19] for EPO), or on P17 (day 7 [P17–P23] for EPO). At 2 months of age, animals underwent novel object recognition, cylinder rearing, and open field testing to assess recognition memory, sensorimotor function, and anxiety in adulthood.

RESULTS: MSC, MSC/EPO, and EPO3 improved brain volume when administered at 3 or 7 days after middle cerebral artery occlusion. MSC/EPO also enhanced long-term recognition memory with either day 3 or day 7 treatment, but EPO3 had the most long-term benefit, improving recognition memory and exploratory behavior and reducing anxiety.

CONCLUSIONS: These data suggest that single-dose MSC/EPO and multidose systemic EPO improve long-term neurobehavioral outcomes even when administration is delayed, although EPO was the most effective treatment overall. It is possible that EPO represents a final common pathway for improved long-term repair, although the specific mechanisms remain to be determined.

GRAPHIC ABSTRACT: An online graphic abstract is available for this article.

Key Words: anxiety ■ erythropoietin ■ infarction, middle cerebral artery ■ mesenchymal stem cell ■ treatment outcome

Stroke in the newborn period is a common cause of mortality and morbidity, with an estimated incidence of ≈1 in 4000 live births.[1] The causes of perinatal stroke are multifactorial, and many survivors deal with life-long motor or cognitive dysfunction.[2] Injury progression following a focal ischemic insult evolves over a period of days to weeks,[3] and there are no accepted therapies specific for this disease process. In addition, diagnosis is often delayed, making the identification of late treatment strategies crucial.[4]

Arterial ischemic stroke in full-term infants is by far the most common type of perinatal stroke, occurring in up to 80% of cases.[5] A fixed period of ischemia is followed by reperfusion that leads to oxidative stress, excitotoxicity, reduced blood flow, and altered cell fate.[6,7] Upregulation of HIF-1α (hypoxia-inducible factor-1α) increases downstream release of EPO (erythropoietin) and VEGF (vascular endothelial growth factor) that promote survival and repair.[8,9] We previously demonstrated that multiple doses of EPO given over a 1-week period

Correspondence to: Fernando F. Gonzalez, MD, University of California, San Francisco, 1550 4th St, Box 2924, San Francisco, CA 94158. Email fernando.gonzalez@ucsf.edu

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Nonstandard Abbreviations and Acronyms

CCM	cell-conditioned media
CR	cylinder rearing
eNOS	endothelial NO synthase
EPO	erythropoietin
EPO-R	erythropoietin receptor
MCAO	middle cerebral artery occlusion
MSC	mesenchymal stromal cell
NOR	novel object recognition
NOS	NO synthase
OF	open field
P	postnatal day
PI3K	phosphatidylinositol 3-kinase
VEGF	vascular endothelial growth factor

immediately following neonatal stroke enhance neurogenesis and cognition in adulthood.[10,11] A multidose EPO regimen initiated as late as 1 week following neonatal stroke also improves short-term histology and function.[12]

Mesenchymal stromal cells (MSCs) are promising candidates to repair early brain injury because of their availability and favorable preclinical data, but their mechanism of benefit is controversial. We previously showed that intranasal MSC significantly reduces infarct size after neonatal stroke, even when treatment is delayed.[13] This occurs even in the absence of engrafted cell survival, suggesting transplanted cells may stimulate secretion of growth and differentiation factors that provide an environment to enhance repair.[14]

Since paracrine effects, and secretory factors, appear to be central to the beneficial response, MSCs have also been engineered to secrete specific growth factors, with possible additional benefit.[13,14] MSCs express EPO-R (EPO receptor), and EPO has been shown to facilitate secretion of angiogenic factors and enhance repair in limb ischemia models.[15,16] Exosomes secreted from MSC have also been shown to promote VEGF secretion and functional recovery.[17] Delayed administration of hypoxia-preconditioned MSC also improved short-term neurovascular regeneration and behavior in neonatal stroke and hypoxia-ischemia models.[18–20] We have demonstrated improved histological and behavioral outcomes with MSC,[13] EPO,[10] and VEGF[21] alone, and it is likely that cross talk between these pathways is important to maximize long-term outcomes, and a combination strategy may provide the most benefit.

To be a candidate for translation into the clinic, an effective treatment strategy must demonstrate long-term efficacy in preclinical models. Here we use a clinically applicable model of full-term equivalent neonatal focal ischemia-reperfusion injury in postnatal day (P) 10 rat pups.[12,22,23] This involves complete occlusion of blood flow to the MCA territory, followed by a reperfusion phase where blood flow restoration activates a number of pathways responsible for injury progression. In this study, our focus is on identifying an effective cell-based treatment strategy to improve long-term brain volume and neurobehavioral function. Combining 2 desirable interventions that have each shown promise as delayed treatment options into 1 treatment modality may widen the therapeutic window and further improve outcomes for a disease process where diagnosis is often delayed. We compare intranasal MSC preexposed to EPO as a delayed, single-dose treatment strategy, to single-dose MSC prepared in standard culture conditions and multidose systemic EPO as treatments for neonatal stroke. Our hypothesis is that treatment with MSC preexposed to EPO will provide more long-term functional benefit than MSC or EPO for neonatal stroke.

METHODS

Data supporting the findings of this study not shown are available from the corresponding author upon request. This protocol received approval from the University of California, San Francisco Institutional Animal Care and Use Committee, and studies were conducted in accordance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals. Every effort was made to minimize animal suffering and to reduce the numbers of animals.

Transient Middle Artery Occlusion or Sham Surgery

P10 Sprague-Dawley rats underwent focal ischemia-reperfusion via transient right middle cerebral artery occlusion (MCAO) for 3 hours, as described previously.[12,22] MCAO or sham surgery was performed in spontaneously breathing animals anesthetized with 1.5% to 3% isoflurane in 100% O_2. Rectal temperature was monitored and maintained at 36 °C to 37 °C with a combination of heating blanket and overhead light. The right internal carotid artery was dissected, and a silicone-coated 6-0 nylon filament (Doccol) was inserted 9 to 10.5 mm (based on animal weight) to occlude the MCA and secured, and awake pups were returned to their dam. Injury was confirmed by severe left frontal/hindlimb paresis during the occlusion period, and animals without this finding were excluded. We previously demonstrated a consistent injury pattern involving the striatum and parietotemporal cortex with diffusion-weighted magnetic resonance imaging during occlusion and tetrazolium chloride staining at 24 hours following MCAO.[12,23] For reperfusion, animals were anesthetized, and suture ties and the occluding filament were removed. Avitene Hemostat (Davol) was placed over the arteriotomy, and the skin incision was closed. Sham animals were anesthetized, and the internal carotid artery was dissected, after which the skin incision was closed, and 3 hours later, shams were again anesthetized for 5 minutes. Weight was monitored for 1 week following surgery to ensure adequate weight gain.

MSC Preparation and Intranasal Treatment

Rat Sprague-Dawley adipose-derived MSCs (Creative Bioarray, Shirley, NY) were seeded at a density of 3×10^6 cells per 25-cm^2 flask and cultured as published previously.[13] The commercially sourced MSCs were chosen based on their capacity for multipotential differentiation along osteogenic, chondrogenic, and adipogenic lineages, with the following markers negative for CD11b, CD34, and CD45 and positive for CD29, CD44, CD90, and CD105.[24] We further confirmed identity by flow cytometry on arrival (data not shown). For MSCs preexposed to EPO (MSC/EPO), cells were exposed to recombinant human EPO (R&D Systems; 1 IU/mL of medium) for 24 hours before administration (dose was established by a dose-response curve in an in vitro slice culture model).[25]

At 3 days (P13) or 7 days (P17) following surgery, MSC, MSC/EPO, or vehicle was administered intranasally.[13] Thirty minutes prior, 2 doses of 5-µL hyaluronidase (total 100 U; Sigma-Aldrich) were applied to each nostril and spontaneously inhaled.[26] Subsequently, a total of 0.5×106 MSC or MSC/EPO resuspended in media was administered as 2 doses of 5 µL to each nostril. This dose was based on a previous dose response of intranasal MSC for short-term efficacy in neonatal brain injury.[27] Vehicle-treated animals received unconditioned media. Animals were randomly divided into groups that received (1) intranasal treatment at 3 days after surgery (Figure 1, cohort A) or (2) intranasal treatment at 7 days following MCAO or sham surgery (cohort B). Additional cohorts of animals were treated with cell-conditioned media (CCM) from cultured MSC (CCM MSC, 20 µL total) or MSC/EPO (CCM MSC/EPO, 20 µL total), absent cells, on day 3 or 7 after MCAO or sham surgery.

EPO Intraperitoneal Treatment

Separate cohorts of rats underwent surgery at P10 and were treated with 3 doses of vehicle (BSA, 0.125 g/dL concentration; 2 µL/g body weight administered intraperitoneally) or EPO (1000 units/kg body weight, 2 µL/g in 0.125 g/dL BSA IP), at P13, P16, and P19 (3-day [3d] EPO3; Figure 1, cohort C) or at P17, P20, and P23 (7-day [7d] EPO3; Figure 1, cohort D) as published previously.[14]

Treatment Groups

A total of 189 animals were studied. Seventy-nine underwent MCAO (2 deaths in vehicle-treated MCAO group), and 60 underwent sham surgery for cell-based and EPO therapies (Figure 1). With an estimated effect size of Cohen f^2 of 0.30 based on prior experimentation between control/experimental groups, α=0.05, power(1−β)=0.95, we calculated a sample size of 9 to 11 animals/group. Sixteen vehicle-treated MCAO animals survived, and remaining MCAO animals were randomized into 3 treatment groups at either 3 days post-MCAO (3d MSC, n=10; 3d MSC/EPO, n=10; 3d EPO3, n=10) or 7 days post-MCAO (7d MSC, n=10; 7d MSC/EPO, n=10; 7d EPO3, n=11). There were separate sham groups representing each treatment and time point, n=5 per group. Since no differences were observed, they were combined into a single sham group for analysis. Sex was equally distributed between groups and time points (Table I in the Data Supplement). Finally, a separate cohort of animals underwent MCAO (n=30; 4 deaths: two 7d CCM MSC, one 3d CCM MSC/EPO, one 7d CCM MSC/EPO) or sham surgery (n=20) and was treated at day 3 or 7.

Behavioral Analysis

Novel object recognition (NOR) examined recognition memory at 8 weeks of age, when animals reached young adulthood.[28] Habituation was performed on days 1 and 2 (P56 and P57) for 10 minutes each day, in a 40×40-cm box with 30-cm-tall walls. A 5-minute Familiarization trial was performed on day 3 (P58), with 2 identical objects inside the box. A 3-minute Testing phase was performed on day 4 (P59), 24 hours following the Familiarization trial, where one familiar object was replaced by a similarly sized novel object. Novel object exploration time was recorded: novel object preference index=novel object exploration time/(NOR time+familiar object exploration time).

Open field (OF) assessed locomotion, exploratory behavior, and anxiety for the day 7 treatment cohorts. Each animal was placed in a 45×45-cm plexiglas box with opaque, 35-cm-tall walls for a single 10-minute trial at P54. A painted grid divided the floor into 9 identical squares. Animals were placed in a random corner,

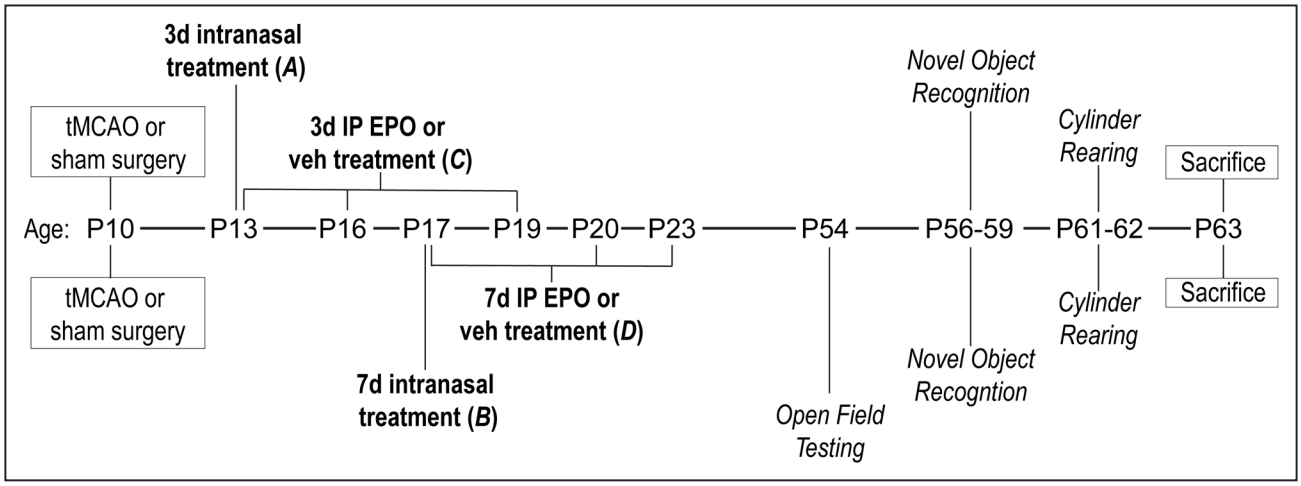

Figure 1. Experimental protocol.
Rats were divided into 4 cohorts: cohort A (intranasal or treatment on day 3), cohort B (intranasal treatment on day 7), cohort C (intraperitoneal [IP] EPO [erythropoietin; 1000 units/kg per dose] or vehicle [veh] initiated on day 3), or cohort D (IP treatment starting day 7). Cohorts A and C underwent novel object recognition (NOR) and cylinder rearing (CR) testing, and cohorts B and D underwent open field, NOR, and CR testing before euthanasia at postnatal day (P) 63.

and the following behavior was recorded: center square entries, duration in center square, rearing behavior, and total locomotion.

Cylinder rearing (CR) measured forelimb use as a function of sensorimotor bias.[29] Rats were placed in a transparent plexiglas cylinder measuring 20 cm in diameter and 30 cm in height for two 3-minute trials conducted on consecutive days (P61 and P62). Forepaw initiation for each weight-bearing lift from the ground and lower back to the ground was recorded as right, left, or both forepaws. Results were expressed as the percentage use of the impaired (left) forepaw relative to the total number of forepaw movements (left+right+both). The results for all behavioral tests were analyzed by 2 independent, blinded raters, and the average scores were used for data analysis.

Histology

Animals were anesthetized with sodium pentobarbital (100 mg/kg; nembutal; Abbott Laboratories) and euthanized at P63. Brains were harvested by transcardiac perfusion with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were removed and postfixed, equilibrated in 30% sucrose, and left at 4°C in 0.1 M PBS until sectioning. The entire brain was sectioned at 50-µm intervals on a sliding microtome (Thermo Fischer Scientific). Mounted sections were air-dried, stained with cresyl violet, dehydrated, cleared, and cover slipped.

Stereological Volumetric Analysis of Brain Volumes

Using systematic random sampling, every 12th section was selected, stained, and analyzed. Sections encompassed the whole brain from the genu of the corpus callosum to the occipital lobes. Blinded volumetric quantifications were on a Zeiss AxioScope Imager Z.2 with a motorized *xyz* axis computer-controlled stage, Neurolucida, and StereoInvestigator software (MicroBrightField, Inc). The cross-sectional area of the right (ipsilateral) and left (contralateral) hemispheres was calculated according to the Cavalieri principle.[30] Injury was quantitated by calculating the ratio of the ipsilateral, or injured, hemisphere versus the contralateral, control hemisphere volume as described previously.[10]

Statistical Analysis

Data are presented as mean±SD. *P* values of <0.05 were considered significant. Repeated measures ANOVA was performed on NOR and OF results and multivariate ANOVA on CR. One-way ANOVA with Newman-Keuls post hoc testing and calculation of COV and COE was performed on volumetric analyses. Mixed random-effects models were used to analyze the data and comparisons between groups to determine whether sex-based differences existed, as sex was equally distributed between groups. All analyses were performed in a blinded manner. Statistical analyses were performed using SAS Enterprise Guide (SAS Institute).

RESULTS

Day 3 Intranasal MSC/EPO Improves Long-Term Sensorimotor Function

To compare the efficacy of delayed, single-dose intranasal therapy on long-term motor outcomes, CR measured forelimb use after surgery and day 3 treatment (Figure 2). Only MCAO+3d MSC/EPO animals showed improvement, with more symmetrical use of forepaws when lifting from the ground (Figure 2A; MCAO+media, 0.662±0.118 versus MCAO+MSC/EPO, 0.4601±0.127; $P<0.001$). For lowers back to the ground, the majority of touches in all groups were bilateral, and no differences were noted (Figure 2B).

Day 3 MSC/EPO and EPO3 Improve Long-Term Cognition

NOR testing evaluated long-term learning and recognition memory following surgery and treatment. There was no long-term improvement in recognition memory, as measured by the NOR Index, in MCAO+3d MSC compared with the MCAO+media animals (Figure 2C; MCAO+media, 0.492±0.105 versus MCAO+MSC, 0.612±0.072; $P=0.064$). There was a significant improvement in long-term recognition memory in both the MCAO+3d MSC/EPO and MCAO+3d EPO3 animals compared with the MCAO+media animals (MCAO+media versus MCAO+MSC/EPO, 0.669±0.049; $P<0.01$. MCAO+media versus MCAO+EPO3, 0.666±0.081; $P<0.01$).

Day 3 MSC, MSC/EPO, and EPO3 Improve Long-Term Brain Volume

Following MCAO at P10, there is a significant decrease in ipsilateral hemispheric brain volume at 2 months of age in MCAO animals (Figure 3; MCAO+media, 0.620±0.098 versus shams, 0.998±0.026; $P<0.0001$). There was a significant improvement in hemispheric brain volume in MCAO+3d MSC (MCAO+media versus MCAO+3d MSC, 0.775±0.081; $P<0.01$) and MCAO+3d MSC/EPO groups (MCAO+media versus MCAO+3d MSC/EPO, 0.816±0.079; $P<0.0001$), although still different from shams.

To compare the efficacy of delayed, single-dose intranasal cells with multiple-dose systemic EPO, an additional cohort of animals was treated with 3 doses of systemic EPO over 1 week, starting 72 hours after surgery. There was a significant improvement in hemispheric brain volume with multidose systemic EPO (Figure 3; MCAO+media versus MCAO+3d EPO3, 0.779±0.122; $P<0.001$), which did not differ from MCAO+3d MSC or MCAO+3d MSC/EPO groups.

Day 7 MSC Improves Sensorimotor Function, and MSC/EPO and EPO3 Improve Long-Term Cognition

To compare the relative efficacy of MSC, MSC/EPO, and systemic EPO with later treatment, CR and NOR were once again used to assess sensorimotor function and cognition in animals treated on day 7 (Figure 4). For CR, only the MCAO+7d MSC group had improved forelimb

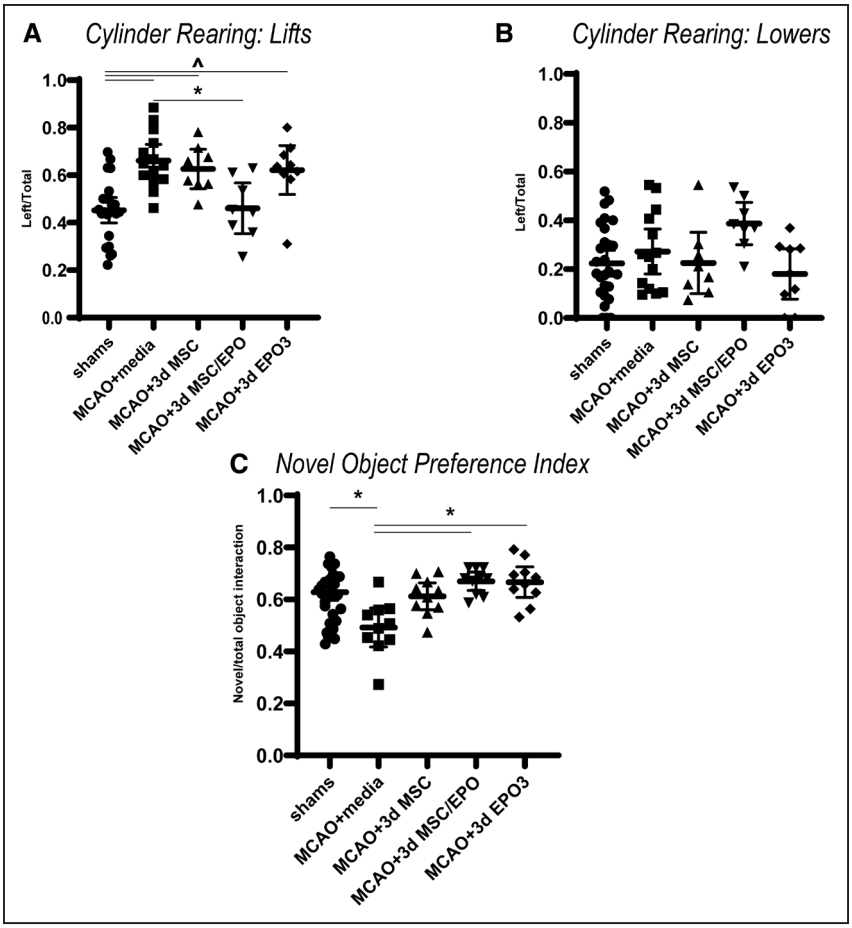

Figure 2. Behavioral testing after day 3 treatment.
Cylinder rearing assessed sensorimotor function via quantification of lifts (**A**) and lowers (**B**) averaged over 2 trials. Middle cerebral artery occlusion (MCAO)+3d mesenchymal stromal cell (MSC)/EPO (erythropoietin) animals had more symmetrical forelimb lifts compared with MCAO+media animals. There was no difference in lowers. Novel object recognition (**C**) assessed recognition memory as a function of cognition at 2 mo (postnatal days 56–59). Following Habituation and Familiarization trials, a Testing trial was performed, and novel object exploration was calculated: novel object preference index=novel object exploration time/(novel object recognition time+familiar object exploration time). MCAO+3d MSC animals did not show a significant improvement (P=0.067), but both MCAO+3d MSC/EPO and MCAO+3d EPO3 animals had improved performance. Data shown as mean with interquartile rage. ^P<0.05 vs shams; *P<0.05 vs MCAO+media.

use compared with MCAO+media animals (Figure 4B; MCAO+media, 0.661±0.118 versus MCAO+7d MSC, 0.484±0.140; P<0.01). For NOR, there was no improvement in MSC-treated animals (Figure 4C; MCAO+media, 0.504±0.107 versus MCAO+7d MSC, 0.614±0.089; P=0.087), but both MCAO+7d MSC/EPO and MCAO+7d EPO3 groups had improved recognition memory (MCAO+media versus MCAO+7d MSC/EPO,

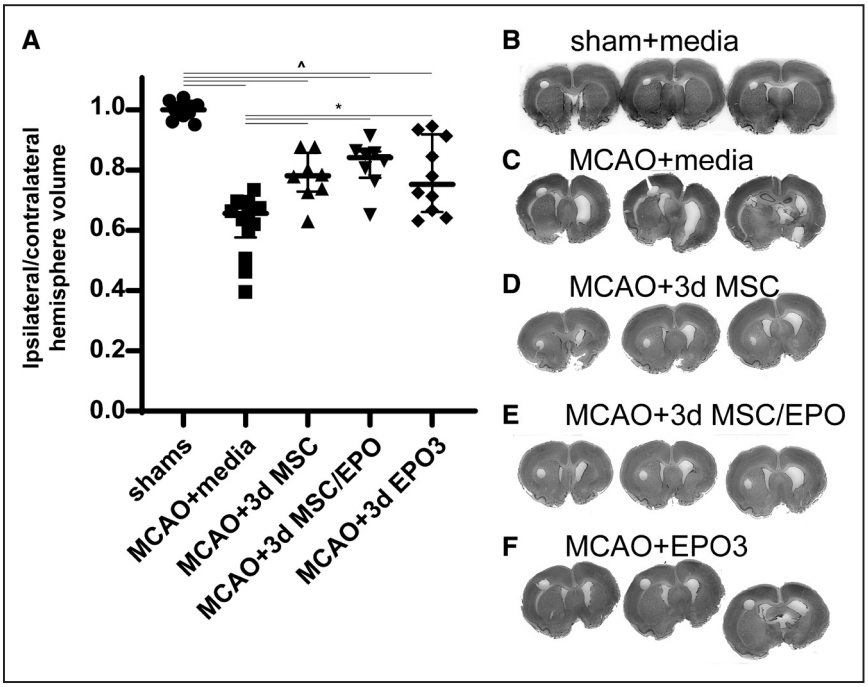

Figure 3. Stereological volumetric quantification of brain injury at 2 mo of age.
Middle cerebral artery occlusion (MCAO) at postnatal day 10 caused a significant reduction in hemispheric volume ratio (**A** and **C**). Mesenchymal stromal cell (MSC; **D**), MSC/EPO (erythropoietin; **E**), and EPO3 (**F**) at day 3 improved hemispheric brain volume relative to MCAO+media animals but were still decreased compared with shams. **B**–**F**, Examples of sham and MCAO injury in 3 serial sections (50 µm each, 600 µm apart) from a single animal in each group. Hole in contralateral (left) hemisphere used as an identifier of uninjured side. ^P<0.05 vs shams; *P<0.05 vs MCAO+media.

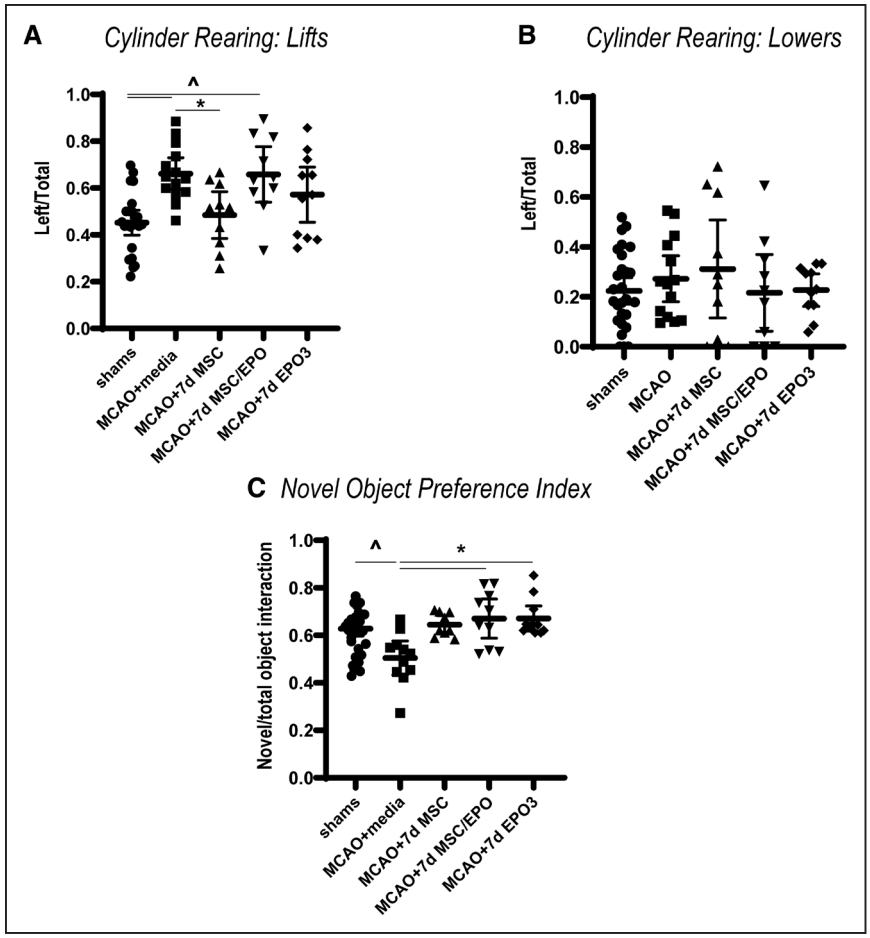

Figure 4. Sensorimotor and cognitive testing after day 7 treatment.
A and **B**, Middle cerebral artery occlusion (MCAO)+7d mesenchymal stromal cell (MSC) had improved forelimb symmetry compared with MCAO+media. **C**, For novel object recognition, while MCAO+7d MSC did not show improvement (P=0.08), there was an improvement in both MCAO+7d MSC/EPO (erythropoietin) and MCAO+7d EPO3 animals. ^P<0.05 vs shams; *P<0.05 vs MCAO+media.

0.670±0.115; P<0.01. MCAO+media versus MCAO+7d EPO3, 0.671±0.079; P<0.01).

Day 7 EPO3 Treatment Improves Exploration and Locomotion and Reduces Anxiety

To further assess long-term neurobehavioral outcomes with day 7 treatment, these animals underwent OF testing before NOR and CR to analyze locomotion, exploratory behavior, and anxiety (Figure 5). There was significantly improved exploration and reduced anxiety in MCAO+7d EPO3 compared with MCAO+media animals, with increased center square entries (Figure 5A; MCAO+media, 3.5±3.1 versus MCAO+7d EPO3, 8.4±3.2; P<0.01), increased center square duration (Figure 5B; MCAO+media, 4.6±4.9 versus MCAO+7d EPO3, 14.9±8.6; P<0.05), increased rearing frequency (Figure 5C; MCAO+media, 22.11±9.5 versus MCAO+7d EPO3, 36.8±8.7; P<0.01), and increased locomotion (Figure 5D; MCAO+media, 31.4±13.3 versus MCAO+7d EPO3, 43.6±10.5; P<0.05).

Day 7 MSC, MSC/EPO, and EPO3 Treatment Improves Long-Term Brain Volume

Finally, we assessed long-term volume changes with day 7 treatment (Figure 6). While all MCAO groups differed significantly from shams, we found persistent improvement in MCAO+7d MSC (0.726±0.076; P<0.05), MCAO+7d MSC/EPO (0.793±0.103; P<0.0001), and MCAO+7d EPO3 (0.777±0.088; P<0.001) groups relative to MCAO+media animals (0.620±0.098). Cell-free, conditioned media (CCM) obtained after culturing MSC or MSC/EPO was administered into separate cohorts of animals at each time point to see whether factors secreted into media may play a role in recovery or repair. There was improved brain volume with administration of CCM from MSC/EPO at 3 days post-MCAO (Figure I in the Data Supplement) but no improvement in behavioral function with CCM at either time point (data not shown). There were also no sex-based differences in any analysis (brain volume analysis shown in Tables I and II in the Data Supplement).

DISCUSSION

This study demonstrates for the first time that a delayed, single-dose treatment strategy preexposing MSC to EPO before intranasal administration results in long-term functional improvement following neonatal rodent stroke, lasting until adulthood. While there was subtle sensorimotor benefit with standard MSC therapy, there was more long-term cognitive improvement with this preexposure

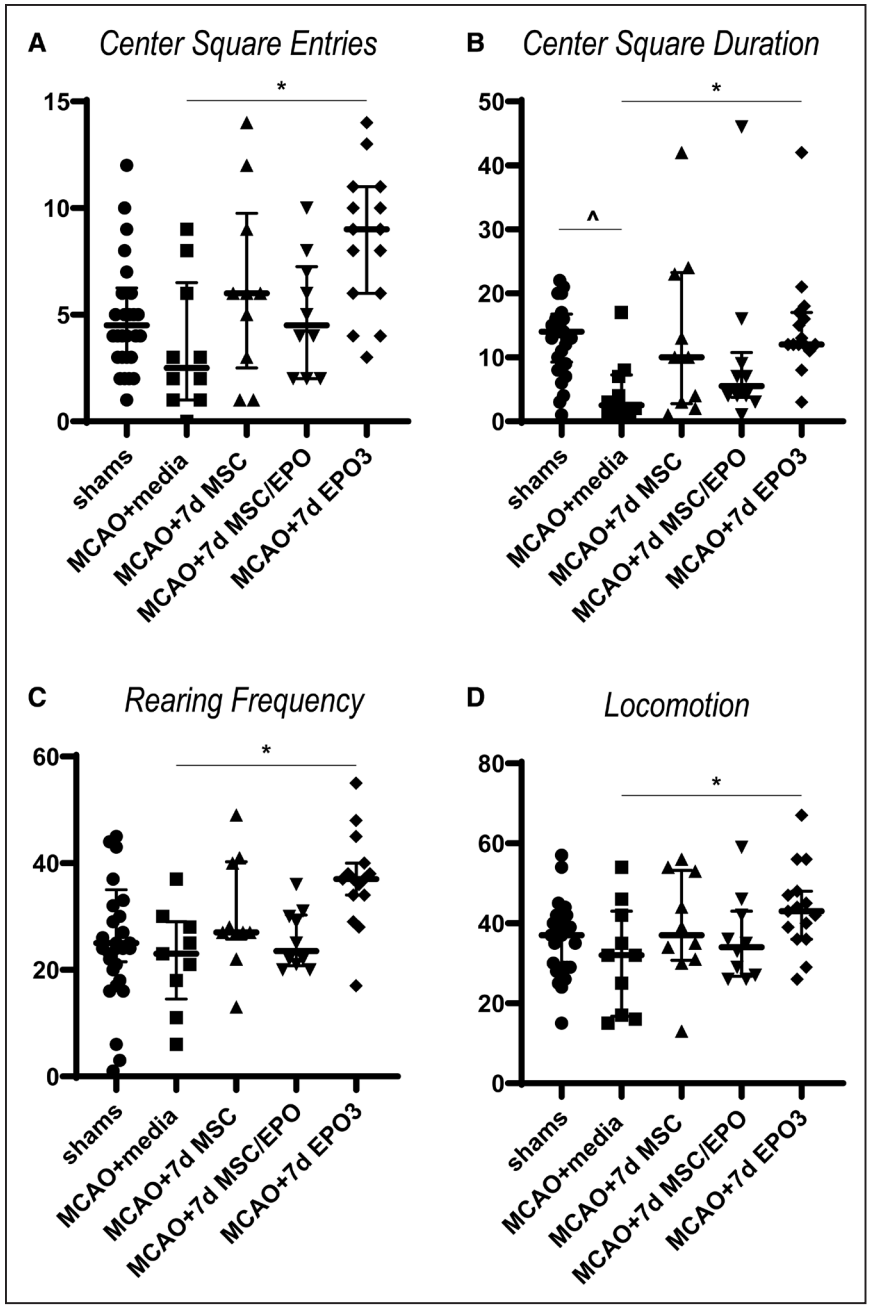

Figure 5. Exploration and locomotion in open field as marker of anxiety for cohorts B and D.
Middle cerebral artery occlusion (MCAO)+7d EPO3 animals showed significant improvement in exploration (**A** and **B**, center square entries and duration), rearing (**C**), and total locomotion (**D**) compared with MCAO+media group, indicating reduced anxiety. EPO indicates erythropoietin; and MSC, mesenchymal stromal cell. *P<0.05 vs MCAO+media, ^P<0.05 vs shams.

strategy. This suggests a possible role for a single-dose combination strategy at delayed time points; however, multidose systemic EPO similarly improved long-term brain volume and recognition memory. Direct EPO administration was also the only treatment to improve exploratory behavior and reduce anxiety in adulthood. Multidose systemic EPO may have the most long-term cognitive benefit for early stroke, even when administered in a delayed fashion.

For a therapy to be effective, it must result in long-term functional improvement. Previous attempts to understand the treatment response after brain injury have focused on preclinical models that are less clinically applicable because of differences in brain region, etiology, or timing in relation to perinatal stroke. Many studies suggesting neuroprotection have not translated to clinical efficacy because of inadequate modeling. Therefore, it is necessary to evaluate long-term neurobehavioral efficacy following early stroke. We chose to treat on day 3 or 7 because similar time points have previously demonstrated short-term benefit following neonatal stroke.[12,13] This follows the initial periods of primary and secondary energy failure leading to cell injury and death but where later stages of injury progression continue to evolve. They also represent reasonable time points for identifying babies at risk for focal ischemic injury, transfer to a referral center, diagnosis, and treatment.

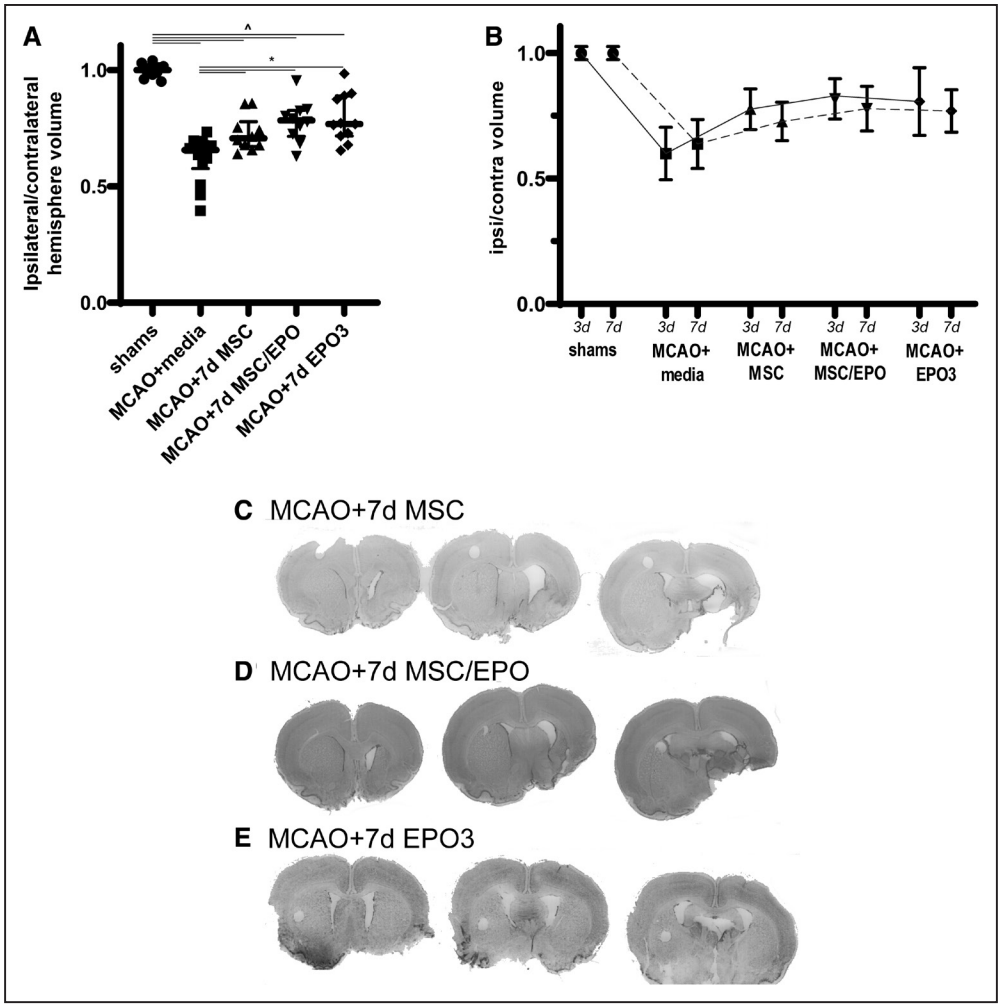

Figure 6. Stereological volumetric quantification of brain injury after day 7 treatment.
A, Delayed mesenchymal stromal cell (MSC), MSC/EPO (erythropoietin), and EPO3 significantly improved hemispheric brain volumes relative to middle cerebral artery occlusion (MCAO)+media animals but were still decreased compared with sham group. **A**, ^ $P<0.05$ vs shams; * $P<0.05$ vs MCAO+media. **B** compares volume measurements for 3- and 7d treatment groups. **C**–**E**, MCAO injury after 7d treatment.

MSC plays a number of roles in the developing and injured brain, including secretion of growth factors critical for brain development and repair in brain injury.[14] Benefit of exogenous MSC occurs even in the absence of survival or differentiation of transplanted cells, suggesting that the paracrine effect is paramount.[27] Human MSCs express EPO-R, and EPO may facilitate secretion of specific growth factors that are critical for the beneficial response.[14–16] We, therefore, hypothesized that a combination strategy that preexposes MSC to EPO would further enhance the paracrine effects and long-term repair, likely via activation of EPO-specific downstream pathways.

First, we found a significant improvement in gross hemispheric brain volume with both intranasal MSC and MSC/EPO treatment administered on day 3 following MCAO, similar to previously published studies of intranasal MSC.[13] Sensorimotor improvement was less definitive, with improvement only in the MSC/EPO group. There was, however, significant improvement in recognition memory with MSC/EPO treatment, demonstrating long-term cognitive improvement. We then pushed the treatment out to day 7 to help define the therapeutic window and possibly further differentiate efficacy of cell-based therapies. While there was still improved brain volume with both MSC and MSC/EPO treatment on day 7, there was less long-term behavioral improvement (only in CR:lifts) with standard MSC therapy. There was long-lasting improvement in recognition memory with MSC/EPO treatment even at this later time point. OF testing was also performed in the day 7 treatment cohorts to further interrogate behavioral outcomes by evaluating exploratory behavior and anxiety. In MCAO animals, there was no significant difference among media-, MSC-, or MSC/EPO-treated groups.

We previously demonstrated that multidose systemic EPO has long-term histological and cognitive benefits when treatment is initiated immediately following MCAO,[10] and delayed administration improves short-term histology and sensorimotor function.[12] A

secondary aim of this study was to directly compare delayed cell-based treatment with systemic EPO, which has consistently shown preclinical benefit and is currently being studied for human perinatal arterial ischemic stroke (phase II DINOSAUR trial; https://www.clinicaltrials.gov; unique identifier: NCT03171818). We chose to administer 3 intraperitoneal EPO doses, as opposed to a single dose, because this was previously shown to have the most long-term neurobehavioral benefit after neonatal stroke.[10] In this study, EPO enhanced long-term brain volume and recognition memory when initiated at either day 3 or 7, similar to MSC/EPO, but only EPO3 animals had improved locomotion, exploratory behavior, and reduced anxiety in OF testing with day 7 treatment.

While there was improved neurobehavioral function with both MSC/EPO and EPO3 treatment at day 3 or 7, not all results showed a consistent benefit. CR assesses forepaw preference as a measure of sensorimotor deficit. Animals with unilateral ischemic injury generally favor the nonimpaired (right) limb for weight-bearing lifts from and lowers to the ground, exhibiting differences as early as P21.[29] We observed a lot of variability within each group and frequent bilateral lowers in all groups. A number of postinjury variables may impact long-term gross motor function, such as standard environmental enrichment, and CR may not accurately measure fine motor deficits during adulthood following early injury.

We did see long-term cognitive improvement with both MSC/EPO and EPO3 groups, even with later treatment. NOR assesses cognition by determining the rat's ability to recognize a familiar object over a specific length of time. This relies on the animal's intrinsic exploratory drive to investigate novel stimuli, and animals with impaired recognition are less likely to familiarize with standard objects and then subsequently recognize the placement of a novel object, resulting in decreased novel object exploration time.[28] Finally, OF provides emotionality testing by quantifying locomotion and exploration as markers of anxiety.[31] EPO3 animals were the only ones to demonstrate increased exploration and reduced anxiety in adulthood. While few previous studies focused specifically on the role of EPO on anxiety-related behavior, EPO has been shown to reduce exploratory impairments and anxiety in different models of mood disorders and hypoxia.[32,33]

We previously showed that EPO increases neurogenesis and MSC reduces histological damage,[11,13] but there is still a large gap in our knowledge regarding the mechanisms of repair. For example, VEGF administration delayed by 3 days enhances angiogenesis and recovery after neonatal stroke,[21] while early VEGF administration worsens injury in adult ischemia.[34] The reasons for these temporal or age-related differences are not clear, and understanding the specific pathways involved in injury and repair is vital to determining the most effective delayed therapeutic strategies. EPO binding to EPO-R leads to phosphorylation and activation of a number of downstream pathways, including the PI3K (phosphatidylinositol 3-kinase)/Akt (protein kinase B) pathway that limits inflammation and cell death while enhancing blood vessel formation and neural precursor proliferation.[35] NO is synthesized from NOS (NO synthase) and plays roles in cerebral perfusion and synaptic plasticity. There is increased eNOS (endothelial NOS) expression/activation in EPO overexpressing mice[36] and increased expression of eNOS from MSC-derived endothelial progenitor cells.[37] EPO-treated MSCs also appear to secrete proangiogenesis factors more readily than untreated MSCs in in vitro models.[15] While there was a subtle benefit in brain volume with early administration of cell-free CCM from MSC/EPO, this did not result in behavioral improvement. In this study, we focused on long-term changes in behavior and brain volume, but cell type–specific effects on endothelial, neuronal, glial, and immune subtypes, as well as the role of secreted factors and growth factor release, will need to be investigated to confirm long-term histological improvement and to focus mechanistic studies. In addition, the effects of additional MSC dosing on long-term function in this model are not known.

This study is a first step in determining the efficacy of a single-dose, cell-based combination strategy for a common cause of perinatal brain injury and comparing to an alternative systemic treatment regimen undergoing clinical trials. While efficacy was demonstrated with standard MSC, there was more benefit with MSC/EPO and EPO3 treatment. Stimulating the EPO pathway directly may enhance not only cell type–specific changes but also circuit and network formation critical for neurodevelopment. It is possible that EPO represents a single common pathway for long-term repair. The relative risk/benefit of administering enhanced MSC, as opposed to multiple systemic doses of a drug currently in use and being studied, is not clear. The specific mechanisms and pathways to maximize long-term protection and repair remain to be determined.

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Affiliation

Department of Pediatrics (A.L., P.P., S.O., A.R., D.F., F.F.G.) and Department of Neurology (D.F.), University of California, San Francisco.

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Disclosures

None.

Supplemental Materials

Figure I
Tables I and II

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