Erythropoietin in stroke: quo vadis

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Importance of the field: Recombinant erythropoietin (rEPO) failed in a recent clinical study to protect from damages induced by ischemic stroke. The lack of acute treatments in ischemic stroke and the promising outcome in numerous preclinical studies in vivo demands a more critical evaluation of the future use of EPO as an acute treatment.

Areas covered in this review: The current use and administration of rhEPO and its analogs in animal models and the future use of this cytokine in the treatment of ischemic stroke.

What the reader will gain: In this review the potential reasons for the failure of EPO in the clinical trial are analysed and whether the preclinical trials sufficiently evaluated the true potential of recombinant EPO and its analogs is assessed. Alternative methods for administration of EPO to enhance its potential as a neuroprotective drug in ischemic stroke are discussed.

Take home message: Failure in clinical trial does not necessarily indicate the lack of therapeutic potential of EPO. This review encourages further investigation of the true potential of EPO as a candidate drug for the treatment of ischemic stroke by improved preclinical experimental design and utilization of alternative administration methods.

Keywords: blood–brain barrier, drug targeting, erythropoietin, intranasal administration, ischemia, neuroprotection, rtPA, stroke, systemic administration, therapeutic strategies

1. Introduction

Ischemic stroke is a major cause of disability and death in the USA. Disabilities and neurological impairments in stroke patients cause mild to severe levels of loss of quality of life, with severe burden on the patients, their families and society [1]. In 2008, ischemic stroke represented 87% of all strokes, affecting over 795,000 people annually. The cost for stroke-related treatments and disabilities with the associated loss of productivity is estimated to be $68.9 billion for 2009 [1].

Despite tremendous efforts and significant progress in understanding the etiology of ischemic stroke, currently, the only FDA-approved treatment for ischemic stroke is thrombolytic treatment with intravenous or intrarheal administration of recombinant tissue plasminogen activator (rTPA) within 3 – 4.5 h of onset of stroke symptoms. This treatment is successful in eligible patients and provides significant benefits by restoring the blood flow to the affected brain regions. However, due to prehospital treatment delays [2] along with medical factors such as previous strokes, advanced age, hyperglycemia, elevated systolic blood pressure, pregnancy, history of intracranial bleeding, bleeding diathesis etc. only an estimated 1.8 – 3.0% of ischemic stroke patients in the US receive rTPA [3]. Hence, more than 97% of all ischemic stroke patients are precluded from rTPA therapy, leaving them without any treatment options and therefore again emphasizing the dire need for alternative strategies for ischemic neuroprotection.
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Article highlights.

- In preclinical trials erythropoietin provided significant improvement in neurological outcome after ischemic stroke.
- In clinical trials systemic administration of erythropoietin did not provide any benefits in patients suffering from acute ischemic stroke.
- Novel safe and efficient administration methods for erythropoietin must be evaluated to deliver this drug to the brain while minimizing systemic contamination.
- The minimum dosage of erythropoietin required to exert a protective effect in the rodent or human brain suffering from ischemic stroke must be established and neurological outcome tested in adherence to the Stroke Therapy Academic Industry Roundtable (STAIR) guidelines.

The development of therapeutic concepts for acute ischemic stroke has proven to be a very difficult task. Numerous neuroprotective drugs are effective in rodent models of ischemia in brain by preventing neuropathological effects and restoring neurological functions. However, the translation of these results to clinical settings has overall been disappointing. This is despite the fact that many drugs were successfully tested in preclinical trials and showed a great potency. Among those tested, erythropoietin (EPO) is a promising candidate drug for the acute and chronic treatment of various neurological diseases. EPO is a glycoprotein hormone (30 kDa) that is produced mainly in adult kidney and in a smaller amount in the liver. The main biological function of EPO is to regulate the proliferation, maturation and the survival of erythroid progenitor cells in human bone marrow, resulting in an oxygen-dependent regulation of red blood-cell mass. EPO is also expressed in the brain, mainly by astrocytes and to a lesser extent by neurons (for an excellent review see [4]).

EPO exerts its cellular effects by binding to the EPO-receptor (EPO-R), which is expressed in erythroid progenitor cells, but also in neuronal tissue, mainly on neurons [5]. However, expression of the EPO-R in neuronal tissue and the specificity of the antibodies used for its detection have been questioned [6-8]. Despite this debate numerous other reports have confirmed the presence of the EPO-R not only in neurons but also in oligodendrocytes and in the endothelial cells of the blood–brain barrier (BBB) [9].

The expression of endogenous EPO and its receptor in the brain and adult liver is induced by hypoxia. However, local expression of normoxic and hypoxia-induced expression in brain is magnitudes lower than that outside of the CNS [10]. Due to significantly higher concentrations of EPO in the blood than in the CNS, a hematopoietic function of brain-derived EPO is doubtful. It is more likely that endogenous EPO produced in the CNS acts locally in a paracrine or autocrine fashion, but its specific function(s) in the brain remain unclear.

The expression of EPO and its receptor are crucial during early embryonic development of the neural system. Lack of EPO and its receptor in EPO–/– and EPO-R–/– mice prevented complete closure of the neuronal tube [11]. In addition, lack of EPO or its receptor results in significant neuronal apoptosis in the embryonic brain [12,13], and ultimately results in the in utero death of the embryo due to severe anemia [9]. It has also been shown that EPO drives the development, differentiation and migration of neuronal progenitor cells in rodents [13,14]. The use of mice with brain-specific deletion of EPO and EPO-R expression revealed decreased proliferation of cells in the subventricular zone, which is the main supplier for neuronal progenitor cells used for neurogenesis and neurorestoration [11,15,16]. Interestingly, even though the post-stroke recovery of these animals was significantly impaired, potentially due to the lack of neurogenesis, no significant differences in acute stroke volume were observed between control and EPO-R-deficient mice. This suggests that endogenous EPO and EPO-R expression in adult mice is an important factor for neurorestoration but has only a partial role in acute neuroprotection. This might be explained by the insufficiency of relatively low levels of endogenous EPO produced in the brain. Therefore, in acute neurodegenerative diseases, a boost of EPO concentrations can be achieved by administration of exogenous recombinant EPO or its analogs.

A great body of evidence indicates that among other functions, exogenously administered recombinant EPO and its hematopoietic and non-hematopoietic analogs are potent neuroprotectants in vitro and in vivo (Table 1). Numerous in vitro studies show that cultured primary neurons or neuron-like cell lines are protected under neurodegenerative conditions when treated with EPO or its analogs. Recombinant EPO protects cultured neurons against glutamate-induced neurotoxicity [17,18], synaptic degeneration [19] and hypoxia [20] by activating several anti-apoptotic mechanisms. Moreover, EPO exerts its protective effects on endothelial cells forming the BBB [21,22] and glial cells [23].

In rodent models of ischemia, EPO or its analogs have been shown to be capable of preserving neurological function in animals subjected to ischemic stroke. Similarly, exogenous EPO or its analogs were neuroprotective in mouse or rat models of traumatic brain injury (TBI) [24], or spinal cord injury (SCI) [25,26]. In contrast, another group reported the lack of any EPO-mediated neuropathological improvement in SCI [27]. Furthermore, use of EPO protected the integrity of the BBB under neurodegenerative conditions [28,29].

In summary, there is a significant amount of evidence in preclinical studies that treatment with recombinant EPO or its analogs provide protective effects under neurodegenerative conditions in animal models. Moreover, in an earlier randomized clinical pilot study intravenously administered recombinant EPO reduced the ischemic stroke volume in patients and raised no safety concerns [30]. Unfortunately, no favorable and protective effects of EPO could be observed in a larger clinical trial involving 522 intent-to-treat patients (460 patients treated as per-protocol) with ischemic stroke [31]. However, analysis of the data from subgroups treated with EPO or...
Table 1. Overview of selected preclinical studies using EPO in neurodegenerative models.

<table>
<thead>
<tr>
<th>Reference</th>
<th>(pmid)</th>
<th>Disease</th>
<th>Species</th>
<th>n</th>
<th>Delivery method</th>
<th>EPO applied</th>
<th>Applied volume</th>
<th>Actual units applied</th>
<th>Experimental groups</th>
<th>Treatment regimen</th>
<th>Outcome measure</th>
<th>Target concentration and measurement method</th>
<th>Measured (EPO)brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>[29]</td>
<td>18434762</td>
<td>1 h MCAO</td>
<td>Rat</td>
<td>n/a IP</td>
<td>2500 IU/kg</td>
<td>n/a</td>
<td>700 - 825 U</td>
<td>EPO or saline</td>
<td>24 h before MCAO</td>
<td>Transfer coefficient (K) of 14C-alpha-aminoisobutyric acid</td>
<td>Used dose has been reported to be effective in preconditioning or neuroprotection</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>[52]</td>
<td>17624681</td>
<td>Permanent focal MCAO</td>
<td>Mouse</td>
<td>n/a IP</td>
<td>3000, 10,000, 30,000 U/kg</td>
<td>n/a</td>
<td>78 - 90 U, 260 - 300 U, 780 - 900 U</td>
<td>EPO or saline</td>
<td>24 h before MCAO</td>
<td>Infarct volume</td>
<td>n/a</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>[104]</td>
<td>1707815</td>
<td>Permanent focal MCAO</td>
<td>Mouse</td>
<td>6</td>
<td>5000 U/kg</td>
<td>n/a</td>
<td>100 - 125 U</td>
<td>EPO or saline</td>
<td>30 min before MCAO and once daily after ischemia</td>
<td>Infarct volume, TUNEL, caspase 3, angiogenesis, blood flow, HCT</td>
<td>n/a</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>[28]</td>
<td>17562165</td>
<td>Permanent right MCAO</td>
<td>Mouse</td>
<td>6</td>
<td>5000 U/kg</td>
<td>n/a</td>
<td>100 - 125 U</td>
<td>EPO or saline</td>
<td>30 min before MCAO and once daily after ischemia</td>
<td>HCT, LCBF, BBB leakage, brain edema</td>
<td>n/a</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>[105]</td>
<td>15178821</td>
<td>Embolic stroke</td>
<td>Rat</td>
<td>14</td>
<td>5000, 10,000 U/kg</td>
<td>n/a</td>
<td>5000, 10,000 U/kg</td>
<td>EPO or saline</td>
<td>24 h after MCAO and once daily for 7 days</td>
<td>Infarct volume, neurological outcome, neurogenesis, angiogenesis, HCT</td>
<td>n/a</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>[106]</td>
<td>12975460</td>
<td>MCAO</td>
<td>Rat</td>
<td>n/a IP</td>
<td>5000 U/kg body weight</td>
<td>n/a</td>
<td>1250 - 1400 U</td>
<td>EPO</td>
<td>At the time of occlusion</td>
<td>Infarct volume, inflammation</td>
<td>n/a</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>[45]</td>
<td>10984541</td>
<td>Permanent MCA and carotid occlusion; 1 h left carotid occlusion</td>
<td>Rat</td>
<td>n/a IP</td>
<td>250 – 5000 U/kg body weight</td>
<td>n/a</td>
<td>62.5 - 1250 U</td>
<td>EPO</td>
<td>24 h before simultaneously 3 or 6 or 9 h after</td>
<td>Infarct volume</td>
<td>Biotinylated 5000 U/kg body weight, rHu-EPO, IP, brain sections analyzed 5 and 17 h after</td>
<td>Peroxidase product: surrounding capillaries and in the brain parenchyma; a distance 3 - 4 times that of the thickness of the capillary wall</td>
<td>No</td>
</tr>
</tbody>
</table>

| Reference | (pmid) | Disease | Species | n | Delivery method | EPO applied | Applied volume | Actual units applied | Experimental groups | Treatment regimen | Outcome measure | Target concentration and measurement method (EPO) | Measured (EPO) | BBB: Blood–brain barrier; CEPO: Carbamylated EPO; CSF: Cerebrospinal fluid; CV: Cerebral ventricle; HCT: Hematocrit; ICV: Intra cerebral ventricle; IN: Intranasal; LCBF: Local cerebral blood flow; MCA: Medial cerebral artery; MCAO: Medial cerebral artery occlusion; MWM: Morris water maze; pmid: PubMed identifier; SC: Subcutaneous; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling. |
|-----------|--------|---------|---------|---|----------------|-------------|-----------------|----------------------|-------------------|----------------|----------------|---------------------------------------------|--------------| Expert Opin. Biol. Ther. Downloaded from informahealthcare.com by Ashley Publications Ltd on 04/19/10 For personal use only. |
| [53]      | 17603558 | Embolic MCAO | Rat (350 – 400 g) | 10 | IV (tail vein) | 50, 500, 1150 or 5000 IU/kg 50 µg/kg | 0.32 – 0.37 ml per rat | 17.5 – 20 U, 175 – 200 U, 402.5 – 460 U, 1750 – 2000 U | rhEPO CEPO | 6, 24, and 48 h after embolization | Infarct volume, TUNEL, HCT, neurological score | Plasma, CSF, brain | 0.044, 0.06, 0.1, 0.06% of total measured found in ipsilateral cortex |
| [63]      | 16511866 | 12 min global ischemia | Rat | 8 – 10/ group histology, 3/group immunohistochemistry | ICV 5 µl | 10 µl | 50 U | EPO | 20 h before ischemia 20 min after ischemia 1 h after ischemia | CA1 neuron loss | n/a | No |
| [62]      | 11891794 | 3 min forebrain ischemia | Gerbil | 8 | Osmotic MiniPump | 0.5, 5 U/day | n/a | 0.5, 5 U | EPO | 0.16, 1.6 U immediately after ischemia 0.5 or 5 U/day continually infused to CV for 28 days | Passive avoidance, CA1 neuronal density (1 h after) | n/a | No |
| [107]     | 9875214  | Permanent MCAO | Rat | 8 | SC infusion to left lateral ventricle | 0.2, 1, 5 U | n/a | 0.2, 1, 5 U | EPO | Once daily for 28 days | MWM, neuron density | n/a | No |
| [19]      | 9539790  | 3 min ischemia | Gerbil | 8, 8, 11, 8 n/a, n/a | Osmotic MiniPump infusion to left lateral ventricle | 0.5, 2.5, 5, 25, 50, 5000 U/day | n/a | 0.5, 2.5, 5, 25, 50, 5000 U | EPO | Continuously infused to left lateral ventricle for 7 days | Response latency, CA1 neuron density, synapse integrity | n/a | No |
| [71]      | 16054296 | 60 min focal MCAO | Rat (250 – 350 g) | n/a | IN IP | 4, 8, 12, 24 U 5000 U/kg | 20 µl/nosntril (2 U/20 µl per nostril) | 4, 8, 12, 24, 1250 U | Compare IN EPO to IP EPO | 10 min after MCAO and 1 h after reperfusion | Infarct volume, cell density | Cites lit. for effective doses for EPO | No | Erythropoietin in stroke: quo vadis | Expert Opin. Biol. Ther. Downloaded from informahealthcare.com by Ashley Publications Ltd on 04/19/10 For personal use only. |
placebo showed an improved neurological outcome in patients that received EPO. This result was similar to the beneficial effects of rTPA. However, a higher death rate was observed in patients treated with EPO when compared with those in the placebo group, mainly due to intracerebral hemorrhage, brain edema and thromboembolic events. In addition, this study revealed that systemic administration of EPO could have fatal outcomes in patients pretreated with the thrombolytic drug rTPA.

In a recent study, safety concerns were raised for systemically administered EPO even when administered in the dose range determined to be ‘safe’ [32]. The main source of these concerns is the risk of cardiovascular events, uncontrolled increase of hemoglobin concentrations and elevated hematocrit levels in patients treated with systemic administered EPO.

The present paper does not claim to be a comprehensive review and does not cover all aspects of existing preclinical studies involving EPO in ischemic stroke. In this review we try to analyze the potential reasons for the failure of EPO and assess whether the preclinical trials sufficiently evaluated the true potential of recombinant EPO and its analogs. In addition this paper reiterates the necessity of future research in this area to adhere more strictly to published guidelines for the evaluation of candidate drugs in ischemic stroke. Moreover, we discuss alternative methods for administration of EPO to enhance its potential as a neuroprotective drug in ischemic stroke.

### 2. Development of therapeutic strategies in ischemia

Current treatment options in ischemic stroke are limited to thrombolytic therapy using intravenously or intraarterially administered rTPA. The immediate target of this treatment is the restoration of blood flow to the ischemic areas by dissolving the clot clogging the artery. However, rTPA has no neuroprotective properties. The necessity for a neuroprotective strategy is further emphasized by the inherent neurotoxic properties of rTPA, which must be counteracted [33].

#### 2.1 Neuroprotective concepts

Treatment of ischemic stroke is highly time-dependent and follows the paradigm ‘time is brain’. This notion is based on the time-dependent progress of pathophysiological processes that expand the infarct core into surrounding areas by converting viable cells in the penumbra into necrotic cells. The ischemic penumbra contains potentially salvageable tissue within the area compromised by an ischemic event. This area contains neuronal cells sufficiently viable for recovery that are the main target for neuroprotective treatments.

The ultimate goal of any treatment in ischemic stroke must be the normalization of brain functions and neurological behavior by protecting neuronal function and integrity from degeneration. This can be achieved by developing neuroprotective concepts to prevent the demise of neurons, the main functional units in the CNS. As defined by Ginsberg [34], the concept of ischemic neuroprotection includes the use of treatment modalities, drugs or a combination thereof to prevent irreversible neuronal damage in ischemic stroke. The search for new potential therapies and drug candidates resulted in the identification of many concepts such as mechanical vessel recanalization [35] and ischemic neuroprotection [36] using various drugs, among them recombinant EPO and its analogs.

#### 2.2 Guidelines for preclinical testing of neuroprotectants

In 2006, O’Collins and colleagues evaluated the use of drug candidates in preclinical trials and compared the experimental efficacy of various drugs to their clinical potential [37]. As a result of their study, the authors suggested a more thorough evaluation of the preclinical data and the adherence to common guidelines for the evaluation of the drug candidates in order to facilitate the development of new drugs for stroke treatment. Further evaluation of the development of novel treatment options for acute stroke revealed that preclinical testing failed to include crucial assessments, such as testing for adequate dosing and complete pharmacological profiles [35]. In order to overcome heterogeneity of experimental protocols for testing candidate drugs for ischemic stroke, the Stroke Therapy Academic Industry Roundtable (STAIR) has published guidelines (STAIR I – IV) [36,38-42] that present the limitations of current treatment options and provide recommendations for the preclinical testing of novel treatments of drug candidates, such as recombinant EPO.

#### 2.3 The blood–brain barrier as an impediment for drug administration to the CNS

As the development of novel drug therapies for ischemic stroke advance, it becomes ever more imperative to determine the best delivery method of therapeutics to the CNS. The absorption and distribution of the candidate drug must be critically evaluated to determine the amount of drug delivered to the target tissue. In addition, it must be assessed whether the dose is sufficient to exert the desired neuroprotective effect under the experimental conditions.

The main impediment for most of the systemically administered drugs trying to reach the brain is the BBB. Brain function relies on the maintenance of ions and the adequate supply of metabolic substrates and exchange of O2 and CO2. The BBB surrounds the CNS, maintaining the homeostasis of the neuronal microenvironment and protecting the CNS from potential neurotoxins circulating in the blood [43]. The BBB is a complex structure that segregates the neuronal milieu from the circulating blood. It tightly regulates the transport and diffusion of ions and molecules from the blood into the brain tissue and vice versa and thereby significantly impairs the direct exchange of molecules between the CNS and the vascular component. The physiochemical properties of a drug determines the
probability for its passage through the BBB. Lipophilic drugs that exceed 600 Da have negligible chances of penetrating through the intact BBB. Hydrophilic drugs, such as EPO, face even more restrictions and rely on receptor-mediated, saturable and therefore slow uptake mechanisms.

Tight junctions (TJ) are important structures that seal the BBB and establish its function as a selective gate for the transport of proteins and non-proteins. Transmembrane proteins, such as occludins and claudins, and adhesion molecules are anchored to the cytoskeleton of the endothelial cells and are the key structural elements in the sealing properties of TJs. The expression of TJs strongly limits the paracellular flow of ions between the endothelial cells. The low conductivity for ions through the BBB by paracellular diffusion is reflected in the high transendothelial electrical resistance, which is 100-fold greater than in peripheral capillaries elsewhere [44]. Large molecules circulating in blood cannot travel into the brain using the paracellular path but must cross the BBB using the transport mechanism known as transcellular transport. Transcellular transport requires the molecules to cross at least two lipid bilayer membranes of the endothelial cells. These molecules must first enter the endothelial cells via endocytosis at the apical (luminal) membrane and then exit by exocytosis at the basal site of the same cell in order to enter the brain tissue. In addition, the carrier-mediated influx of amino acids and glucose into the brain occurs using specific transport molecules. Peptide transport systems are responsible for the transfer of certain peptides and proteins across the BBB into the brain, such as arginine vasopressin (AVP) and enkephalins. Large proteins, such as insulin and IGFs, arrive in the brain by utilizing receptor mediated transport systems. This transport modus was also suggested for EPO because EPO receptors were found in the endothelial cells of the brain capillaries [45].

### 2.4 EPO as a neuroprotectant

During the last decade, many studies showed beneficial and protective effects for EPO and its derivates in animal models of acute and chronic neurological diseases. A meta-analysis examined the efficacy of EPO and its hematopoietic and non-hematopoietic derivates in animal models of stroke. It confirmed the potential of EPO as a protective agent in stroke models but also revealed that EPO must be administered within 6 h after stroke onset to exert its effects [46].

Under physiological conditions BBB excludes the access of most small peptides and all large molecules, such as EPO to the CNS [47,48]. BBB permeability significantly increases under ischemic stroke conditions [4] and in traumatic brain injury [49]. In all studies the penetration of intravascular or intraperitoneally administered EPO relied on the disease-dependent disruption and subsequent hyperpermeabilization of the BBB. Neuroprotective effects observed after intravenous, intraperitoneal or subcutaneous EPO led to the conclusion that this cytokine is capable of penetrating through the compromised BBB in pharmacologically sufficient amounts to provide neuroprotection in an animal model of ischemic stroke [45].

However, only a few studies have determined the acute concentration of systemic administered EPO in the brain. The only study specifically designed to quantify the capability of systemically administered recombinant human EPO to cross the BBB in non-injured mice concluded that this passage is unsaturable and hence likely to be receptor-independent [50]. Therefore, the authors suggested that a transport system for EPO across the intact BBB exists. Using radiolabeled recombinant EPO this study found that 0.05 – 0.1% of the intravenously injected dose can be found per g of the unjured mouse brain. In a different study in uninjured rats, 100 mU of intraperitoneally administered EPO (5000 U/kg) reached the cerebrospinal fluid (CSF) peaking at 4 h after the injection. Using an average weight of 200 – 250 g per rat this would indicate that 0.01 – 0.008% of the administered dose was capable of reaching the rat CSF. The same study also presented results indicating a similar efficiency of EPO penetration into the CNS in humans. None of these human subjects were suffering from ischemic stroke and therefore the BBB can be considered intact. However, in an embolic rat model of ischemic stroke 0.02 – 0.07% of the intravenously administered recombinant EPO arrived in the brain within 6 h after the onset of the occlusion. Previous studies suggest that hyperpermeabilized BBB as a result of ischemic stroke is the main access route for EPO into the CNS [45]. In accord with this hypothesis, more EPO should have arrived in the injured brain than in the control animals. However, when comparing the EPO levels in the brain in either study, there is no significant difference in EPO levels in the injured and uninjured brain. This may be attributed to the different species used in the two studies (mouse vs rat), but it is also possible that the injury-related disruption of the BBB 6 h after the onset of the occlusion is no longer present and the BBB regained its functionality. Another possibility is that the model of thrombosis-induced ischemic stroke has less disrupting effect on the BBB than other models of ischemic stroke. Several studies also showed that pretreatment with EPO 0.5 – 24 h prior to the onset of the ischemic injury provides significant reduction in infarct volume [28,45,51,52]. Unfortunately, no data is available from these studies to evaluate the uptake of the systemic administered EPO (intraperitoneal in all studies). The extent of neuroprotection in these studies is remarkable since EPO administered before the onset of the injury has to penetrate into the CNS through fully functional and non-hyperpermeabilized BBB. Moreover, considering that EPO concentration in intact mouse brain peaks within 3 h [50] and EPO has a half-life of 5 – 6 h in circulating blood [26] these results indicate the potency of EPO as a neuroprotectant in various conditions. Carbamylated EPO, a non-hematopoietic analog of EPO, resulted in 8 – 2.4% of the original dose in the brain [53]. In another study biotinylated EPO was used for the quantification of systemically administered recombinant EPO in the CNS.
However, biotin is transported very rapidly through the BBB[55,56]. Therefore, even a small portion of degraded and uncoupled biotin from EPO penetrating into the brain tissue can result in false positive detection. Currently, there are no studies available profiling a dose response in rodent and human brain. Therefore, the minimum dosage of EPO required to exert a protective effect in the rodent or human brain is not known.

Recent studies have shown that the hyperpermeabilization of the BBB is a complex event that exhibits heterogeneous temporal and spatial characteristics[57]. BBB hyperpermeabilization in animal models of ischemic stroke strongly depends on the type, severity and duration of insult and complete permeabilization is not a definite event in ischemic brain[58]. Studies using MRI confirmed the biphasic hyperpermeabilization of BBB in rats subjected to transient middle artery occlusion, with the first phase of BBB permeabilization occurring 4 – 5 h following the reperfusion, with a subsequent significant decline in permeability and partial recovery[59]. The second phase of BBB breakdown occurs at 48 h. Any drug targeting the brain, particularly drugs that cannot readily pass a fully functional BBB and administered systemically must respect these characteristics of BBB permeability changes. Moreover, so far it is not known if the characteristics of BBB permeability changes observed mostly in rodent models reflect the actual events in humans. Every ischemic stroke in humans has individual characteristics in its severity, duration and location. BBB hyperpermeabilization varies greatly with these parameters, making a standard clinical dosing regimen relying on BBB breakdown impossible, resulting possibly in inadequate or overdosing. Therefore, relying on BBB breakdown as the main access to the brain for potent neuroprotectants is highly speculative and risky.

Interestingly, systemic administration of EPO has also been described as protecting the integrity of the BBB by preventing its hyperpermeabilization[28,60]. This notion would imply that systemic EPO would reduce its own access through the compromised BBB into the ischemic brain by preventing hyperpermeabilization of the BBB in stroke and require an alternate path for EPO to enter the CNS.

### 3. Alternate strategies for administration of EPO

The enormous amount of preclinical studies using EPO in ischemic stroke and other neurodegenerative diseases clearly demonstrate the potency of this cytokine as a neuroprotective drug. However, the failure of systemically administered EPO as a treatment option in stroke patients and the heterogeneity of preclinical studies require a novel approach for reliable and quantifiable administration of EPO.

Drug delivery into the brain that bypasses the BBB can be achieved by methods other than systemic administration. Delivery of drugs under normal and pathological conditions have been covered by previously published studies[58,61]. Here we will briefly describe intracerebral, intraventricular and intranasal delivery as well as discuss their suitability for targeted and efficient delivery of EPO in ischemic stroke.

#### 3.1 Intracerebral delivery

The most direct method of delivering a therapeutic into the brain tissue is intracerebral delivery. This method permits the direct administration of the substances into the brain parenchyma, a specific target area, by various means[58]. However, there are several restrictions that lessen the theoretical advantage of this method. First, access to brain tissue requires surgical or other invasive, expensive and risky methods of delivery potentially resulting in undesired and harmful complications. Second, the capability of the drug to diffuse beyond its site of injection is strongly limited by its size and the restricted diffusion potential of the drug in brain tissue. This limits the size of brain volume that can be treated, which can be large in an ischemic stroke. In addition, due to restricted space for expansion, the liquid volume of intracerebral administration is limited. However, in preclinical studies the intracerebral delivery of EPO into the brain parenchyma resulted in significant neuroprotection in animal models of transient ischemia in rats and gerbils[19,62].

#### 3.2 Intraventricular delivery

The cerebrospinal fluid has unrestricted access to the brain tissue, allowing small and large molecules to move freely between these two compartments. Based on this rational, intraventricular delivery of drugs offers another promising delivery path that bypasses the BBB. Similar to intracerebral delivery, this method also requires surgical expertise and its invasive character carries the same potential danger. In addition, drugs injected into the liquid CSF still need to diffuse through the brain tissue to the target area, which is a slow process and not suitable for acute treatments. Time-consuming diffusion combined with the rapid turnover of the CSF within 5 – 6 h, which further reduces the availability of the administered drug, makes this administration method unsuitable for acute treatment in humans. However, in rodent models intraventricular administered EPO was capable of protecting brain tissue from degeneration induced by ischemia[63].

#### 3.3 Intranasal delivery

Among the alternative administration methods, intranasal administration of EPO is the most promising. Intranasal application of therapeutics dates back as far as the Han Dynasty (150AD) during which this method was used in Traditional Chinese medicine[64]. In the last two decades, the potential of intranasal drug administration has been explored[65] and successfully used to deliver a wide variety of neuroprotective drug candidates into the brain with corresponding beneficial effects[66-68].

The intranasal administration of drugs received increasing attention due to the difficulties of delivering drugs into the CNS by using the bloodstream. Anatomical studies give...
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evidence for the presence of nerves connecting the nasal cavity directly with the brain. Most prominent are the trigeminal and the olfactory nerve (Figure 1). The olfactory epithelium is located at the roof of the nasal cavity in mice and humans and contains bipolar sensory cells. The axons of these olfactory receptors form small nerve bundles and enter the CNS via the cribriform plate of the ethmoid bone. These nerve bundles may provide a direct and less limited access route into the CNS due to the reduced restrictions in BBB permeability in this area [65,82,86]. A few studies in humans are available describing the access of drugs into the CNS using this route [87,88].

The capability of a drug to penetrate through barriers into the brain tissue is dependent on its size and physiochemical properties (hydrophilic vs lipophilic). Lipophilic drugs can utilize paracellular transport (endocytosis and exocytosis) as required in intact BBB. Hydrophilic drugs, such as EPO, cannot use the transcellular route as required by the BBB.

The intranasal administration of drugs bypasses the BBB and affords rapid and efficient delivery to the brain [89]. The accessibility of the brain parenchyma via the intranasal pathway also avoids unnecessary high dosing in order to achieve therapeutic levels of systemically administered drugs in brain tissue. Moreover, due to reduced systemic leakage, undesired side effects as observed in systemic administration can be avoided. Among the problems to face with systemic administration of EPO is the promotion of tumor angiogenesis. In addition to its hematopoietic and neuroprotective role, EPO induces the growth of new blood vessels under normal and pathological conditions [90]. Under normal conditions, induction of vascularization by systemic EPO might be desirable and can induce increased tissue oxygenation and perfusion with oxygen and nutrients, however it can also generate side effects. For example, in cancer patients treated with EPO to counteract anemia, neoplastic disease and metastasization can be promoted due to the vasculogenic effect of this cytokine [91,92]. Recently the FDA issued a warning emphasizing the potential side effects of intravenously administered EPO in cancer patients, emphasizing the potential for tumor growth, increase probability of strokes, and heart failure [93]. In addition, other potential side effects of elevated EPO levels due to systemic administration mentioned by the FDA include hypertension and the risk of developing erythroblastopenia.

The systemic administration of EPO requires sustained blood flow in the vicinity of the target area. However, blood flow is compromised in ischemic stroke, particularly in embolic stroke unresponsive to thrombolysis. In these cases, drugs contained in the blood cannot reach the affected areas. Intranasal application does not require sustained blood flow, though specifics about how intranasally administered drugs reach the target area are not known. One possibility is the transport within the trigeminal nerve that extends into the nasal cavity and offers a connection to the brain.

The intranasal administration is capable of directly transporting drugs [65], among them EPO [71] and IGF-I [67,83], and even cells [94] into the CNS. The non-invasive method of intranasal delivery does not depend on the breakdown of BBB (see above) under neuropathological conditions. Intranasally administered therapeutics bypass the BBB and enter the CNS via transcellular pathways [95] and do not require any modification such as co-administration of synthetic peptides [79].

However, there are limitations to intranasal drug delivery, which were discussed in earlier studies [64,80,89,96,97]. The nasal cavity in rodents is easier to access than in humans. Moreover, while the olfactory tissue covers over 50% of the nasal cavities lining in rodents, human olfactory tissue is restricted to 3 – 5%. Therefore drug delivery through the nasal cavity and olfactory tissue in rodents is likely to be more efficient than in humans [96]. In addition, the differences in CSF volume in rodents and humans and the turnover time for CSF in these species can also lead to reduced efficiency of the intranasal drug delivery to brain in humans when compared with rodents.

In recent years various forms of EPO were generated and discussed as a non-hematopoietic alternative for treatment of stroke. Among the EPO derivatives are most notably the low-sialic acid EPO (Neuro-EPO), non-sialic EPO (asialoEPO) and carbamylated EPO (cEPO). However, as with EPO, due to their molecular weight their passage through the BBB is restricted when administered systemically. The intranasal administration route is likely to offer a desirable and efficient alternative for the transport of these EPO analogs to the CNS.

Despite the large number of preclinical studies demonstrating the neuroprotective potential of EPO as a monotherapy, the quest to increase the efficacy of this cytokine must continue. Co-administration of EPO with IGF-I has been reported to exert a synergistic effect in vitro, protecting the neurons more efficiently than either cytokine alone [98]. Moreover, intranasal administration of this synergistic combination proved to be superior to administration of either cytokine alone in a mouse model of ischemic stroke [83]. The combination of EPO with methylprednisolone protects neuronal cells and structures in a rodent model of multiple sclerosis [99], and co-administration of EPO with olmesartan improved neurological outcome after ischemic stroke [100]. In contrast, the combination of the iron chelator deferoxamine with EPO failed to provide synergistic protection from hypoxia-induced brain damage [101]. These contradictory results indicate that further preclinical testing is necessary to identify synergistic beneficial effects of EPO with other drugs.

3.4 Nanoparticles and nanosystems for target delivery

The field of drug targeting is improving rapidly. New technologies such as nanoparticle and nanosystem drug delivery are beginning to emerge as new methods to administer drugs to the target area. However, this technology is still in the early stages and currently focused on the development and
reproducible production of consistent nanocarriers for drugs [102]. As with systemic administration, the transportation of these carriers into the area affected by neuronal injury requires intact circulation of blood, which is interrupted in stroke. Once at the destination, the carriers still have to permeate through the brain tissue in order to reach the desired location and release their load in a reasonable time period to exert their therapeutic effect.

4. Expert opinion

During the last two decades EPO has emerged as a potent inhibitor of neuronal demise under acute and chronic pathological conditions. To study the protective effects of EPO, mostly rodent models of neurodegenerative diseases, such as ischemic stroke, were used. Moreover, various administration methods such as intravascular, intraperitoneal and intracerebral were examined. The efficacy of EPO treatment was measured by examining the stroke volume, behavioral testing and animal survival. In addition, signaling pathways induced by EPO were also studied. Meta-analysis comparing the results of eligible published studies statistically confirmed the efficacy of EPO as a treatment option in ischemic stroke. Moreover, a pilot study in human subjects suffering from ischemic stroke further established the potential of EPO as a therapy in human patients. However, another meta-analysis emphasized that experimental bias can lead to overstatement of therapeutic efficacy of EPO in ischemic stroke [103].

Despite EPO’s potential observed in animal models and a small population of patients, EPO failed to show beneficial therapeutic effects in stroke patients in a larger clinical trial. Increased complications and elevated death rates were observed in patients suffering from ischemic stroke treated with intravenous EPO and in the presence or absence of rtPA pretreatment. Therefore, the question that must be asked is whether EPO is one of many drugs that showed promising results in preclinical trials but failed in clinical testing? To answer this question, pre-clinical data that encouraged this trial must be re-evaluated. Examination of published studies reveals a wide heterogeneity of administration methods, dosing, animal models of disease and evaluation of the outcome. Most importantly, there is no evidence for the required disease-specific minimum-effective dose of EPO in brain to exert the hypothesized protective effect. Furthermore, it is not known whether a therapeutically efficient minimum-dosage of EPO in the injured brain can be achieved by systemic administration in humans. However, in a subpopulation of stroke patients intravascular EPO provided similar benefits to rtPA treatment [31].

Many drug candidates have failed to reach clinical testing, mainly due to safety issues. However, EPO has been used for treatment of anemic patients after kidney failure for a long period of time. When used with precaution, EPO is one of the safer drugs currently available to patients. Its reasonable safety record together with very promising results in preclinical testing and the serious lack of other promising drug candidates for treatment of ischemic stroke necessitates reexamining the potential of EPO as a neuroprotectant.

To fairly examine its potential in future studies, preclinical testing of EPO and its analogs should adhere strictly to STAIR guidelines [36,38-42] and remove any sources of experimental bias [103]. Moreover, the efficacy of administration methods for EPO and its analogs should be further examined and compared to find out the best method to deliver this drug in a most efficient and economical way to the target tissue. As an outcome measure, a combination of behavioral tests and histology should be utilized. Behavioral tests are not universal and can be insensitive or unsuitable in certain animals. Therefore the tests must be tailored to the species and the objectives to be tested. Moreover, animal groups to be tested must have
sufficient power to deliver statistically significant results. In addition, results obtained from rodent models should be reexamined in non-human primates. The neuropathological impact of each animal model varies and can distort the outcome measures when compared with other models of brain injuries. Therefore the limitations of each model should be carefully examined and considered in the analysis of data.

Despite strong commercial interest and the desperate need for novel treatment options in ischemic stroke, the re-emergence of EPO and its analogs as a future drug to counteract the neurodegenerative effects of brain injuries in clinical settings must be preceded by prudent and methodical analysis of its potential in studies that strictly adhere to STAIR guidelines. Based on the heterogeneity of current data and the lack of dose–response information for EPO, the failed clinical trial was premature and should be repeated in the future using novel parameters and approaches and tested in non-human primates. To coordinate the research efforts and to streamline and facilitate preclinical testing protocols, an EPO roundtable should be considered.

Declaration of interest

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