

No Neuroprotective Effect of Erythropoietin Under Clinical Treatment Conditions in a Rabbit Model of *Escherichia coli* Meningitis

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ABSTRACT: Despite effective antibiotic treatment, neuronal injury is frequent among children and adults with bacterial meningitis resulting in a high rate of death and neurologic sequelae. The hematopoietic cytokine erythropoietin (EPO) provides neuroprotection in models of acute and chronic neurologic diseases. We studied whether recombinant EPO (rEPO) reduces neuronal damage in a rabbit model of *Escherichia coli* meningitis. Inflammation within the central nervous system (CNS) was monitored by measurement of bacterial load, pleocytosis, protein, and lactate in the cerebrospinal fluid (CSF). Neuronal damage was measured by quantification of the density of apoptotic neurons in the hippocampal dentate gyrus and the concentration of the global neuronal destruction marker neuron-specific enolase (NSE) in CSF. To increase clinical relevance, rEPO was applied as adjunctive therapy from the beginning of antibiotic therapy 12 h after infection. EPO treatment applied as an intravenous injection at a dose of 1000 IU/kg body weight resulted in plasma concentrations of 6993 ± 1406 mIU/mL, CSF concentrations of 1291 ± 568 mIU/mL, and a CSF-to-plasma ratio of 0.18 ± 0.07 (mean \pm SD) 6 h after injection. Under these treatment conditions, no anti-inflammatory or neuroprotective effect of EPO was observed. (*Pediatr Res* 62: 680–683, 2007)

In children and adults, bacterial meningitis continues to be associated with high rates of death and neurologic sequelae. Neuronal injury in bacterial meningitis is caused by diverse mechanisms including the local and systemic inflammatory host response to bacterial invasion and direct toxicity of bacterial compounds (1).

The hematopoietic cytokine erythropoietin (EPO) is a major component of the endogenous tissue-protective system. In the adult brain, EPO and EPO receptors are constitutively expressed only at a low level but are rapidly up-regulated as a response to metabolic stress of neurons (2) and provide neuroprotection in a multimodal manner including the Jak2 (3) and the NF κ B (4) systems. Extrinsic application of human recombinant erythropoietin (rEPO) has been shown to be neuroprotective in various models of acute and chronic neurologic diseases, including ischemic stroke, chronic neuroinflammatory disorders such as experimental autoimmune encephalitis (5), and a rabbit model of subarachnoid hemorrhage (6). It acts in an antiapoptotic (3), antioxidative (7,8), anti-

inflammatory (9,10), and glutamate-inhibitory (11) manner. Since neuronal injury in bacterial meningitis is mediated by inflammation, reactive oxygen species and toxicity of excitatory amino acids, these properties of rEPO appeared promising with respect to a neuroprotective effect of rEPO in the therapy of this disease. EPO is a body's own hormone and rEPO has been applied for long time in the therapy of different types of anemia. The side effects of rEPO are limited and mainly occur during long-term administration. As bacterial meningitis is an acute infection, a potentially neuroprotective treatment would be administered for a short period and should be well tolerated. Therefore, EPO appeared to be an ideal candidate for adjunctive treatment in bacterial meningitis.

The earliest possible starting point for adjunctive therapy in the treatment of patients suffering from bacterial meningitis is the time of admission to a medical center. A multitude of experimental studies have been published demonstrating a neuroprotective effect of the investigated pharmaceutical compounds in animal models, when applied before the impairing event (e.g. ischemic stroke) yet failing to be neuroprotective when applied under clinical conditions (12,13). For this reason, we investigated the effect of rEPO in a rabbit model of Gram-negative bacterial meningitis with rEPO administered from the onset of antibiotic treatment.

MATERIALS AND METHODS

Pathogen. An *Escherichia coli* K1-strain isolated from a child with neonatal meningitis (gift of Dr. G. Zysk, Institute of Medical Microbiology, Düsseldorf, Germany) was cultured on blood agar plates at 37°C, harvested with 0.9% saline, and preserved in aliquots containing 10^{10} colony-forming units per milliliter (CFUs/mL) at -70°C . Minimal inhibitory concentration and minimal bactericidal concentration for ceftriaxone determined by broth microdilution were 0.0625 and 0.125 mg/L, respectively.

Animal model. Animal experiments were performed in accordance with national animal experimentation guidelines and were approved by the District Government of Braunschweig, Lower Saxony. Anesthetized rabbits were infected by intracisternal injection of 300 μL containing \log_{10} 4.0 CFUs (median, \log_{10} ; range, 3.9–5.3 CFUs) *E. coli*. Cisternal cerebrospinal fluid (CSF) and arterial blood were drawn at 0, 12, 14, 18, and 24 h. At each time point, CSF leukocytes were counted and bacterial titers were determined. The remaining CSF as well as the arterial blood anticoagulated with ethylenediamine tetraacetic acid were immediately centrifuged at $3000 \times g$ for 10 min, and the supernatants were frozen in liquid nitrogen and stored at -70°C . At 23.5 h post-infection, an arterial blood gas analysis was performed (Radiom-

Received June 12, 2007; accepted July 21, 2007.

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This work was supported by the Else Kröner-Fresenius-Stiftung.

Abbreviations: CFU, colony-forming unit; CSF, cerebrospinal fluid; EPO, erythropoietin; rEPO, recombinant human erythropoietin; HE, hematoxylin-eosin (staining); NSE, neuron-specific enolase

eter ABL 605, Diamond Diagnostics) to determine arterial oxygen (P_{O_2}) and carbon dioxide tension (P_{CO_2}) and pH. The animals were killed at 24 h post-infection (14).

Treatment. Before infection, animals were randomized for treatment with rEPO or an equal volume of saline. At 12 h post-infection, all animals ($n = 26$) received intravenous antibiotic treatment (ceftriaxone, 125 mg/kg body weight, Hoffmann LaRoche, Grenzach-Wyhlen, Germany). In 10 animals, rEPO (a kind gift of Janssen-Cilag, Neuss, Germany) was administered as bolus injection intravenously (1000 IU/kg body weight) at the time point of onset of antibiotic treatment and 6 h later. This dose was chosen in accordance with a study describing a neuroprotective effect in a rabbit model of subarachnoid hemorrhage (6).

Analysis of CSF. Protein content was quantified using the BCA Protein Assay (Pierce, Rockford, IL) with bovine serum albumin as standard according to the manufacturer's instructions. CSF lactate was determined enzymatically (Rolf Greiner Biochemica, Flacht, Germany). Plasma and CSF concentrations of EPO were quantified using a commercially available enzyme immunoassay (R&D Systems, Minneapolis, MN). The global neuronal destruction marker neuron-specific enolase (NSE) was quantified in CSF by enzyme immunoassay with mouse anti-human monoclonal antibody against the γ -subunit of NSE (DRG Instruments, Marburg, Germany). CSF bacterial titers were quantified by plating serial 10-fold dilutions on blood agar plates, which were subsequently incubated for 20 h at 37°C (15).

Histopathology. Immediately after sacrifice, the animals' brains were removed and fixed in 4% paraformaldehyde and embedded in paraffin. *In situ* tailing and hematoxylin-eosin (HE) staining were performed as described previously (16).

Quantification of apoptotic neurons. Four coronal sections from each rabbit's left hemisphere were histologically analyzed using the Analysis Software Imaging System (BX51; Olympus, Hamburg, Germany; software AnalySIS 3.2; Soft Imaging System GmbH, Münster, Germany). In coded sections, a blinded observer counted dentate granule cells labeled by *in situ* tailing. Adjacent sections stained by hematoxylin-eosin (staining) (HE) showed morphologic features of apoptosis in the same neurons. The density of apoptotic neurons was expressed as the number of marked cells per square millimeter of the granule cell layer of the hippocampal dentate gyrus, measured in the adjacent HE-stained section.

Statistical analysis. In the presence of normal gaussian distribution, parameters of two groups were compared using an unpaired *t* test. In the absence of normal distribution, statistical analysis was performed using the two-tailed Mann-Whitney *U* test; $p < 0.05$ was considered significant.

RESULTS

Course of disease. In this rabbit model of *E. coli* meningitis, one of 10 in the rEPO-treated group and three of 16 rabbits in the control group died before the end of the experiment. Only animals surviving at least 23 h after infection were included into the analysis to avoid effects of survival time on the rate of neuronal apoptosis (10 animals in the rEPO-treated

group and 14 in the control group). Animals dying prematurely did not differ substantially from the surviving animals with respect to CSF pleocytosis and bacterial load. Twelve hours after intracisternal inoculation of *E. coli*, rabbits developed full clinical presentation of meningitis [bacterial CSF load \log_{10} 7.93 CFUs/mL (5.30–9.00) (median, min, max)] and septicemia [bacteremia of \log_{10} 3.00 CFUs/mL (\log_{10} 1.48, 5.48 (median, min, max))] without relevant differences between the two treatment groups. Systemic inflammation parameters in *E. coli* meningitis including arterial P_{O_2} , P_{CO_2} , and pH at 23.5 h post-infection did not differ significantly between the rEPO-treated and the control group (Table 1).

CSF analysis. At 12 h after intracisternal inoculation of *E. coli*, all rabbits developed meningeal inflammation (pleocytosis, elevated CSF levels of protein and lactate, high bacterial CSF load; Table 1). There was no significant difference between the rEPO group and the controls before treatment. CSF leukocytes, protein, and lactate further increased between 12 h and 24 h. The 24-h CSF protein and lactate concentrations did not differ between the rEPO-treated and the respective control animals. rEPO did not affect bacterial elimination (Table 1). Plasma and CSF levels of EPO were determined 18 h after infection. EPO levels of meningitic control animals ($n = 3$) treated with ceftriaxone only were below the detection limit of the assay at 6 mIU/mL in CSF and plasma. In the animals treated with intravenous rEPO at a dose of 1000 IE/kg body weight, EPO concentrations in CSF were 1291 ± 568 mIU/mL ($n = 10$) and 6993 ± 1406 mIU/mL in plasma (mean \pm SD, $n = 9$) 6 h after injection. The CSF-to-plasma ratio of rEPO was 0.18 ± 0.07 in the rEPO-treated animals.

Global neuronal damage. NSE in CSF as an indicator of neuronal destruction was quantified at 12 h and 24 h after infection (Table 1) (14). The NSE concentration at 12 h and 24 h post-infection did not differ significantly between rEPO-treated and control animals (Table 1, $p = 0.60$ and 0.72 , respectively, both *U* test).

Neuronal apoptosis in the hippocampal dentate gyrus. Neuronal apoptosis was regularly induced by *E. coli* menin-

Table 1. Parameters of CSF inflammation, global neuronal damage, and blood gas analysis in rEPO-treated rabbits and controls

	Time after infection (h)	<i>E. coli</i> meningitis	
		Controls	rEPO-treated
Bacterial load (log CFUs/mL)*	12	7.65 (5.30–9.00)	8.00 (7.00–9.00)
	24	2.70 (<2.00–3.30)	2.00 (<2.00–6.00)
Leukocytes (cells/ μ L)*	12	581.5 (120–2261)	1424 (99–3783)
	24	9483 (1578–26450)	4992 (501–61870)
Protein (mg/mL)*	12	2750 (1500–5600)	2550 (1100–4200)
	24	5700 (3300–10000)	5400 (3200–11000)
Lactate (mmol/L)†	12	9.08 (\pm 4.13)	11.90 (\pm 4.98)
	24	15.18 (\pm 4.60)	15.71 (\pm 2.93)
NSE*	12	26.0 (11.0–219.0)	26.0 (11.0–296.0)
	24	303.0 (32.0–702.0)	143.0 (26.0–1060)
Arterial pH†	23.5	7.57 (\pm 0.11)	7.53 (\pm 0.06)
Arterial P_{O_2} †	23.5	105.8 (\pm 12.7)	121.6 (\pm 27.7)
Arterial P_{CO_2} †	23.5	17.96 (\pm 8.9)	15.8 (\pm 7.1)

* Median (minimum; maximum), no significant difference between rEPO-treated and respective control group, $p > 0.05$ (*U* test).

† Mean (\pm SD), no significant difference between rEPO-treated and respective control group, $p > 0.05$ (*t* test).

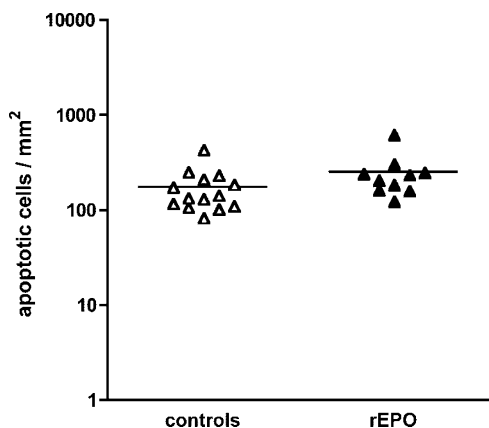


Figure 1. Rate of hippocampal apoptotic granule cells per area of the granule cell layer of the dentate gyrus in rabbit bacterial meningitis with or without adjunctive rEPO treatment. No significant difference was found in the rate of apoptotic neurons between rEPO-treated animals and respective controls. Symbols represent measurements of each individual animal (rEPO-treated *E. coli* meningitis: ▲, controls: Δ), and horizontal bars represent means.

gitis in the granule cell layer of the dentate gyrus at 24 h after infection. Apoptotic cells were identified by *in situ* labeling of DNA double-strand breaks and confirmed by morphologic criteria (14). The rate of apoptotic neurons ranged from 85 to 633/mm² (area of the granule cell layer, 208 ± 120, mean ± SD). Adjunctive rEPO treatment administered from the time point of antibiotic treatment did not reduce the rate of neuronal apoptosis in the hippocampal dentate gyrus in comparison to control animals ($p = 0.12$, unpaired *t* test, Fig. 1).

DISCUSSION

Bacterial meningitis is a life-threatening infection of children and adults with high rates of neurologic sequelae including epileptic seizures, mental retardation, and learning disorders. Despite effective antibiotic treatment, stimulation of the host's systemic and local immune defense and direct toxicity of bacterial products result in impairment of the CNS. Neuropsychological deficits and cognitive sequelae in bacterial meningitis are at least in part caused by neuronal cell death detected in the hippocampal formation of patients (17). Bilateral hippocampal atrophy is seen by magnetic resonance imaging in patients who survived meningitis (18). In search of neuroprotective treatment strategies, we used a rabbit model of bacterial meningitis, which regularly exhibits neuronal apoptotic cell death in the granular cell layer of the hippocampal formation. In the absence of meningitis, the density of apoptotic neuronal cells/mm² in the hippocampal dentate gyrus was found to be approximately 10 apoptotic neurons/mm² in previously healthy rabbits killed immediately without long-term anesthesia (19). In uninfected control rabbits subjected to 24 h of urethane long-term anesthesia, antibiotic treatment, and repeated suboccipital punctures the density of apoptotic neuronal cells was approximately 40 apoptotic neurons/mm² of the dentate gyrus (14). The greatly increased rate of apoptotic neurons in the hippocampal dentate gyrus of rabbits suffering from meningitis (in the present study: 208 ± 120 apoptotic neurons/mm², mean ± SD) can be attributed to the

infection and serves as a parameter for the evaluation of putatively neuroprotective therapeutic agents.

In search of neuroprotective treatment strategies, we evaluated the hematopoietic cytokine EPO. The recombinant human cytokine rEPO is currently one of the most promising neuroprotective agents: it has been shown to be beneficial in various models of neurologic diseases. Neuroprotective effects have been found in models of acute neuronal damage such as ischemic stroke (20), neonatal hypoxia-ischemia (21), traumatic brain injury (22), subarachnoid hemorrhage (6), and pilocarpine-induced status epilepticus (23). Likewise rEPO was neuroprotective in chronic models of neuroinflammation such as experimental autoimmune encephalomyelitis (10) or in a model of Parkinson's disease (24). In animal models, recombinant human EPO exhibited its neuroprotective properties in different mammalian species including rabbits (6). Hippocampal neuronal cells, which are especially vulnerable during bacterial meningitis, were also protected from neuronal death in animal models (23). This prompted us to investigate the neuroprotective effect of rEPO in experimental bacterial meningitis.

Many studies revealed neuroprotective effects of a variety of substances when applied in a narrow time window before or shortly after the induction of neuronal impairment but failed to be beneficial under clinical treatment conditions (12,13). In clinical practice, the earliest time point of adjunctive treatment in bacterial meningitis is the time of a patient's admission to a medical center. Hence, in the present study, a design was chosen in which rEPO was administered from the time of antibiotic treatment, a treatment that should be administered as quickly as possible after diagnosing bacterial meningitis. To date, no data have been published on the pharmacokinetics of rEPO in bacterial meningitis. Only very few data are available about the penetration of EPO in the CSF in patients with noninflamed meninges (25) and in the brain tissue of rats after cerebral hypoxia (26). In our rabbit model of *E. coli* meningitis, we investigated the pharmacokinetics of rEPO and could demonstrate a high penetration rate with a CSF-to-plasma ratio of 0.18 ± 0.07 (mean ± SD) 6 h after injection.

Additional rEPO treatment did not affect the course of the disease in our model of *E. coli* meningitis in a statistically significant manner. One of 10 rabbits in the rEPO-treated group *versus* three of 16 rabbits in the control group died before the end of the experiment, *i.e.* mortality was not significantly different ($p = 1.00$, Fisher exact test). Even though we cannot fully exclude a type II error, we assume that no true difference in mortality was present. Accordingly, no significant differences could be found for parameters of inflammation in CSF or systemic circulation and for bacterial elimination (Table 1).

Despite high rEPO concentrations reached in the CSF in bacterial meningitis, no neuroprotective effect of rEPO was found under clinical treatment conditions, neither with respect to the rate of apoptotic neuronal cells in the hippocampal dentate gyrus nor to the global neuronal injury marker NSE in CSF.

In conclusion, no neuroprotective effect of rEPO could be found in this rabbit model of *E. coli* meningitis and therefore

rEPO is not recommended as an adjunct to antibacterial therapy in human bacterial meningitis.

Acknowledgments. The authors thank Stefanie Bunkowski for excellent technical support.

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