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Erythropoietin in Stroke Therapy: Friend or Foe

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Abstract

Recombinant human erythropoietin (rhEPO), over the past decade, was hailed as an auspicious therapeutic strategy for various types of brain injuries. The promising results from experiments conducted in animal models of stroke led to a hurried clinical trial that was swiftly aborted in Phase II. The multiple neuroprotective modalities of rhEPO failed to translate smoothly to human adult ischemic brain injury and provided limited aid to neonates. In light of the antithetical results, several questions were raised as to why and how this clinical trial failed. There was bolstering evidence from the preliminary studies that pointed to a bright future. Therefore, the objective of this review is to address these questions by discussing the signaling pathways of rhEPO that are reported to mediate the neuroprotective effect in various animal models of brain injury. Major biomedical bibliographical databases (MEDLINE, ISI, PubMed, and Cochrane Library) were searched with the use of keywords such as erythropoietin, stroke, neonatal hypoxia ischemia, intracerebral hemorrhage, etc. This article will discuss the confounding factors that influence the efficacy of rhEPO treatment hence challenging its clinical translatability. Lastly, rhEPO may still be a promising therapeutic candidate for neonates in spite of its shortcoming in clinical trial if caution is taken with the dose and duration of its administration.

Keywords

Erythropoietin signaling; janus kinase 2; signal transducers and activators of transcription 3

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

INTRODUCTION

Stroke is the altering of brain blood supply either by the rupturing or obstruction of a vessel (WHO). The manner in which the blood supply is interrupted determines the category of stroke. Strokes can be hemorrhagic or ischemic. Hemorrhagic strokes are less common but more detrimental than ischemic strokes. Alternately, a hemorrhagic stroke could lead to an ischemic stroke (ASA; WHO). Thus, we will focus primarily on cerebral ischemia. The two main types of stroke can be further broken down into subcategories depending on the region affected and clinical manifestation. Subarachnoid hemorrhage, intracerebral hemorrhage, global ischemia, focal ischemia, neonatal hypoxia ischemia were all modeled in laboratory animals and was attenuated by erythropoietin (EPO) treatment. Systemic administration of a single or multiple doses of recombinant EPO pre- or post- insult was shown to confer neuroprotection in subarachnoid hemorrhage [1–4], intracerebral hemorrhage [5, 6], global ischemia [7–9], focal ischemia [10, 11], neonatal hypoxia-ischemia [12, 13], traumatic brain injury [14, 15] and even spinal cord injury [16], Parkinson's disease [17] and multiple sclerosis [18]. Thus, EPO was hailed as the panacea in stroke therapy and rapidly advanced to clinical trials.

Clinical trials for the use of EPO in SAH treatment yielded inconclusive results due to a small population size; but showed promising results in Phase I for the treatment of neonatal hypoxia-ischemia and adult cerebral ischemia [19]. These findings served to bolster confidence in EPO's potential for stroke therapy and accelerated its advance to Phase II clinical trial [20]. Hence, it was a resounding blow to EPO researchers and the stroke community at large when Ehrenreich and colleagues reported that EPO not only failed to improve injury but also promoted tumor formation and reduced quality of life for stroke patients in a multicenter clinical trial [21]. The failure of EPO in Phase II/III clinical trials raised several questions: Was the route of administration too traumatic? Was the potential seen in animal models over-interpreted/exaggerated? Were the primary mechanisms of action unclear? Or is EPO, like its primary regulator HIF-1 α a biphasic molecule, that is beneficial or detrimental depending on the dose and time of administration? EPO was previously administered intravenously for treatment of anemia over the past ten years with no major complication. Additionally Taylor and colleagues showed that there were no difference in the prevalence of thrombotic and hypertensive side effects between patients administered EPO intravenously compared to subcutaneously [22]. Thus if the route of administration affected the outcome it would have played a minor role in EPO's failure in Phase II/III clinical trial. A comprehensive review of EPO treatment in animal models of stroke with Meta analysis still predicts that EPO should show positive result in stroke therapy [23, 24]. Since two of the four critical questions were previously answered, we will focus on the critical non-hematopoietic functions of EPO and the primary mechanisms and mediators of EPO-induced neuroprotection.

ERYTHROPOIETIN REGULATION IN CEREBRAL HYPOXIA ISCHEMIA

Cerebral hypoxia ischemia promotes an increase of hypoxia inducible factor 1- α (HIF-1 α) in neurons. HIF-1 α has over 70 target genes most of which are upregulated in response to hypoxia [25]. HIF-1 α downstream gene EPO expression increases in mammalian brains

following hypoxia ischemia [26]. The expression of EPO and EPO-receptor (EPOR) is regulated by HIF-1 α . There is evidence showing an increase in both HIF-1 α and HIF-2 α [27]. However, our findings show that only HIF-1 α was upregulated in NGF-differentiated PC-12 cells following oxygen and glucose deprivation (OGD) and in the brain of hypoxic ischemic neonatal rats. HIF-1 α regulates both pro and anti-apoptotic genes [28, 29]. Our findings have shown that intraperitoneal injection of EPO inhibits HIF-1 α in a dose-dependent manner and is associated with increased prolyl hydroxylase (PHD)-2 expression and decreased ROS formation [29].

Erythropoietin and its Receptor

Hematopoietic growth factor, EPO is a physiological oxygen sensor that stimulates red blood cell (RBC) production and is released in response to low oxygen [30–32]. EPO secretion switches from the liver in the fetus to the kidney at approximately 2 years of age, through adulthood and is the primary regulator of erythropoiesis in mammals [33]. EPO is a part of the hypoxic response element and a known downstream gene of HIF-1 α . Under physiological conditions, serum EPO levels range from 4–27 mU/mL and many times is undetectable in adult serum [34]. However during hypoxia ischemia or asphyxia associated condition, there is more than a hundredfold increase in serum EPO, which in turn increases circulating reticulocytes, oxygen carrying capacity of the blood and overall tissue oxygenation [31, 32]. In addition to hematopoietic tissue, EPOR expression was observed on ovarian [35], testicular [36, 37], intestinal, pancreatic, cardiovascular [38–40] and neuronal tissues [41, 42]. Additionally, EPO is also associated with cellular delineation, and propagation as well as prevents/limits apoptosis [8, 11, 43]. Moreover, EPO treatment protects against blunt force trauma, excessive glutamate and NMDA, and inflammation in the brain [8, 11, 43]. Whether or not the non-hematopoietic functions of EPO are transmitted via the same receptor and signaling pathway as the hematopoietic functions is heavily debated. Casals-Pascual speculated that the erythroid functions are carried out via the homodimer form of the EPOR whereas cellular proliferation, survival and differentiation in non-hematopoietic cells are carried out by the β -receptor subunit (β cR) that is a heterodimer [44]. A clearer understanding of EPO signaling in hematopoietic and non-hematopoietic events is pertinent to the clinical future of EPO in stroke therapy. Should two different isoforms of EPOR be identified the signaling pathway still presents a challenge, because the current dogma states that EPO signals via the Janus Kinase (JAK)-2 pathway and its downstream signaling pathway in both events.

Signaling Pathways of Erythropoietin

EPO binds to the EPOR and phosphorylates the receptor associated tyrosine kinase, JAK-2 [45, 46]. Phosphorylated JAK-2 activates signal transducers and activators of transcription (STAT), which in-turn transcribes cytokines, growth, proliferation and differentiation factors [47–50].

Janus Kinases are receptor-associated tyrosine kinases, which phosphorylates the receptors allied with that kinase [47, 48]. The specific receptor attracts and phosphorylates different STAT [47–50]. The phosphorylated STAT protein is translocated to the nucleus where the gene transcription begins [47, 48]. EPO is known to signal via the JAK-2 tyrosine kinase

[51]. However, the specific STAT that is phosphorylated by each growth factor differs. STAT-3 is associated with EPOR activation [52] whereas STAT-6 and STAT-4 responds to interleukin (IL)-4 [50] and IL-12 [50], respectively. In addition to STAT-3 signaling EPO is also believed to signal via STAT-5, the more heavily studied STAT. Studies have shown that EPO-activated STAT-5 signals via the Ras mitogen activated protein kinase (MAPK), ERK-1/-2, and PI3K/Akt pathways [53]. STAT-3 is associated with propagation of growth in mature neurons and cell survival [54]. Studies have shown that STAT-3 causes an increase in tissue inhibitor of matrix metalloproteinase (TIMP)-1, which promotes cell survival, growth and differentiation [55, 56].

EPO is associated with increases in TIMP-1 mRNA and protein levels [55]. TIMP-1 is an endogenous inhibitor of MMP-9 that works by blocking the proteinase activity of MMP-9. The same pathways utilized in hematopoietic cells via EPO to promote differentiation, maturation, and survival of erythroid cells are utilized during EPO-induced neuroprotection. Thus EPO-induced cell survival is more dependent on EPOR expression than cell type.

Erythropoietin and the Brain

Endogenous EPO signaling is necessary for the development of the central nervous system and neuroprotection. Increased neuronal apoptosis was demonstrated in EPO-null and EPOR-null mice [57, 58]. EPO is regarded as an important regulator of the differentiation of neural progenitor cells (NPCs). Wang and colleagues previously demonstrated that EPOR expression and activation is critical to EPO's neuroprotective effects [59, 60]. EPOR expression on NPCs seems necessary for NPCs differentiation into mature neurons but less important in mature neurons. EPOR-null mice exhibited significant lower levels of NPCs in the subventricular zone compared to the wild-type counterparts. The transgenic mice were more susceptible to hypoxia ischemia and exhibit defect in NPCs maturation and migration to the ischemic penumbra [42]. In addition to its role in NPCs differentiation, maturation and migration, EPO and EPOR also play significant role in neuronal survival in different stroke models [61, 62].

Erythropoietin-Induced Neuroprotection *In vitro*

Cultured hippocampal and cortical neurons were protected against glutamate toxicity [63], oxygen and glucose deprivation (OGD) [64] and chemically induced hypoxia via EPO treatment [31]. Primary neuronal cultures exposed to OGD were protected via EPO administration in a dose- and time-dependent manner. EPO-induced neuroprotection *in vitro* was associated with suppression of Bad and upregulation of JAK-2, STAT-5, PI3K, AKT and Bcl₂ [65]. In addition to the anti-apoptotic properties of EPO, antioxidant properties of EPO were also detected in cultures. Studies have shown that EPO stabilizes mitochondrial membrane potential and decreases ROS in A β (25–35)-induced neuronal toxicity in PC-12 cells, up-regulated anti-apoptotic and down-regulated pro-apoptotic proteins [66]. The protective effects of EPO were not limited to *in vitro* findings.

Erythropoietin-Induced Neuroprotection in Subarachnoid Hemorrhage (SAH)

Despite the difference in the pathophysiology of stroke, EPO was shown to be neuroprotective in both rat and rabbit models of hemorrhagic stroke. EPO was shown to reduce neuronal damage and S-100 expression in cerebrospinal fluid of SAH rabbits 24, 48 and 72 hours after insult. The decrease in S-100 was correlated with improved neurological outcome [3]. In a rat intracisternal blood injection SAH model, a single low dose of EPO (400IU/Kg) was shown to improve arterial blood pressure and cerebral blood flow [67]. Increases in CSF, EPO expression, reduced neurological deficits, vasoconstriction and attenuation of vasospasm were observed in EPO-treated SAH white rabbits [68]. It has been shown that EPO gene administration prompts an elevation in phosphorendothelial nitric oxide synthase (eNOS) and phospho-AKT with a corresponding decrease in eNOS. This finding infers that EPO confers neuroprotection and reduces vasospasm by modulating phosphorylated AKT and eNOS in SAH [69]. Alternately it appears that the neuroprotective mechanism of EPO is conserved between species.

Erythropoietin-Induced Neuroprotection in Intracerebral Hemorrhage (ICH)

Neuroprotection against collagenase induced ICH in rats by EPO was associated with reduced hematoma formation, edema, apoptosis and inflammation and improved neurological outcomes [5, 6]. The dose and form of EPO did not appear to hamper its neuroprotective properties. Intraperitoneal administration of 500 to 5000 IU/kg of recombinant human (rHu) EPO or 1000 IU/kg Darbepoetin alfa conferred neuroprotection after ICH [5, 6]. The neuroprotection observed in ICH was correlated with decreased caspase, 3, 8 and 9 activity, TNF- α and Fas and Fas ligand expression [5, 6]. There was also a notable increase in STAT-3, pAkt, pERK and eNOS activity observed in EPO-treated ICH rats. This observation alludes to an overlap in the mechanisms of EPO induced-neuroprotection with the other animal models of stroke such as neonatal HI, global and focal ischemia and SAH.

Erythropoietin-Induced Neuroprotection in Adult Cerebral Ischemia

Cerebral ischemia is associated with impaired cerebral blood flow (CBF), skewed pH and electrolyte balance and increased free radical formation, intracranial pressure (ICP) and inflammation [70]. Reduced CBF limits energy availability and forces the cell into anaerobic respiration, which creates an acidic environment and shifts the sodium, potassium and calcium equilibrium [71, 72]. Erythropoietin-induced neuroprotection in cerebral ischemia is associated with neurogenesis, angiogenesis, oligodendrogenesis, and enhanced cerebral blood flow and prevent/limit blood brain barrier leakage [73–75]. Sakanaka *et al.* demonstrated that intraventricular infusion of EPO in a gerbil model of forebrain ischemia decreased synaptic degeneration and neuronal loss. The neuroprotective effect of EPO infusion was rapidly reversed by the addition of a soluble EPOR, which increased the ischemic core and total neuronal loss in the animals [8]. EPO-induced neuroprotection in both transient and permanent MCAO appeared to be associated with increased pSTAT-5, Bcl-xL, XIAP and decreased caspase-3 and caspase-9 in neuronal tissue [76, 77]. The upregulation of STAT-5 and the pan inhibition of caspase-3 and -9 appear to be conserved across the different stroke models.

Erythropoietin-Induced Neuroprotection in Neonates

Single or multiple doses of EPO administered pre- or post- HI was shown to promote neuroprotection in a rat model of neonatal hypoxia ischemia [78, 79]. EPO-induced neuroprotection in neonatal rats is associated with decreases inflammation, apoptosis, brain atrophy, caspase-3 and -9 and increase expression of heat shock protein (HSP)-70 expression, JAK/STAT and TIMP-1 activity [80, 81]. Previous studies on the anti-inflammatory properties of EPO-induced neuroprotection in neonates showed that EPO inhibits/ reduces cerebral expression of CD4⁺ and CD68⁺ cells IL-1 α and TNF- α expression [43]. In addition to the anti-apoptotic and anti-inflammatory properties, EPO was also shown to stimulate progenitor cell proliferation in the dentate gyrus and subventricular zone of HI neonates [82]. These findings, along with positive results in phase I/II clinical trial for EPO use in the treatment of neonatal hypoxic ischemic brain injury, have offered a glimmer of hope for EPO in stroke therapy [20].

CLINICAL USE OF ERYTHROPOIETIN IN STROKE THERAPY

Clinical trials in three different categories of stroke yielded divergent results. A single center study in China revealed that intravenous administration of 250 IU/kg of EPO three times per week for four weeks, significantly improved motor function in preterm infants at six months after birth [83]. Subsequent studies were then conducted to determine the safety and efficacy of EPO for used in neonates for the treatment of hypoxic ischemic brain injury. Two independent single center studies revealed that high doses of EPO improved neurodevelopmental outcomes in very preterm infants and was safe and effective in doses ranging from 1000–2500 IU/Kg [84–86]. The dose was extrapolated to adults and yielded inconclusive [87] or negative results as shown by German Multicenter EPO Stroke Trial [88]. However, re-examination of the data and excluding patients receiving recombinant tissue-type plasminogen activator (rtPA) revealed that EPO was beneficial in the absence thrombolysis and promoted better outcomes compared to placebo-treated individuals [88, 89].

Clinical analysis of the neuroprotective effects of EPO in SAH patients' base on age, sepsis and concomitant Statin use revealed that the presences of sepsis attenuate EPO's protection. Considering that sepsis is an attack on the hematopoietic system, it is not surprising that it is difficult to harness the non-hematopoietic function of EPO in the presence of infection [87]. Harnessing the non-hematopoietic benefits of EPO in stroke therapy is a delicate balancing act. Recent studies have revealed that EPO treatment is able to increase brain tissue oxygen tension (PbtO₂) in poor grade (SAH grades 4/5) SAH patients. Thus, more studies in larger cohorts are necessary to determine the efficacy of the use of EPO in the treatment of SAH [90].

The Use of Non-Erythropoietic Derivatives in Neuroprotection

Dissociating the non-hematopoietic function of EPO from the hematopoietic function for treatment of stroke has proven to be challenging. Thus recent efforts have been made to design pharmaceutical analogs that lack the ability to initiate erythropoiesis. Structural analogs asialoerythropoietin and carbamylated-Epo (CEPO) both confers cellular survival

and proliferation with minimal erythropoiesis [91–93]. These analogs appear to promote neuroprotection without initiating notable erythropoiesis, coagulation and thrombosis. However if the derivatives are able to withstand long treatment without the significant side effects is still to be elucidated. These findings suggest that there is potential for EPO derivative in stroke therapy.

Confounding Factors that Limits the Clinical Translatability of EPO Treatment in Adults

EPO forms a negative feedback loop with HIF-1 α , its upstream regulator. Pretreatment of NGF-differentiated PC-12 with EPO inhibited HIF-1 α transcription and translation in a dose dependent manner [29]. This is particularly noteworthy because HIF-1 α regulates over 70 different genes. Some of which are responsible for: angiogenesis, cellular growth, differentiation, proliferation, homeostasis and apoptosis [94]. Thus, continuous inhibition of HIF-1 α that could be facilitated by extended EPO treatment could be detrimental. Additionally, many of the laboratory studies were done using a single EPO treatment or three treatments administered over 48 hours. In most clinical trial EPO is administered over a 3 week period [83, 84, 86]. Previous studies reveal that HIF-1 α expression and benefits are biphasic. At the onset of a stroke an initial spike in HIF-1 α levels is prone to increase transcription of proapoptotic genes. Thereby inhibition by EPO is beneficial. However the late phase/post 24 hours increase in HIF-1 α is associated with increases in prosurvival genes [95]. Thus EPO induced neuroprotection associated with activation of the negative feedback inhibition of HIF-1 α is beneficial in the acute phase and detrimental in the delayed/ recovery phase. The inhibition of biphasic master regulator HIF-1 α limits the clinical translatability of EPO treatment in adults. Therefore, a thorough understanding of the interaction of EPO and its upstream regulator HIF-1 α is necessary to enhance clinical translatability of EPO-induce neuroprotection.

The majority of EPO studies were done with a single complication (stroke) and a single therapeutic intervention (EPO). However most individuals post stroke are on thrombolysis therapy (blood thinner, antiplatelet therapy) [88]. Therefore, more combination studies should be done. Moreover, obesity, diabetes and the metabolic syndrome predates stroke in many individuals [96]. These conditions are associated with increased ROS and decrease HIF-1 α , which is a regulator of EPO [97]. Thus the efficacy of EPO treatment needs to be evaluated in the presence of more than one infirmity.

SUMMARY AND CONCLUSION

Erythropoietin (EPO) is a cytokine with several roles in addition to erythropoiesis, including cell death inhibition, immunomodulation, and angiogenesis. EPO, like inflammation, is a double edged sword. It can be beneficial or detrimental depending on the dose and time of administration [98]. EPO treatment is neuroprotective in several *in vivo* and *in vitro* model of stroke. It was shown to have proangiogenic, anti-inflammatory, apoptotic, and anti-oxidant properties. EPO's neuroprotective effects in animal models are undisputed from its first use in animal models, where it was shown to prevent synapses degeneration to more recent studies where it was shown to have antioxidant properties [8] [98]. EPO has been shown to

preserve brain structure and function in several studies of rodent HI and stroke [99–101] with long-term improvement seen with 3 doses of EPO [102].

The primary signaling pathway of EPO-induced neuroprotection is the JAK/STAT pathway [65]. EPO bound to the EPO-R triggers a conformational JAK-2 that is propagated to the STATs [103]. Activation of the JAK/STAT pathway especially STAT-3 is associated with cell survival and proliferation [52, 54]. STAT-3 activation prompts a robust increase in TIMP-1 expression and activity [80]. Prolonged increases in both STAT-3 and TIMP-1 were shown to promote tumor formation/survival [104, 105]. Additionally STAT-3 was shown to be one of the primary signaling molecule in the proliferation of glioma stem cells and glioblastomas [104]. Thus separating the beneficial effect of EPO-induced neuroprotection from the possible detrimental ones is a delicate balancing act.

The benefits of EPO treatment in stroke therapy are tremendous. It has been shown that systemically administered EPO is readily permeable to the blood–brain barrier thus offering an advantage for its use in human stroke therapy. Additionally non-hematopoietic EPO derivatives have shown much promise in animal models; these analogs should be explored for use in the treatment of adult strokes.

However, cautious optimism should be employed in the use of EPO in human stroke because EPO alone had modest benefits and failed miserably, when used in combination with rtPA in a German multicenter stroke trial. The failure of EPO in combination with rtPA gravely overshadowed the benefits of EPO alone in neonates as well as adults [88, 89]. The information available on the safety and effectiveness of EPO in stroke therapy is sparse. Therefore this needs to be thoroughly investigated prior to condemning EPO use for human stroke therapy

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Biography

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Fig. (1). Schematic representation of the signaling pathway involved in EPO-induced neuroprotection. The primary mechanisms of secondary injury due to hemorrhagic stroke are presented in blue. One of the primary mechanisms of ischemic injury is presented in red. Matrix metalloproteinase serves as the pivotal molecule leading to pathology in both hemorrhagic and ischemic strokes. The black, green, grey and tan show the primary signaling pathway activated downstream of Janus Kinase 2, during EPO induced neuroprotection. Because JAK-2 pathway is activated during neuroprotection in all stroke model the mechanism of neuroprotection is somewhat conserved across models.

Table 1
A Summary of Erythropoietin-induced Neuroprotection in Animal Models of Hemorrhagic Strokes.

Stroke Model	Species	Time of Administration	Outcome Measured	Effect	References
ICH	Male Sprague-Dawley Rats	5 minutes after surgery/ Daily for 14 days	Neurobehavior: Histopathological Computation of Tissue Loss, Immunohistochemistry for NeuN positive cells	Significantly Improved Neurobehavior 1 week after insult. Reduced injury and preservation of parenchymal cytoarchitecture.	Grasso <i>et al.</i> Neurosurgery. 2009 Oct; 65(4):763–9
	Male Sprague-Dawley Rats	2 hours after surgery / Daily for 1 or 3 days	Neurobehavior: Hemorrhage Volume. Hemispheric Atrophy. Brain Edema. Cell Death: TUNEL staining, Caspase Activity	Better functional recovery. Reduced brain water content. Decreased mean hemorrhage volume and hemispheric atrophy. Reduced apoptosis: lower caspase activities, TUNEL positive cells	Lee <i>et al.</i> J Neurochem. 2006 Mar;96(6):1728–39
SAH	Male New Zealand White Rabbits	5 minutes after SAH induction/ every 8 hrs for 72 hrs	Neurobehavior: Ischemic Lesions. Analysis of Basilar Arteries diameter	Improved neurobehavioral outcome. Decreased ischemic neuronal damage. Reduced vasoconstriction of arteries	Grasso <i>et al.</i> Proc Natl Acad Sci USA. 2002 Apr 16;99(8): 5627–31.
	Male New Zealand White Rabbits	* Immediately after injection of autologous blood.	Cerebral Angiography. Vascular Reactivity.	Reduced cerebral vasospasm. Augmented basilar arterial relaxation to acetylcholine	Santhanam <i>et al.</i> Stroke. 2005 Dec;36(12):2731–7
	Male New Zealand White Rabbits	10 minutes after SAH procedure/Day 2, 4, and 6	Computed tomography perfusion. CT angiography. Neurological Function. Histology	Improved neurological outcomes. Improved cerebral blood flow and microcirculatory flow. Improved tissue perfusion	Murphy <i>et al.</i> J Neurosurg. 2008 Dec;109(6):1155–64.

Table 2

A Summary of Erythropoietin-induced Neuroprotection in Animal Models of Ischemic Stroke.

Stroke Model	Species	Time of Administration	Outcome Measured	Effect	Reference
Focal Ischemia	Male Long Evans Rats	6, 7 and 8 days after occlusion	Neurobehavior Lesion Volume, Tissue Loss	Improved infarct volume and neurological outcomes	Belayev <i>et al.</i> Brain Res. 2009 Jul 14;1280:117–23.
	Male C57BL/6 mice	30 minutes before ischemia/ daily for 3, 7, 14 and 21 days	Infarct Volume, Angiogenesis, Endothelial Cell Survival, Local Cerebral Blood Flow (LCBF), Apoptosis : Caspase activities	Decrease cell death and infarct volume 3 days after stroke. Restored CBF 14 days after stroke. neurovascular protection and neurogenesis	Li <i>et al.</i> J Cereb Blood Flow Metab. 2007 May;27(5):1043–54
Global Ischemia	Male Sprague-Dawley Rats	20 hr before or 20 min or 1 hr after ischemia	Histology of neurons, Cell Death; DNA Fragmentation	Protected CA1 neurons of hippocampus, Decreased neuronal death	Zhang <i>et al.</i> J Neurosci Res. 2006 May 15;83(7):1241–51.
	Male Mongolian Gerbils	Immediately after restoration of blood flow	Brain Edema, Hippocampal neuronal death, Brain nitric oxide, Survival	Increased neuronal survival and decreased brain edema. Reduced NO levels after ischemia	Calapai <i>et al.</i> Eur J Pharmacol. 2000 Aug 11;401(3):349–56.
Neonatal Hypoxia Ischemia	P-7 Sprague-Dawley Rats	24 hours after insult	Inflammatory effects, Brain Weight.	Attenuated brain injury Reduced in-inflammatory cytokine and leukocytes infiltration.	Sun <i>et al.</i> Stroke. 2005 Aug; 36(8):1672–8.
	P-7 Sprague-Dawley Rats	20 minutes after insult/ Day 2,4 and 6	Infarct Volume, Revascularization, Neurogenesis, Immunohistochemistry Neurological behavior	Reduced tissue infarct volume. Enhanced revascularization, increased subventricular zone cell proliferation and neuronal migration. Improved neurological outcomes	Iwai <i>et al.</i> Stroke. 2007 Oct; 38(10):2795–803