Human Recombinant Erythropoietin in Asphyxia Neonatorum: Pilot Trial

WHAT’S KNOWN ON THIS SUBJECT: Neuronal apoptosis that follows hypoxia-ischemia is triggered by upregulation of NO synthase, with excessive accumulation of NO and release of excitatory amino acids such as glutamate. Animal studies demonstrated the ability of erythropoietin to attenuate these mechanisms.

WHAT THIS STUDY ADDS: The administration of erythropoietin to infants with asphyxia with mild/moderate HIE was associated with favorable decreases in endogenous NO production, decreases in seizure activity, and improved neurodevelopmental outcomes to 6 months of age.

abstract

OBJECTIVE: The goal was to examine biochemical, neurophysiologic, anatomic, and clinical changes associated with erythropoietin administration to neonates with hypoxic-ischemic encephalopathy (HIE).

METHODS: We conducted a prospective case-control study with 45 neonates in 3 groups, a normal healthy group (N = 15), a HIE-erythropoietin group (N = 15; infants with mild/moderate HIE who received human recombinant erythropoietin, 2500 IU/kg, subcutaneously, daily for 5 days), and a HIE-control group (N = 15; did not receive erythropoietin). Serum concentrations of nitric oxide (NO) were measured at enrollment for the normal healthy neonates and at enrollment and after 2 weeks for the 2 HIE groups. The 2 HIE groups underwent electroencephalography at enrollment and at 2 to 3 weeks. Brain MRI was performed at 3 weeks. Neurologic evaluations and Denver Developmental Screening Test II assessments were performed at 6 months.

RESULTS: Compared with normal healthy neonates, the 2 HIE groups had greater blood NO concentrations (P < .001). At enrollment, the 2 HIE groups did not differ in clinical severity, seizure incidence, NO concentrations, or electroencephalographic findings. At 2 weeks of age, electroencephalographic backgrounds improved significantly (P = .01) and NO concentrations decreased (P < .001) in the HIE-erythropoietin group, compared with the HIE-control group; MRI findings did not differ between groups. At 6 months of age, infants in the HIE-erythropoietin group had fewer neurologic (P = .03) and developmental (P = .03) abnormalities.

CONCLUSION: This study demonstrates the feasibility of early administration of human recombinant erythropoietin to term neonates with HIE, to protect against encephalopathy. Pediatrics 2010;125: e1135–e1142
Brain injury secondary to perinatal asphyxia is a common cause of severe, long-term, neurologic deficits in newborns. Hypoxic-ischemic encephalopathy (HIE) occurs with an incidence of 1 to 4 cases per 1000 live births.

During HIE, free radicals are generated within mitochondria and also as byproducts in the synthesis of prostaglandins. These free radicals initiate a secondary phase of damage to the brain by attacking membranal fatty acids. Nitric oxide (NO) is involved in the cascade of metabolic events that contribute to HIE. It mediates, in part, the cytotoxic activity of macrophages, induces relaxation of blood vessels, and acts as a neurotransmitter in the central and peripheral nervous systems. Therefore, the therapeutic value of NO synthase inhibitors, among many other agents used to ameliorate the course of HIE, is currently under investigation in experimental animals.

Erythropoietin is a cytokine that originally was identified for its role in erythropoiesis and more recently was found to inhibit metabolic events that occur during HIE. The potential immediate protective effects of erythropoietin include decreased NO production, activation of antioxidant enzymes, reduction of glutamate toxicity, inhibition of lipid peroxidation, and reduction of inflammation. Long-term protective effects of erythropoietin include generation of neuronal anti-apoptotic mechanisms, stimulation of angiogenesis, and modulation of neurogenesis.

Preliminary data on exogenous erythropoietin has been shown to inhibit metabolic events that occur during HIE. The potential immediate protective effects of erythropoietin include decreased NO production, activation of antioxidant enzymes, reduction of glutamate toxicity, inhibition of lipid peroxidation, and reduction of inflammation.

In this prospective trial, we planned to study the efficacy of the administration of human recombinant erythropoietin as a therapeutic agent for neonates with mild/moderate HIE. The aim was to examine biochemical, neurophysiologic, anatomic, and clinical changes at up to 6 months of age associated with the systemic administration of erythropoietin in neonates with mild/moderate HIE.

METHODS

Patients

We conducted a prospective, case-control, pilot trial with 45 neonates in 3 groups, that is, a normal healthy group \(N = 15\), HIE-erythropoietin group \(N = 15\), and HIE-control group \(N = 15\). The normal healthy group \(N = 15\) included neonates without any history findings suggesting perinatal asphyxia or other diseases, who were enrolled for measurement of baseline NO concentrations. The HIE-erythropoietin and HIE-control groups included newborns with HIE who fulfilled the following inclusion criteria: (1) inborn infants at term gestation (38–42 weeks), (2) Apgar scores of ≤3 at 5 minutes and/or delayed first breath (>5 minutes after birth), (3) profound metabolic or mixed acidosis with serum bicarbonate levels of <12 mmol/L in initial arterial blood gas analyses, and (4) evidence of mild or moderate encephalopathy, such as lethargy, seizures, abnormal reflexes, or hypotonia, in the immediate neonatal period. Infants were excluded if they had any of the following: (1) twin gestation, (2) maternal diabetes mellitus, (3) congenital malformations of the central nervous system, (4) chromosomal abnormalities, (5) chorioamnionitis or congenital infections, or (6) intrauterine growth restriction. Maternal and delivery histories were reviewed thoroughly for all subjects; gestational age was assessed by using Ballard scores.

Patients underwent full neurologic assessments at enrollment, performed by a single coinvestigator. The severity of HIE was graded according to a modification of the system described by Sarnat and Sarnat, which included assessment of the level of consciousness, tone, primitive and tendon reflexes, autonomic function, and the presence of myoclonus and seizures. Neonates were monitored closely for side effects that might be related to the administration of the high dose of human recombinant erythropoietin, including allergic reactions, venous thrombosis, hypertension, electrolyte disturbances, and deterioration of renal and/or liver function.

The study was conducted at Tanta University Hospital (Tanta, Egypt) during the period from October 2007 to De-
Neonates in the HIE-control group (N = 15) received human recombinant erythropoietin (Eprex [Janssen-Cilag, Murarrie, Australia]). The erythropoietin was administered subcutaneously at a dose of 2500 IU/kg. Similar doses were administered daily for a total of 5 doses. Neonates in the HIE-erythropoietin group (N = 15) did not receive human recombinant erythropoietin; otherwise, the 2 groups received similar care. When seizures occurred, phenobarbital was used first. Phenytoin was added if seizures did not respond to phenobarbital.

**Intervention**

Within 4 to 6 hours after birth, after baseline blood samples had been obtained, neonates in the HIE-erythropoietin group (N = 15) received human recombinant erythropoietin (Eprex [Janssen-Cilag, Murarrie, Australia]). The erythropoietin was administered subcutaneously at a dose of 2500 IU/kg. Similar doses were administered daily for a total of 5 doses. Neonates in the HIE-control group (N = 15) did not receive human recombinant erythropoietin; otherwise, the 2 groups received similar care. When seizures occurred, phenobarbital was used first. Phenytoin was added if seizures did not respond to phenobarbital.

**Determination of Plasma NO Levels**

For all infants, blood samples were obtained for measurement of NO levels shortly after birth, before any intervention. This process was repeated at 2 weeks of life only for the 2 HIE groups. The total nitrite and nitrate levels were determined by using spectrophotometry, as described by Miranda et al. Blood samples were centrifuged for 10 minutes at ~1000 rpm. Sera were separated and stored at −20°C. Plasma samples were deproteinized with absolute ethanol to reduce turbidity. Quantitation of NO levels was achieved by measuring the stable NO metabolites nitrite and nitrate, which provided a reliable estimate of NO output in vivo. These anions were detected colorimetrically by using the Griess reagent. Because of the inability of the Griess reaction to detect nitrate, vanadium(III) was added first, as a reducing agent, to reduce nitrate to nitrite. The mixture was maintained at room temperature for 30 minutes. The absorbance was measured at 540 nm by using a double-beam spectrophotometer (Shimadzu UV-PC 1601 [Shimadzu, Kyoto, Japan]). A standard curve was obtained by plotting absorbance against concentration (micromoles per liter).

**Electroencephalography**

Electroencephalography (EEG) was performed at enrollment and was repeated at 2 to 3 weeks of age with a digital computerized apparatus (Neurofax EEG-9000 [Nihon Kodhen, Tokyo, Japan]). Recording was performed with a 16-channel, EEG polygraph system; bipolar montage was used, with electrodes placed on the basis of the 10–20 system, as modified for newborns. The recording speed was 30 mm/second. The state of the newborn and all movements during recording were noted. The single neurologist who interpreted all EEG tracings (Dr El-Gohary) was not aware of the clinical data or the treatment group. In each EEG tracing, background and paroxysmal abnormalities were evaluated. Three different types of background abnormalities were observed, that is, constant low voltage, constant discontinuity, and dysmaturity. A tracing was defined as constant low voltage when the detected background activity amplitude was constantly <20 μV. Constantly discontinuous tracings, defined when there was constant alternating of relatively high-amplitude bursts and low-voltage (≥45 μV) intervals, were further classified as exhibiting extreme discontinuity (maximal interval duration of >40 seconds), severe discontinuity (maximal interval duration of 20–40 seconds), or moderate discontinuity (maximal interval duration of <20 seconds). A dysmaturity tracing was defined when the observed maturational features were immature for ≥2 weeks, compared with the postmenstrual age of the patient. In addition, the incidences of paroxysmal abnormalities (abnormal EEG transients) and ictal EEG discharges were evaluated and were scored as described by Biagioni et al.24,25

**MRI**

Infants were transported to the MRI unit after 3 weeks, when they were in clinically stable condition. They were accompanied by a pediatrician and were monitored with pulse oximetry throughout the procedure. Chloral hydrate (50 mg/kg) was administered orally, to sedate subjects during the procedure. Imaging of the brain was performed by using a Siemens Magnetom Symphony 1.5-T system (Siemens, Munich, Germany). Images were obtained in the transverse plane, with T1-weighted spin echo (TR: 860 milliseconds; TE: 20 milliseconds), T2-weighted spin echo (TR: 3000 milliseconds; TE: 120 milliseconds), and age-related inversion recovery (TR: 3800 milliseconds; TE: 30 milliseconds; TI: 950 milliseconds) sequences. The posterior limb of the internal capsule was assessed as normal, equivocal, or abnormal. The basal ganglia and thalami were assessed as normal or with minimal, moderate, or severe abnormalities. Minimal abnormalities were considered if focal lesions were seen but the posterior limb of the internal capsule was normal. Moderate indicated focal abnormalities involving the posterior lentiform nuclei and ventrolateral nuclei of the thalami, with equivocal or abnormal signal intensity within the posterior limb of the internal capsule. Severe indicated widespread abnormalities in all regions of the basal ganglia and thalami and abnormal signal intensity within the posterior limb of the internal capsule. White matter abnormalities were documented according to which lobes of the brain were involved, whether there was a hemorrhagic element to the lesions, and whether they were subcortical, periventricular, or widespread. Abnormalities in white matter were graded as moderate or severe. Moderate indicated small focal lesions with short T1 and
short T2, consistent with hemorrhage, and/or areas of exaggerated long T1 and long T2 but no loss of gray/white matter differentiation. Severe indicated more-marked areas of abnormality, with larger areas of hemorrhage or exaggerated long T1 and T2 with loss of gray/white matter differentiation, consistent with infarction. All images were assessed by an experienced radiologist (Dr El-Barbary), who was masked to EEG readings, clinical data, and treatment group.

Neurodevelopmental Outcomes

Detailed neurologic examinations at the age of 6 months were performed by a single pediatric neurologist, who was unaware of the EEG and brain MRI readings at the time of evaluation. The patients were further evaluated with the Denver Developmental Screening Test II. The test includes multiple items to examine 4 major categories (gross motor, language, fine motor-adaptive, and personal-social). Infants are scored for each test item as advanced, normal, caution, or delayed. The overall developmental assessment of an infant can be considered failed if there are ≥2 delays, questionable if there is 1 delay and/or ≥2 cautions, and normal if there are no delays and a maximum of 1 caution. The Denver Developmental Screening Test II is a simple screening test that is easily administered and was shown to predict severe adverse outcomes accurately.

Statistical Analyses

We analyzed the data by using SPSS for Windows 11 (SPSS Inc, Chicago, IL). Data were expressed as mean ± SD or number and proportion. For continuous variables, t tests and Mann-Whitney tests were used. For categorical variables, χ² tests and Fisher’s exact tests were used. For repeated measurements of NO concentrations, analysis of variance was used. Differences were considered significant when P values were <.05.

RESULTS

A total of 45 neonates were enrolled in the study and stratified into 3 groups, that is, a normal healthy group (N = 15), HIE-erythropoietin group (N = 15), and HIE-control group (N = 15). The 2 HIE groups were similar in demographic and clinical characteristics (Table 1). The HIE-erythropoietin group received fewer transfusions of packed red blood cells during their hospital stays. Neonates tolerated the high dose of human recombinant erythropoietin without any side effects specific to its use. No neonates in the HIE-erythropoietin group and 2 neonates in the HIE-control group developed necrotizing enterocolitis (NEC) (P = .24). Rates of survival without NEC did not differ between the 2 groups (15 and 12 neonates, respectively; P = .067). Hospital courses and clinical outcomes were similar in the 2 groups (Table 2). The incidences of clinical seizures were similar at enrollment, but episodes of breakthrough seizures were seen more frequently among infants in the HIE-control group at day 3 (P = .033) and at day 5 (P = .025) (Fig 1). EEG backgrounds were similar in the 2 HIE groups at enrollment. In the 2-week repeated EEG tracings, 10 infants in the HIE-erythropoietin group and 3 infants in the HIE-control group (P = .01) had normal backgrounds. The 5 abnormal EEG tracings in the HIE-erythropoietin group were in the form of moderate discontinuity and seizure activity (n = 4) and abnormal EEG discharges (n = 1). In the HIE-control group, abnormal:

| TABLE 1 Demographic Data and Clinical Characteristics for Infants With HIE (N = 50) |
|----------------------------------|-----------------|-----------------|
| **Erythropoietin Group** (N = 15) | **Control Group** (N = 15) | **P** |
| Birth weight, mean ± SD, g* | 3190 ± 267 | 3200 ± 342 | .93 |
| Gestational age, mean ± SD, wk* | 38.7 ± 2 | 38.6 ± 0.9 | .72 |
| Male, n (%)b | 9 (60) | 8 (53) | .54 |
| Apgar score at 1 min, median (IQ range)* | 2 (2–2) | 2 (1–2) | .51 |
| Apgar score at 5 min, median (IQ range)* | 3 (3–3) | 3 (3–3) | .15 |
| Delivery mode, n (%)b | | | |
| Cesarean section | 4 (27) | 4 (27) | .64 |
| Vaginal delivery | 7 (47) | 6 (40) | .54 |
| Instrumental | 4 (27) | 5 (33) | .57 |
| Maternal age, mean ± SD, y* | 26.5 ± 5.1 | 28.5 ± 3.96 | .29 |
| Fetal bradycardia, n (%)b | 12 (80) | 11 (73) | .01 |
| Meconium, n (%)b | 2 (13) | 3 (20) | .5 |
| Resuscitation in delivery room, n (%)b | 3 (20) | 4 (27) | .5 |
| Positive-pressure ventilation | 10 (67) | 10 (67) | .6 |
| Intubation | 4 (27) | 5 (33) | .57 |
| Chest compression | 1 (7) | 0 (0) | .24 |
| HIE grade, n (%)b | | | |
| Mild (grade I) | 3 (20) | 4 (27) | .67 |
| Moderate (grade II) | 12 (80) | 11 (73) | |

* The Mann-Whitney test was used.

b The χ² test was used.

| TABLE 2 Hospital Course for Infants With HIE (N = 30) |
|----------------------------------|-----------------|-----------------|
| **Erythropoietin Group** (N = 15) | **Control Group** (N = 15) | **P** |
| Ventilator support | 7 (47) | 9 (60) | .44 |
| Inotropic support | 2 (13) | 3 (20) | .71 |
| Red blood cell transfusions | 0 (0) | 2 (13) | .24 |
| Platelet transfusions | 2 (13) | 3 (20) | .71 |
| Multiple-organ failure | | | |
| NEC | 3 (20) | 7 (47) | .2 |
| Survival without NEC | 15 (100) | 12 (80) | .07 |

The χ² test was used.
Table 3

<table>
<thead>
<tr>
<th>MRI Findings</th>
<th>Erythropoietin Group</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal EEG Background (N = 10)</td>
<td>Abnormal EEG Background (N = 5)</td>
</tr>
<tr>
<td>Normal</td>
<td>6 (60)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>Minimal basal ganglia and thalami</td>
<td>3 (30)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>Moderate basal ganglia and thalami</td>
<td>0 (0)</td>
<td>2 (40)</td>
</tr>
<tr>
<td>Moderate white matter</td>
<td>1 (10)</td>
<td>1 (20)</td>
</tr>
</tbody>
</table>

The χ²-test was used to compare the overall numbers of subjects with abnormal EEG findings in the HIE-erythropoietin and HIE-control groups.

*Only 11 cases are presented, because 1 patient did not undergo a MRI study.

FIGURE 1
Incidence of seizures at baseline, at 72 hours, and at 5 days among infants with HIE in both groups (N = 30). Black bars indicate the control group; gray bars, erythropoietin group. a P = .035; b P = .025.

DISCUSSION
In this study, we demonstrated the feasibility and potential efficacy of the use of human recombinant erythropoietin for infants with HIE. The use of human recombinant erythropoietin was associated with promising biochemical, neurophysiologic, and clinical improvements, including decreased serum NO concentrations, fewer seizures, improved EEG backgrounds, and favorable neurologic outcomes at 6 months of age; however, MRI findings did not improve.

Infants with HIE displayed higher NO concentrations, compared with healthy control subjects. This finding is consistent with previous studies that reported increased NO levels after perinatal asphyxia, in a way that correlated with the severity of brain damage in infants with HIE. In our study, NO concentrations decreased significantly within 2 weeks in infants with HIE who received human recombinant erythropoietin. This is the first study to report the impact of human recombinant erythropoietin on NO concentrations in infants with HIE. This result suggests that human recombinant erythropoietin ameliorates the production of endogenous NO. In their study with rat oligodendrocytes, Genc et al demonstrated the suppressive effect of erythropoietin on endogenous inducible NO production. It has been suggested that erythropoietin exerts its neuroprotective properties by reducing the formation and antagonizing the toxicity of free radicals mediated by NO.

The use of human recombinant erythropoietin was associated with the development of normal EEG backgrounds, compared with control infants with HIE (10 vs 3 infants; P < .01). Clinical seizures also significantly decreased in the erythropoietin group at the third and fifth days of life. These results were consistent with the results of previous animal studies in which erythropoietin was neuroprotective when administered before or after hypoxic insults. Preconditioning of rats with erythropoietin pro-
tected against brain injury during the acute phase of seizures and suppressed apoptotic neuronal cell death.\textsuperscript{34} Erythropoietin administered directly after an acute hypoxic insult increased the latency and reduced the duration of seizures; it also protected against seizure-induced cell loss and programmed cell death caused by subsequent status epilepticus.\textsuperscript{35} In human adults with stroke, erythropoietin was shown to reduce infarction size slightly and to improve clinical outcomes slightly.\textsuperscript{36} In their study of neonatal rats with focal cerebral ischemia, Chang et al\textsuperscript{37} noted markedly preserved hemispheric volume in the erythropoietin-treated group.

Neurodevelopmental outcomes according to the Denver Developmental Screening Test II and neurologic examinations at 6 months of age significantly improved in the erythropoietin group, compared with the HIE-control group.

Multiple animal studies explored the mechanisms through which erythropoietin exerts its neuroprotection. It has been shown that erythropoietin has anti-apoptotic and antiinflammatory roles during the acute postinjury period; it also has neurogenic and vasculogenic effects in the recovery period.\textsuperscript{37–40} We enrolled infants with mild/moderate HIE and did not include any subjects with severe HIE, on the basis of previous studies on head cooling that demonstrated a lack of efficacy to preserve the brain after severe HIE. Interestingly, a recent trial demonstrated a neuroprotective role for human recombinant erythropoietin only in moderate, and not severe, HIE.\textsuperscript{17}

In addition to neuroprotection, human recombinant erythropoietin may have other potential benefits. All patients survived without NEC in the erythropoietin group, whereas 1 patient died and 2 patients had NEC in the HIE-control group. These differences were not significant ($P = .067$), which might be attributable to small numbers, and further investigations are needed to examine these outcomes. Of note, a recent trial demonstrated potential benefits in many aspects of health among extremely low birth weight infants who received high doses of human recombinant erythropoietin, including less severe intracranial hemorrhage and white matter injury, more hemodynamic stability, and less severe NEC.\textsuperscript{41} Erythropoietin receptors are known to be present in the developing intestine, and the administration of erythropoietin was shown previously to elicit trophic and protective effects in this system.\textsuperscript{42}

**CONCLUSION**

Early administration of erythropoietin to infants with mild/moderate HIE was efficacious in controlling seizures, improving EEG backgrounds, and producing favorable neurodevelopmental outcomes at 6 months of age.
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