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### Authors

Larphaveesarp, Amara  
Georgevits, Margaret  
Ferriero, Donna M  
et al.

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## Delayed Erythropoietin Therapy Improves Histological and Behavioral Outcomes after Transient Neonatal Stroke

Amara Larphaveesarp, BS<sup>1</sup>, Margaret Georgevits, BS<sup>1</sup>, Donna M. Ferriero, MD<sup>1,2</sup>, and Fernando F. Gonzalez, MD<sup>1</sup>

<sup>1</sup>Department of Pediatrics, University of California San Francisco, San Francisco, CA, 94143, United States

<sup>2</sup>Department of Neurology, University of California San Francisco, San Francisco, CA, 94143, United States

### Abstract

**BACKGROUND and PURPOSE**—Stroke is a major cause of neonatal morbidity, often with delayed diagnosis and with no accepted therapeutic options. The purpose of this study is to investigate the efficacy of delayed initiation of multiple dose erythropoietin (EPO) therapy in improving histological and behavioral outcomes after early transient ischemic stroke.

**METHODS**—32 postnatal day 10 (P10) Sprague-Dawley rats underwent sham surgery or transient middle cerebral artery occlusion (tMCAO) for three hours, resulting in injury involving the striatum and parieto-temporal cortex. EPO (1000 U/kg per dose × 3 doses) or vehicle was administered intraperitoneally starting one week after tMCAO (at P17, P20, and P23). At four weeks after tMCAO, sensorimotor function was assessed in these four groups (6 vehicle-sham, 6 EPO-sham, 10 vehicle-tMCAO and 10 EPO-tMCAO) with forepaw preference in cylinder rearing trials. Brains were then harvested for hemispheric volume and Western blot analysis.

**RESULTS**—EPO-tMCAO animals had significant improvement in forepaw symmetry in cylinder rearing trials compared to vehicle-tMCAO animals, and did not differ from sham animals. There was also significant preservation of hemispheric brain volume in EPO-tMCAO compared to vehicle-tMCAO animals. No differences in ongoing cell death at P17 or P24 were noted by spectrin cleavage in either EPO-tMCAO or vehicle-tMCAO groups.

**CONCLUSIONS**—These results suggest that delayed EPO therapy improves both behavioral and histological outcomes at one month following transient neonatal stroke, and may provide a late treatment alternative for early brain injury.

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Corresponding author and address: Fernando F. Gonzalez MD, 1550 4<sup>th</sup> St, Room RH-382C, Campus Box 2711, San Francisco, CA 94158, Tel: (415) 502-8932, Fax: (415) 514-0235, Fernando.Gonzalez@ucsf.edu.

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**Disclosures:** None

## Introduction

Stroke during the neonatal period is a significant cause of death and long-term disability, occurring in as many as 1 in 2300 live births<sup>1</sup>. Most survivors have long-term motor or cognitive dysfunction, yet despite these lifelong effects no accepted post-injury treatment exists. In addition, many cases are identified outside of the perinatal period, which further complicates therapeutic strategies as effective treatments initiated remotely from the insult would be necessary to benefit many affected infants.

It is clear that injury continues to progress over a period of days to weeks following the primary insult<sup>2</sup>. This involves a variety of mechanisms and pathways that result in early necrosis and later programmed cell death, as well as decreased cell proliferation and altered cell fate. Of many potential therapies that have been studied in an effort to both suppress early cell death but also enhance later proliferation and repair, erythropoietin (EPO) has shown promise in a number of brain injury models. EPO is a pleiotropic cytokine with a number of erythropoietic and non-erythropoietic roles<sup>3</sup>. EPO and EPO receptor (EPO-R) expression are elevated in the brain during gestation but decline rapidly after birth, with cell-specific endogenous EPO/ EPO-R upregulation after injury<sup>4</sup>. Following hypoxia there is stabilization of HIF-1, with increased expression of downstream targets and growth factors that include EPO and VEGF<sup>5, 6</sup>. This results in specific expression of EPO and its receptor on neurons, astrocytes and microglia at different time points that initiate endogenous mechanisms for neuroprotection and repair<sup>4</sup>. Initiation of these intracellular processes lead to anti-apoptotic, anti-inflammatory and pro-angiogenic effects, and play a significant role in neurogenesis and cell fate outcome<sup>7</sup>.

We have previously described a non-hemorrhagic ischemia-reperfusion stroke model in the immature rat using transient middle cerebral artery occlusion (tMCAO)<sup>8, 9</sup>. This is similar to the most common cause of stroke in the perinatal period<sup>10, 11</sup>. We have demonstrated increased cell proliferation and migration from the subventricular zone (SVZ), with altered cell fate favoring newly born neurons and oligodendrocyte precursors in the injured brain following EPO treatment<sup>9</sup>. Single dose EPO therapy given immediately following tMCAO preserved short-term histological and sensorimotor outcomes<sup>12, 13</sup>, while three doses administered over a 1-week period were required for long-term improvement in both histologic brain volume and cognitive function<sup>14</sup>.

Identification and diagnosis of perinatal stroke is often delayed in neonates, who initially present with seizures<sup>1</sup>. Given the anti-apoptotic, pro-angiogenic and neurogenic effects of EPO in ischemia models, and the knowledge that the injury continues to evolve beyond the acute phase of ischemia, the question arises regarding the benefits of EPO initiated at more remote time periods. While multiple dose EPO has demonstrated long-term benefit in this model when initiated immediately following occlusion, and hypothermia has demonstrated benefit for hypoxic-ischemic injury within a tight therapeutic window<sup>15</sup>, late treatment alternatives for ischemic brain injury do not exist. For this reason, we examined the efficacy of a three dose exogenous EPO regimen initiated at one week following stroke, hypothesizing that treatment starting at a late time point would still have significant histological and functional benefit.

## Materials and Methods

The protocol for this study received approval from the University of California, San Francisco Institutional Animal Care and Use Committee, and all studies were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals. Every effort was made to minimize animal suffering and to reduce the numbers of animals used.

### Transient Middle Cerebral Artery Occlusion

Postnatal day 10 (P10) Sprague-Dawley rats, each weighing 19–21g, underwent focal ischemia-reperfusion with transient right middle cerebral artery occlusion (tMCAO) for three hours, or sham surgery<sup>8, 9</sup>. This age was chosen to approximate development of the full-term human newborn brain<sup>16</sup>. Female rats with 7-day old litters (approximately 10 pups per litter) were purchased from Simonson Labs (Gilroy, CA, USA). Mothers were housed in a temperature and light-controlled facility and given ad libitum access to food and water until pups were 10 days old. tMCAO or sham surgery was performed in spontaneously breathing animals anesthetized with 3% isoflurane in 100% O<sub>2</sub>. Following induction, rectal temperature was monitored and maintained at 36°–37°C with a combination of heating blanket and overhead light until recovery from anesthesia. The right internal carotid artery (ICA) was dissected and a temporary ligature was tied using a strand of 6-0 suture at its origin. This ligature was retracted laterally and posteriorly to prevent retrograde blood flow. A second suture strand was looped around the ICA above the pterygopalatine artery and an arteriotomy was made proximal to the isolated ICA. A silicone coated 6-0 nylon filament from Doccol Corporation (Sharon, MA, USA) was inserted 9–10.5 mm (based on animal weight) to occlude the MCA and the second suture strand was tied off to secure the filament for the duration of occlusion. Following recovery from anesthesia, pups were returned to their dam for the duration of the occlusion. Injury was confirmed by severe left frontal/hindlimb paresis resulting in circling movements during the occlusion period. We have previously demonstrated a consistent pattern of injury involving the striatum and parieto-temporal cortex with this model using MRI during occlusion and TTC staining at 24 hours following tMCAO<sup>9, 13, 14</sup>. For reperfusion, each animal was anesthetized and all suture ties and the occluding filament were removed. Avitene Microfibrillar Collagen Hemostat (Warwick, RI, USA) was placed over the arteriotomy and the skin incision was closed. Sham animals were anesthetized and the ICA was dissected, after which the skin incision was closed. At the time of reperfusion, the sham animals were once again anesthetized for 5 minutes, equivalent to the reperfusion procedure time for tMCAO animals. 20 animals underwent tMCAO and 12 animals received sham surgery. Animal sex was equally distributed amongst the four groups, and there were no deaths.

### Erythropoietin Treatment

Rats were treated with intraperitoneal (IP) doses of EPO (1000 units/kg) at three time points: 7 days (P17), 10 days (P20), and 13 days (P23) after injury. Sham animals received vehicle (0.1% BSA) IP at these same time points. Weight was monitored for one week following tMCAO or sham surgery to ensure adequate weight gain. There were four experimental

groups: vehicle-sham (n=6), EPO-sham (n=6), vehicle-tMCAO (n=10), and EPO-tMCAO (n=10).

### Behavioral testing

Cylinder rearing was used to assess the effects of ischemic injury and EPO treatment on forelimb use as a function of sensorimotor bias. Animals with unilateral ischemic brain injury exhibit forelimb preference shown by favoring use of the non-impaired limb for touching or bracing the side of the cylinder, and rats are capable of exploring the walls of the cylinder as early as P21<sup>17</sup>. Forelimb movements for each rat were analyzed during exploratory activity in a transparent Plexiglas cylinder measuring 20-cm in diameter and 30-cm in height in two trials conducted on consecutive days at ~4 weeks after tMCAO (P37 and P38). The size of the cylinder allowed free movement but was small enough to encourage exploration and touches/braces onto the side of the cylinder, while its height prevented the rat from reaching the top edge and its heavy weight prevented its movement during braces. Animals were handled for about 5 minutes per day for three days prior to testing. Each animal was then individually placed in the cylinder in a quiet room without distinctive markings and observed for three minutes in each trial, with results averaged per animal. Initial forepaw placement of each weight-bearing contact with the wall was recorded as right, left, or both forepaws<sup>18</sup>. Results were expressed as the percentage use of the non-impaired (right) forepaw for braces relative to the total number of forepaw initiations. The results were analyzed by two independent raters and the average scores of the two raters blinded to group were used for data analysis.

### Histology

Immediately following behavioral testing at P38, animals were anesthetized with sodium pentobarbital (100 mg/kg; Nembutal, Abbott Labs, Abbott Park, Ill., USA) and sacrificed. Brains were harvested by transcardiac perfusion with 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered saline (pH 7.4). Brains were carefully removed and postfixed overnight, equilibrated in 30% sucrose in 0.1M PBS and left at 4° C in 0.1M PBS until sectioning and staining. The olfactory bulbs and cerebellum were removed, and the entire brain was sectioned at 50- $\mu$ m intervals on a sliding microtome (Thermo Scientific, Waltham, MA, USA). The mounted sections were air-dried, stained with cresyl violet, dehydrated in graded ethanol solutions, cleared in Citrisolv (Fisher Scientific, Pittsburgh, PA, USA) and cover slipped in Permout (Fisher Scientific).

### Stereological Volumetric Analysis of Brain Volumes

Using systematic random sampling, a series representing every 12<sup>th</sup> section was selected, cresyl violet stained, and analyzed. Sections encompassed the whole brain rostrally from the genu of the corpus callosum through the posterior portion of the hippocampus to the occipital lobes caudally. All volumetric quantifications were performed in a blinded manner on a Zeiss AxioScope Imager Z.2 (Zeiss Inc., Thornwood, NY) with a motorized XYZ axis computer-controlled stage, and NeuroLucida and Stereoinvestigator software (MicroBrightField Inc., Colchester, VT, USA). When calculating the volume, the cross-sectional area of the region of interest (ROI) was calculated according to the Cavalieri principle<sup>19</sup>. For the ROIs, the right (ipsilateral) and left (contralateral) hemispheres were

traced. Damage secondary to stroke was determined quantitatively by calculating the percent volume of the ipsilateral, or lesioned, hemisphere versus the contralateral, control hemisphere.

### Western Blot Analysis

Western blot analysis was performed in lysates obtained from ipsilateral injured and control cortices collected at P11 (24 hours following tMCAO), P17 and P24. Fresh-frozen tissue was homogenized in ice-cold buffer containing 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L sodium orthovanadate, 1 µg leupeptin, and 1 mmol/L Pefablok, and homogenates were centrifuged at 12000g for 10 minutes. Protein concentration was normalized in supernatant from each brain sample (Pierce kit). Samples were boiled for 5 minutes, subjected to SDS-PAGE (20 µg of protein per lane), and transferred to nitrocellulose (Amersham, Marlborough, MA). Blots were rinsed with 1× Tris-buffered saline (TBS) and 0.1% Tween (TTBS), blocked with 5% milk/TBST for 1 hour, and probed with polyclonal mouse anti-spectrin (1:1000, overnight, Millipore, Billerica, MA) antibody and normalized to B-actin expression in the same samples (1:5000, Thermofisher). Secondary horseradish-peroxidase-conjugated antibodies (1:3000, 1 hour, room temperature, Cell Signaling, Danvers, MA) were used, and signal was visualized with ECL (Amersham).

### Data Analysis

Data are presented as mean ± SD. For statistical analysis, nonparametric methods were used. Significance was set at  $p < 0.05$ . The Kruskal-Wallis test was applied for comparisons of multiple groups followed by the Wilcoxon rank sum test including Bonferroni correction for comparison between two groups. Evaluation of the relationship between the hemispheric volume ratio, as well as the percentage of non-impaired forepaw initiation was performed by linear regression. All statistical analyses were done using SAS Enterprise Guide, version 5.0 (SAS Institute, Cary, NC, U.S.A.).

## Results

### Delayed EPO Therapy after Transient Neonatal Stroke Improves Brain Volume

A total of 32 rats underwent tMCAO for a three hour occlusion period, resulting in ipsilateral hemispheric damage and associated acute behavioral changes, or sham surgery. The rats were allocated to either vehicle treatment following sham surgery (vehicle-sham, n=6), EPO treatment following sham surgery (EPO-sham, n=6), vehicle treatment following tMCAO (vehicle-tMCAO, n=10), or EPO treatment following tMCAO (EPO-tMCAO, n=10). EPO (1000 U/kg) or vehicle treatment was initiated on post-tMCAO day 7 (P17), with additional doses administered at 72 hour intervals (P20 and P23) for three doses total [Fig. 1].

Animals that underwent tMCAO had significant tissue loss at four weeks following stroke (P38) [Fig. 2]. In animals that underwent sham surgery, there was no difference in hemispheric brain volume between vehicle and EPO treatment. In the vehicle-treated tMCAO group, there was a significant decrease in the ipsilateral/contralateral hemispheric

brain volume ( $p < 0.001$  vs. sham groups). In the EPO-treated tMCAO group, delayed EPO treatment significantly preserved hemispheric brain volume on the ipsilateral side compared to vehicle-treated tMCAO animals ( $p < 0.001$  vs. vehicle-tMCAO), but this group still had a significant decrease compared to sham animals.

### **Delayed EPO after Transient Neonatal Stroke Improves Sensorimotor Function**

To evaluate the sensorimotor deficit, the percentage of weight-bearing episodes (braces) onto the side of the cylinder that were initiated with the non-impaired (ipsilateral), impaired (contralateral), and both forepaws simultaneously were calculated in each animal at four weeks after tMCAO [Fig. 3]. Sham animals primarily showed symmetrical use of their forepaws when bracing, with the percentage of ipsilateral and contralateral paw use around 30%.

Vehicle-treated tMCAO animals showed marked asymmetry in forepaw preference, favoring the non-impaired forepaw when bracing the side of the cylinder ( $p < 0.001$  vs. sham groups). Delayed EPO treatment significantly improved this asymmetry in tMCAO animals ( $p < 0.001$  vs. vehicle-tMCAO). In addition, EPO-treated tMCAO animals did not differ from either sham group in forepaw preference ( $p = 0.19$  vs. vehicle-sham;  $p = 0.16$  vs. EPO-sham). We then compared the ratio of ipsilateral to contralateral hemisphere volume with the percentage of non-impaired limb contacts in the cylinder rearing test, and found a direct inverted linear relationship with coefficients of simple determination ( $r^2$ ) of 0.395.

### **Delayed EPO therapy has no effect on ongoing cell death at 7 days and 14 days following tMCAO**

The structural protein spectrin is cleaved during acute brain injury by both caspase-3-dependent and calpain-dependent mechanisms<sup>20,21</sup>. At 24 hours after tMCAO there was presence of both caspase-3-dependent (120-kD) and calpain-dependent (160-kD) products of spectrin degradation in injured cortex, but there were no differences between any group in spectrin cleavage at 7 days (P17) and 14 days (P24) following tMCAO [Fig. 4].

## **Discussion**

This study shows for the first time that delayed EPO therapy, initiated one week after transient neonatal stroke, preserves brain volume and behavioral function when compared to vehicle-treated animals. EPO therapy started at seven days (P17) after tMCAO performed at full-term equivalent age (P10), and administered at 72-hour intervals (P20 and P23) for three doses total, resulted in improved sensorimotor function as measured by forepaw preference at four weeks after stroke. EPO-treated animals did not differ from sham animals in sensorimotor function, and their functional performance correlated with hemispheric brain volume after stroke.

The literature regarding EPO treatment in neonatal rodent brain injury is quite heterogeneous regarding timing and dosage. Some studies have demonstrated short-term benefit of single dose EPO (500–1000 U/kg)<sup>22</sup>. Another study comparing high-dose EPO protocols reported benefit with doses ranging from 5000 to 30000 U/kg, with most benefit arising from multiple doses of 5000 U/kg<sup>23</sup>. We have previously demonstrated that single-



dose EPO (5000 U/kg) given immediately after injury significantly preserved hemispheric brain volume and improved behavioral function in the short-term, but this effect did not persist in animals that reached adulthood<sup>12, 14</sup>. In contrast, animals that received three doses of EPO over a one week period (each 1000 U/kg, with the first dose given at the time of reperfusion) did demonstrate long-term improvement in brain volume and cognitive function<sup>14</sup>. The total amount of EPO administered was less than the single dose protocol, suggesting the importance of timing of administration in pathways responsible for possibly both protecting or salvaging cells but also enhancing neurogenesis and long-term repair. Interestingly, the histological improvement in the current study is similar to those seen with early multi-dose administration. Early EPO may have greater effects on cell survival but may be unable to maintain intracellular signaling pathways or alter perfusion disturbances to generate long-lasting change.

Our goal in the current study was to identify a late therapeutic option for a common cause of neonatal brain injury that is often not diagnosed in the acute period<sup>24</sup>. We chose to initiate EPO starting at one week after tMCAO, the same time point as the final dose in our previously published multiple dose treatment protocol, in an effort to push the window out as far as possible while still providing a therapeutic benefit. This would follow the periods of primary and secondary energy failure and initial stages of cell injury and death that occur during the acute post-injury period, but where the later stages of injury continue to evolve. Other studies have demonstrated ongoing damage and tissue loss in animal models of hypoxia-ischemia at later time points following injury<sup>25, 26</sup>, an injury pattern distinct from tMCAO in that there is permanent occlusion of the carotid artery without a reperfusion phase resulting in a more global injury pattern<sup>20</sup>. Brain injury and cell death is an ongoing process, and penumbral injury may result in eventual cell loss over a prolonged period of time due to disruption of intracellular signaling pathways, receptor expression, growth factor release or other structural or paracrine factors that result in eventual degradation of tissue. We investigated the role of ongoing cell death at later time points in this model of tMCAO by examining caspase-3-dependent and calpain-dependent cell death via spectrin cleavage during the acute injury period, at initiation and at completion of EPO or vehicle treatment. While there was a significant increase in spectrin cleavage at 24 hours following tMCAO in the injured cortex, there was no significant difference between tMCAO and sham groups at P17 and P24. While this does not completely exclude later cell death and tissue degradation, and more specific local effects in the penumbra cannot be ruled out, there were no significant differences between EPO and vehicle-treated animals at these later time points.

This model of tMCAO is distinct from hypoxia-ischemia, or the Rice-Vannucci model<sup>27</sup>, because there is transient focal ischemia without systemic hypoxia, followed by a reperfusion phase when the obstruction is removed and blood flow is restored. Reperfusion is an important component of injury progression in stroke, with increased excitotoxicity, free radical formation, and nitric oxide production leading to delayed cell death. This model enables us to evaluate the effects of neuroprotective therapies on different behavioral and histological outcomes in the injured core and peri-infarct cortex. In addition, this model is similar to the most common cause of stroke in neonates, a transient occlusive thrombus that may occur during or prior to the perinatal period<sup>10, 28</sup>. Magnetic resonance imaging studies in human neonates have demonstrated that the onset of injury is usually around the time of



delivery, with tissue breakdown continuing through 6 weeks of age<sup>29</sup>. The etiology is not entirely clear, and most likely multifactorial, but it is presumed in most cases to result from emboli passing from the placenta<sup>10</sup>. In addition, many newborns with presumed perinatal stroke often present with later seizure activity or subtle focal neurological exam abnormalities<sup>24</sup>. This makes the identification of late therapeutic options that can enhance repair and improve outcomes crucial.

We did find that three doses of EPO starting one week post-tMCAO significantly improved histological outcomes. Furthermore, these histological improvements correlated with sensorimotor function at four weeks after stroke. Cylinder rearing is a validated test for sensorimotor performance in brain ischemia models, and is sensitive to both mild and severe deficits of different chronicity<sup>30–32</sup>. Rats that experience unilateral brain injury exhibit ipsilateral (non-impaired) forepaw weight-bearing preference as early as P21<sup>17</sup>. Motor function, specifically hemiparesis, is a major lasting deficit in this model, and animals display a clear forelimb preference according to severity of injury, with increasing asymmetry in forelimb use.

The improved outcomes associated with prolonged, multiple-dose administration of EPO for brain injury may be related to upregulation of EPO-R, synthesis of proteins or activation of cascades that results in a number of beneficial effects. The cellular mechanisms and time course by which EPO exerts neuroprotection and repair are complex and not completely understood. Binding of EPO to EPO-R leads to phosphorylation and activation of Janus Kinase 2 (JAK2), which provides docking sites for intracellular signaling pathways phosphatidylinositol 3-kinase (PI3K) and Akt, STAT5, and extracellular signal-regulated kinase ERK<sup>7</sup>. These affect a number of downstream targets that decrease cell death and limit inflammation. Importantly, EPO enhances neurogenesis and direct multipotent neural stem cells toward a neuronal cell fate<sup>13, 33, 34</sup>. EPO directly stimulates growth factor release<sup>33</sup>, which may play a significant role in the neurogenic and angiogenic effects and lead to long-term plasticity and repair. So EPO may provide early neuroprotective effects but also trophic effects that last well beyond the acute period. However, in untreated or single-dose treated tMCAO animals these newly differentiated neurons do not survive long term, possibly from a lack of paracrine support<sup>35, 36</sup>. Single dose therapy administered during the acute stages of injury may be insufficient to overcome endogenous mechanisms at these later time points. Later doses of EPO may be critical for the continued proliferation, differentiation, incorporation and survival of these cells. While we did not investigate cell proliferation or cell fate in the current study, we previously demonstrated increased cell proliferation in neural precursors in the SVZ with three doses of EPO initiated at the time of reperfusion<sup>9</sup>, with increased production and migration of immature neuroblasts and oligodendrocyte precursors. Post-injury angiogenesis may also be necessary for long-term repair, and may be enhanced by EPO interaction with VEGF. EPO-treated adult rats have increased VEGF expression after stroke, with enhanced angiogenesis and neuroblast migration to injured regions<sup>33</sup>.

In the clinical setting, immediate initiation of EPO treatment at the time of stroke or hypoxia-ischemia is not possible for the majority of cases, and the importance of late treatment alternatives is clear. Other preclinical studies have demonstrated benefit of delayed

EPO therapy after brain injury. For example, Iwai et al. found that EPO initiated at 48 hours after neonatal hypoxia-ischemia in mice improved behavioral outcomes, enhanced neurogenesis and reduced white matter injury at 14 days<sup>37</sup>. More recently, Reitmeir et al. demonstrated improved function, with enhanced neurogenesis, angiogenesis and reduced scar formation when EPO was administered at 3 days following adult rodent stroke<sup>38</sup>. This included enhanced axonal sprouting and remodeling of perilesional tissues, as well as compensatory contralesional responses. The Iwai study did not demonstrate gross histological changes with treatment, and the Reitmeir study did not examine histological changes in volume, but these models also differed from ours in cause, severity of injury, or age of animal.

While initiation of EPO treatment delayed by one week demonstrated short-term benefits in this tMCAO model, it is difficult to equate one week in the rodent to human perinatal stroke or brain injury, and the equivalent therapeutic window in humans is not known. We chose to examine outcomes in rodents at one month following tMCAO as a first step in examining the efficacy of a delayed treatment protocol, and long-term behavioral studies will be necessary to prove long-term benefit. In addition, we made every effort to approximate normothermia during the procedure. While we did monitor the temperature of the rat pups until full recovery from anesthesia, we cannot exclude later alterations in temperature that may be associated with outcomes.

Some other treatment strategies have suggested benefit with delayed initiation for early brain injury in preclinical models, and these include environmental enrichment and stem cell administration. Beneficial effects of mesenchymal stem cells have been observed when transplanted anywhere from 3 hours to 10 days after the onset of injury<sup>39, 40</sup>. Interestingly, transplantation is followed by apoptosis of the majority of transplanted cells, while endogenous neurogenesis continues to be enhanced, suggesting a paracrine/growth factor effect. Multiple injections appear to be more beneficial than single injections, but the exact mechanisms are still not clear. Injection at day 3 stimulated cell proliferation and differentiation, while injection at day 10 stimulated axonal remodeling, and the temporal-specific effects may be similar with EPO, effecting neuronal plasticity and therefore cognitive development and function in the long-term.

In humans, EPO therapy for hypoxic-ischemic encephalopathy in full-term and near term infants has been studied in a few clinical trials which have suggested benefit with early initiation<sup>41, 42</sup>. Additional clinical trials evaluating safety and pharmacokinetics of high-dose EPO therapy in combination with hypothermia have not shown adverse events<sup>43</sup>, and a dose of 1000 U/kg achieved plasma serum concentrations that most closely approximated the neuroprotective levels in animal models<sup>44</sup>. More recently, a phase I trial of three EPO doses for neonatal stroke demonstrated safety when initiated within 4 days of birth<sup>45</sup>, but the efficacy of this therapy and the therapeutic window for initiation need to be clarified.

In conclusion, this study shows that delayed initiation of multiple dose EPO therapy improves both morphological and behavioral outcomes at one month following rodent neonatal stroke. Taken together with previous studies, our observations suggest that EPO may be useful as a late therapeutic option. The effect of continued therapy past the second

week, in combination with other neuroprotective or neuroregenerative therapies, or in different causes or different models of early brain injury requires further study.

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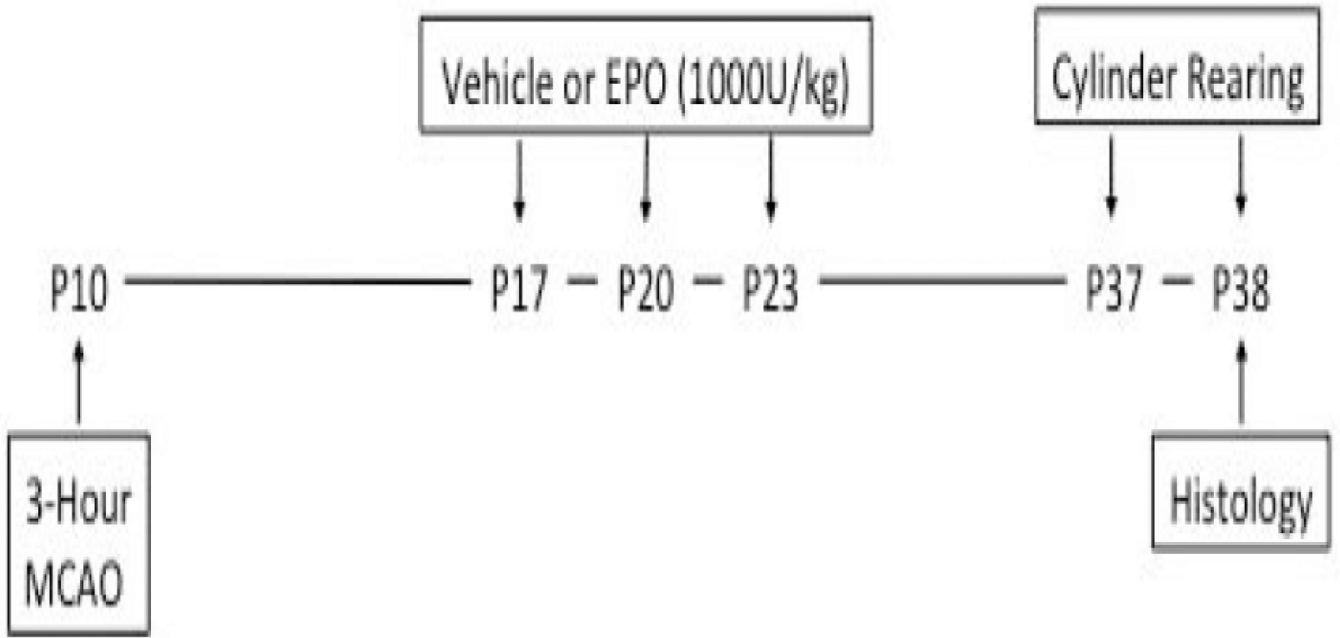
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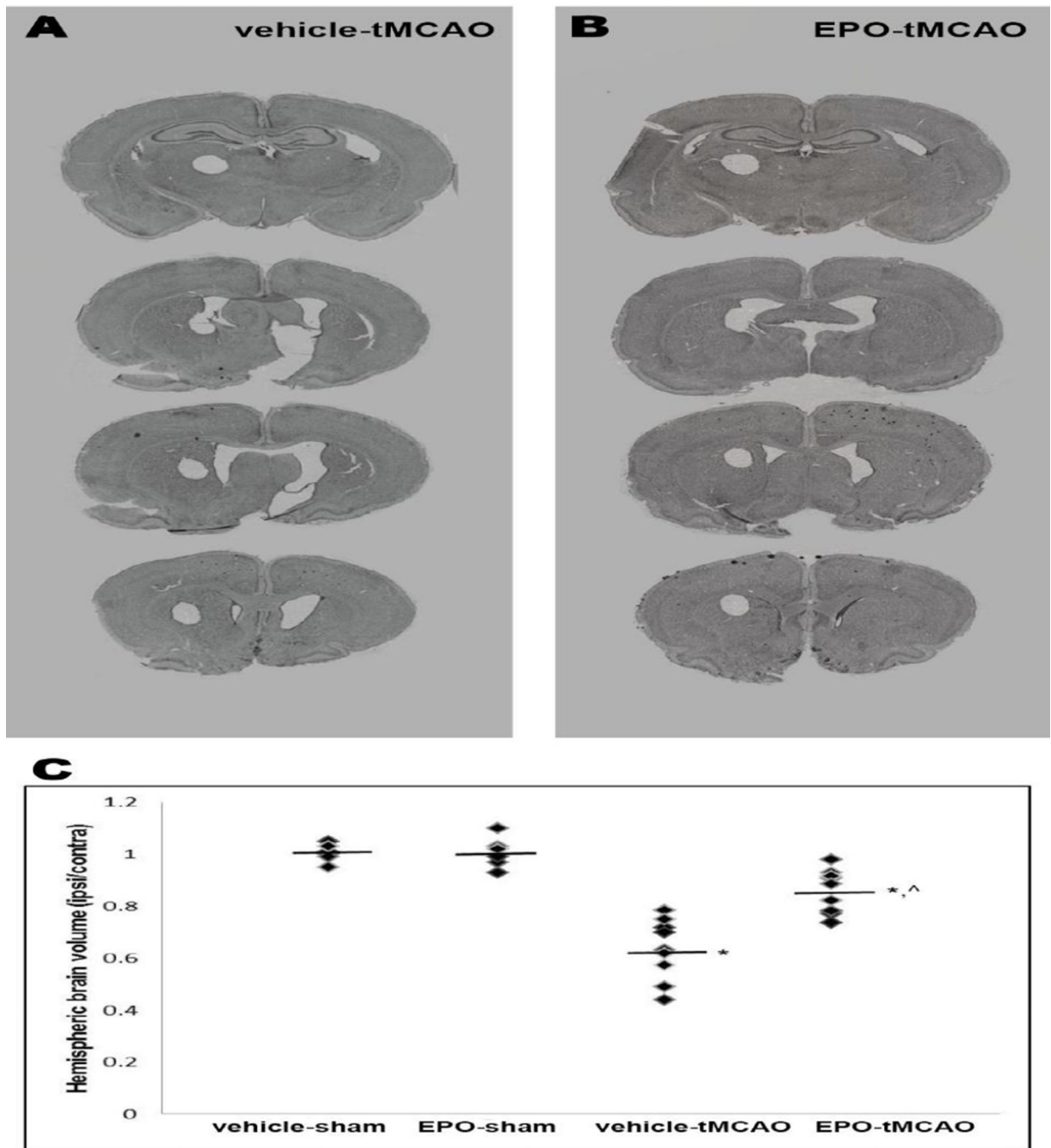
### Highlights

- Transient focal ischemia-reperfusion is a common cause of early brain injury.
- Delayed initiation of multiple dose erythropoietin therapy improves histological and functional outcomes after early transient stroke.
- Erythropoietin may provide a late treatment option for early brain injury.



**Figure 1.**  
Experimental study design





**Figure 2.**

Stereological volumetric quantification of brain injury at four weeks after tMCAO. Examples of posterior to anterior injury pattern seen in vehicle-tMCAO (A) and EPO-tMCAO (B) animals at this time point following tMCAO, demonstrating injury primarily involving striatum and parietotemporal cortex but also involving hippocampus. Small left hole in left hemisphere represents contralateral hemisphere identifier. tMCAO at P10 caused a significant reduction in percentage hemispheric volume (ipsilateral/contralateral ratio) four weeks later (C). Delayed EPO treatment significantly improved ipsilateral volume, which

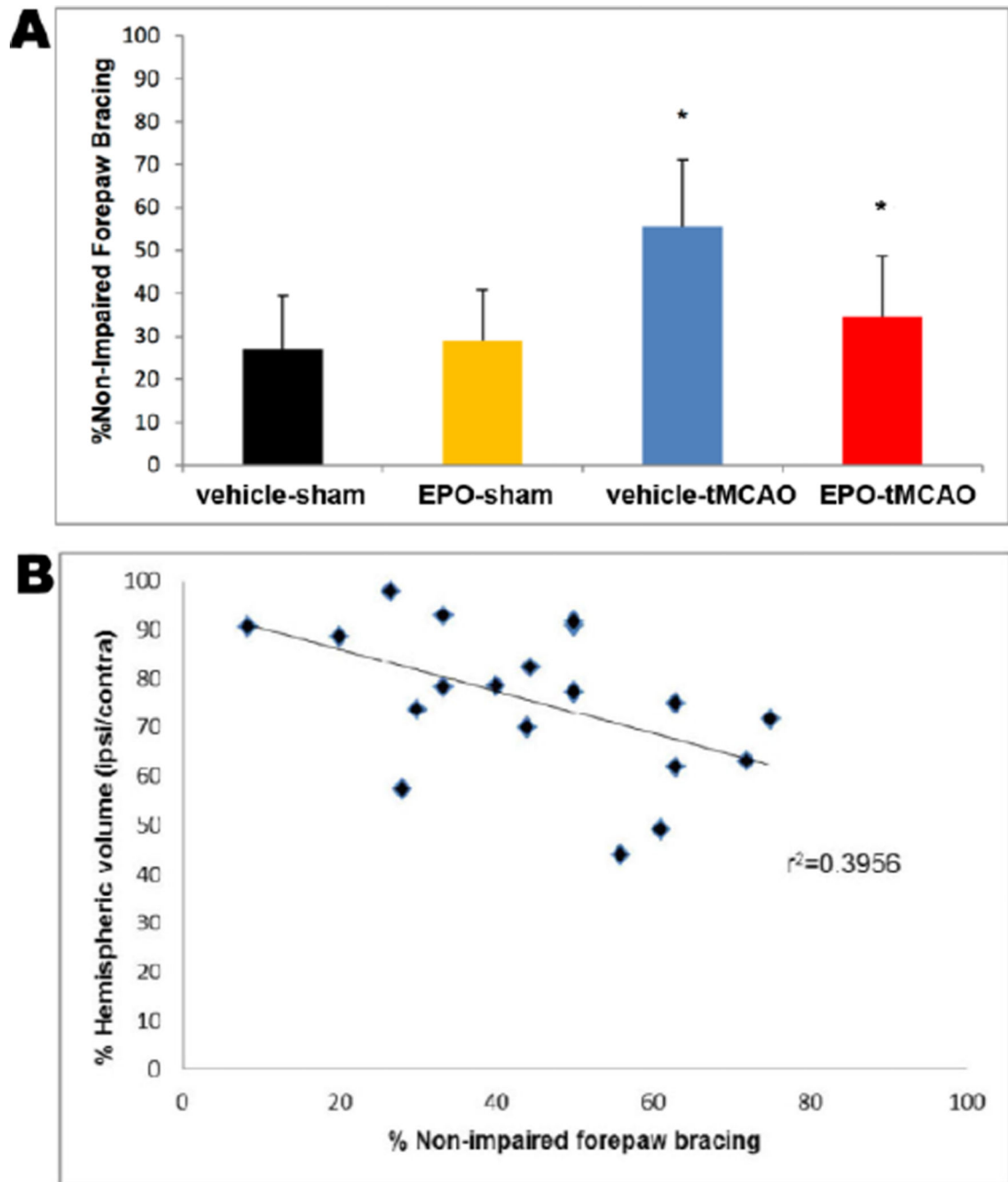
was still decreased compared to vehicle-sham and EPO-sham groups (\*  $p < 0.001$  vs. shams, ^  $p < 0.001$  vs. vehicle-tMCAO). Data shown as mean  $\pm$  SD;  $n=6$  for sham groups,  $n=10$  for tMCAO groups.

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**Figure 3.**

Behavioral testing at four weeks after tMCAO (P37 and P38). Percentage of weight-bearing contacts or braces onto cylinder side initiated with the non-impaired forepaw in each of four animal groups was averaged over two trials for each animal (*A*). Vehicle-tMCAO animals showed marked forelimb asymmetry by demonstrating preferential use of non-impaired (right) limb. EPO treatment significantly decreased this functional asymmetry after tMCAO, and EPO-tMCAO animals did not differ from shams. Regression analysis (*B*) of percentage of non-impaired limb bracing in cylinder rearing and percentage hemispheric brain volume

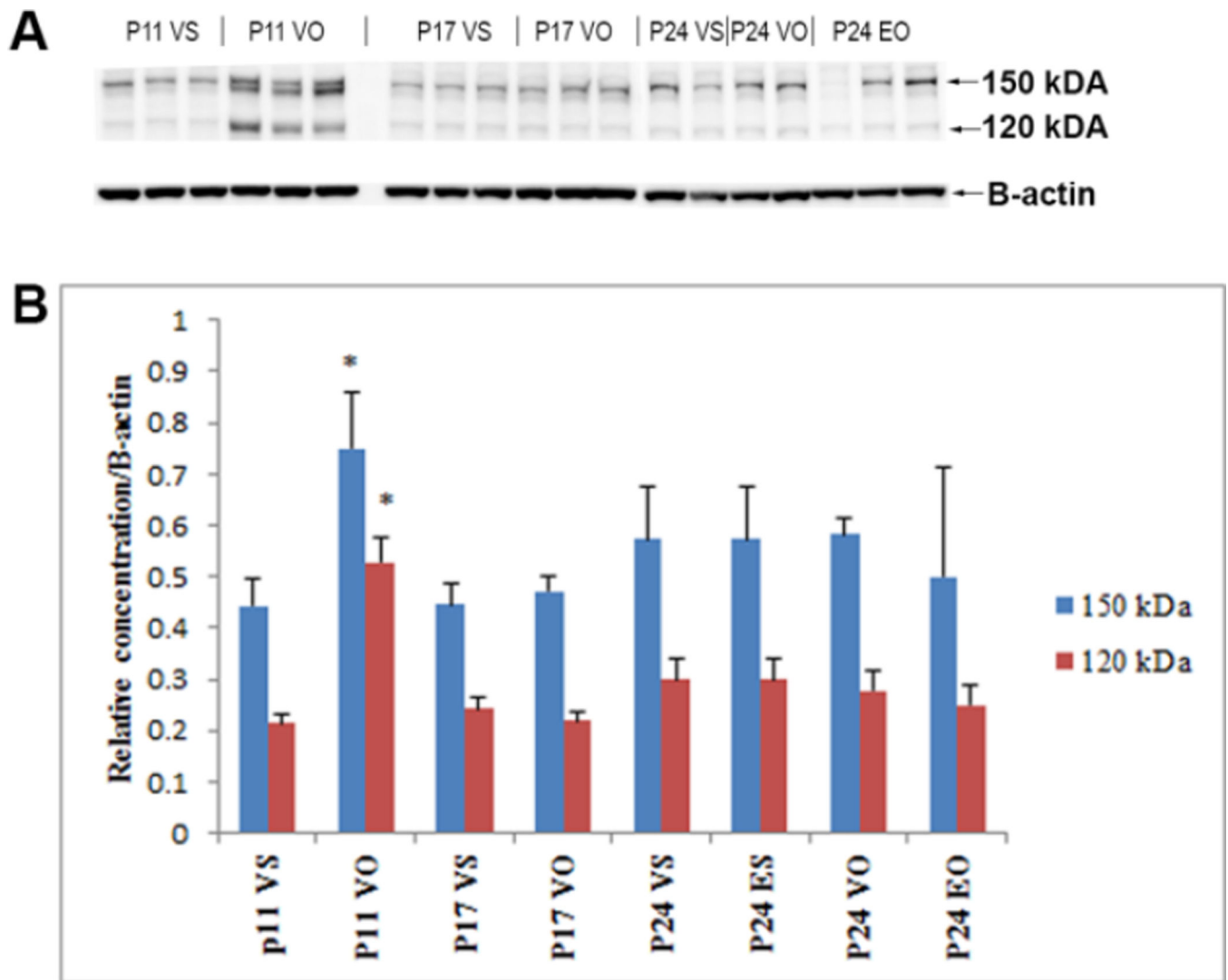
(ipsilateral/contralateral ratio) shows a direct inverse relationship between forelimb use and hemispheric brain volume ( $r^2= 0.395$ ,  $p=0.003$ ).

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**Figure 4.**

Western blot analysis of spectrin cleavage following tMCAO. tMCAO considerably increases spectrin cleavage 24 hours following injury. (A) A Western blot from ipsilateral cortex of representative animals demonstrates spectrin cleavage by caspase-3 (120 kD) and calpain (150 kD). (B) Protein quantification normalized to B-actin demonstrates significant increase in spectrin cleavage at 24 hours, no differences noted in other groups or later time points. (VS= vehicle-sham, ES= EPO-sham, VO= vehicle-tMCAO, EO= EPO-tMCAO; each lane represents different individual animal. \*  $p < 0.001$  vs. sham,  $n = 3$  per group per time point).