A comparison of the effectiveness of erythropoietin and dexamethasone therapy in Streptococcus pneumoniae induced meningitis in rabbits.

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Abstract

Background: Although bacterial meningitis can be seen at all ages, it is more common in childhood. The primary treatment option in bacterial meningitis is antibiotics. Ancillary therapies are required due to inflammation developing secondary to antibiotherapy. The purpose of this study was to investigate whether Erythropoietin (EPO), which has recently come to prominence with its anti-inflammatory effects, can be used as an alternative to steroid therapy, with its known side-effects and debatable efficacy, in the treatment of meningitis.

Methods: Forty female New Zealand rabbits weighing 1900-3100 g were divided into six groups. The study groups consisted of seven animals each and the control group of five. *Streptococcus pneumoniae* at 1×10^7 colony forming units (in 0.2 ml 0.9% NaCl) was injected into the cisterna magna of the induced meningitis groups, while the control group received saline solution. Seven rabbits with induced meningitis received 1000 IU/kg EPO, 7 received EPO+antibiotic, 7 received 0.25 mg/kg dexamethasone+antibiotic and 7 received dexamethasone. Cerebrospinal fluid (CSF) and serum specimens were collected at the end of 24 h and the rats were euthanized. IL-1β, TNF-α and CRP levels were investigated from CSF and serum. SPSS software was used for statistical analysis.

Results: Serum and CSF IL-1β, TNF-α and CRP values in the groups with induced meningitis were significantly higher than those in the control group. No significant difference in terms of serum and CSF CRP, TNF-α and IL-1β values was determined between the group with induced meningitis alone and the group receiving EPO therapy (p values p=0.949, p=0.159, p=0.655, p=0.848, p=0.749 and p=0.655, respectively).

Conclusion: Our study results show that EPO has no effect on serum or CSF IL-1β, TNF-α and CRP in pneumococcal meningitis in rabbits. To the best of our knowledge, this is the first study to investigate the effect of EPO on CRP, IL-1β and TNF-α levels in serum and CSF in meningitis.

Introduction

Cytokines are important in bacterial meningitis in terms of showing severity of meningitis, because cytokines are mediator molecules in the regulation of dimension, structure and duration of inflammatory response [1]. The greatest injury in meningitis occurs with cytokine release occurring in association with intense bacterial death with the start of antibiotic therapy. Proinflammatory cytokine response begins with TNF-α and IL-1 release [2,3]. Administration of anti-TNF-α and anti-IL-1β in experimental models of meningitis in animals has been shown to reduce inflammatory response in CSF [1,4]. Steroid administration before or concurrently with first antibiotic has been shown to reduce cerebral edema in *Streptococcus pneumoniae* and *Haemophilus influenzae* meningitis and to prevent complications, and steroids have for long been used for this purpose.

Erythropoietin (EPO) is a glycoprotein that stimulates erythrocyte formation as a mitosis stimulating factor and differentiating hormone in erythroid stem cells and that

serves as a cytokine for erythrocytes. Studies have shown that at the cellular level EPO reduces programmed cell death in neuronal cells, stimulates release of cell-protecting neurotransmitters such as dopamine, inhibits release of free oxygen radicals, nitric oxide and inflammatory mediators, regulates intracellular calcium and prevents kainic acid intoxication [5]. Experimental studies of autoimmune encephalomyelitis (EAE) have also shown a decrease in clinical severity of encephalomyelitis following systemic administration of EPO [6]. In addition to all these effects, inhibition has been shown in IL-2, IFN-γ, TNF-α, IL-4, IL-5, IL-6 and IL-10 in children and adults with administration of EPO following lipopolysaccharide stimulation [7]. This suggests that EPO may also exhibit anti-inflammatory effects [8]. EPO exhibits its anti-inflammatory effect by inhibiting nuclear factor kappa B (NF-κB) dependent immune-driven cytokine production [9].

The purpose of this study was to investigate the effect of EPO on IL-1β, TNF-α and CRP levels in pneumococcal meningitis, in the light of the fact that neuronal injury in bacterial meningitis emerges in association with inflammation, reactive oxygen radicals and excitatory amino acid toxicity, and to compare that effect with that of dexamethasone.

Materials and Methods

This study was performed with the approval of the Karadeniz Technical University (KTU) Animal Experiments Local Ethical Committee and with the support of the KTU Scientific Research Projects Coordination Unit.

Forty female New Zealand rabbits weighing 1900-3100 g were used. Rabbits were obtained from a certificated private laboratory. After the animals had been transported under appropriate conditions they were kept in quarantine for 15 days in the KTU Animal Experiments Laboratory. During the procedure, anesthesia was induced with intramuscular 50 mg/kg ketamine and 4 mg/kg xylazine. Rabbits were divided into six groups, five study groups and a control group. The study groups consisted of seven animals each and the control group of five:

- Group I: Meningitis

- Group II: Meningitis and EPO treatment

- Group III: Meningitis plus EPO and ceftriaxone treatment

- Group IV: Meningitis and dexamethasone treatment

- Group V: Meningitis plus dexamethasone and ceftriaxone treatment

- Group VI: Control

Following anesthetic procedures, the study groups received 1×10^7 Colony Forming Units (CFUs) of *S. pneumoniae* (in 0.2 ml 0.9% NaCl) injected into the cisterna magna using a 25 gauge spinal needle. The control group received saline solution injected into the cisterna magna. The strain used was previously isolated from the CSF of patients

monitored in our hospital with a diagnosis of meningitis, with a ceftriaxone minimal inhibitory concentration (MIC) value of ≤ 0.5 mcg/ml. At the 12th hour post-injection, polymorphonuclear leukocyte (PMNL) levels were investigated in rabbits' CSF under direct microscopy for confirmation of meningitis. Experimental animals in Group II received 1000 IU/kg EPO intravenously (iv) (from the lateral ear vein), animals in Group III received simultaneous 1000 IU/kg EPO and 125 mg/kg ceftriaxone, animals in Group IV received 0.25 mg/kg dexamethasone iv and animals in Group IV received simultaneous 0.25 mg/kg dexamethasone and 125 mg/kg ceftriaxone iv. In the control group, small CSF samples were again collected at the 12th hour, but no other procedure was performed [10-12].

At the end of 24 h monitoring and following assessment of clinical status, CSF and serum specimens were collected from all animals under ketamine and xylene anesthesia. Hemogram tests were performed as soon as specimens were collected using a fully automatic Beckhman Coulter LH 750 blood count device with its own original solutions in the biochemistry laboratory. Blood specimens were centrifuged for 15 min at 3000 g/min. The sera were then separated, while CSF specimens were stored in a deep freeze at -80 degrees until the day of the study. Serum and CSF IL-1β, TNF-α and CRP were measured with enzyme-linked immunosorbent assay (ELISA) using a commercial kit and a VERSA (Designed by Molecular Devices in California, USA) microplate reader. Data were analyzed on SPSS software. Results were expressed as mean and standard deviation. The Mann-Whitney U test was used in two-group comparisons, and Kruskal-Wallis analysis of variance for comparison of more than two groups. Significance was set at $p<0.05$ for all analyses.

Results

Observation at the 12th hour revealed slowed movements, lack of interest in food and water and increased temperature in rabbits in the groups infected with *S. pneumoniae*. One infected rabbit died following trembling and contraction in the entire body at the 13th hour. No change was determined in the control group.

Growth in CSF was observed in meningitis groups I, II and IV at the 24th hour, while no growth was determined in groups III and V. Clinical improvement (increased movement, interest in feeding, body temperature returned to normal) was observed at the 12th hour post-treatment in groups III and V, while clinical worsening (lethargy, increased temperature, lack of interest in food and water, fur loss and trembling) was observed in groups I, II and IV. No clinical abnormality was observed in the control group at 24 h observation.

A comparison of all groups' BOS, CRP, TNF-α and IL-1β values is shown in Tables 1 and 2.

No significant difference was determined in serum and

CSF CRP, TNF-α and IL-1β values between groups I and II (Table 3).

Group I serum CRP and TNF-α values were significantly higher than those in Group III (p values p=0.002 and p=0.003, respectively). However, no significant difference was determined between the two groups in terms of IL-1β levels. Group I CSF CRP level was significantly higher than that in Group III (p=0.013), while the Group III CSF IL-1β value was higher than that in Group I (p=0.002). No difference was determined between TNF-α values.

No significant difference was determined between Group I serum CRP, TNF-α and IL-1β values and those in Group IV. Although no significant difference was observed in CSF CRP and IL-1β values, Group I CSF TNF-α was significantly higher than that in Group IV (p=0.018).

Serum CRP and TNF-α values in Group I were significantly higher than those in group V (p values p=0.013 and p=0.006, respectively). However, no significant difference was determined between the two groups' serum IL-1β values. While significant differences were determined in CSF CRP and IL-1β values between groups I and V (p=0.006 and p=0.009, respectively), no significant difference was determined in CSF TNF-α levels.

CRP, TNF-α and IL-1β values in serum and CSF in Group I were significantly higher than those in Group VI (p values p=0.004, p=0.004, p=0.004, p=0.004, p=0.004 and p=0.004, respectively)

Serum CRP, TNF-α and IL-1β and CSF TNF-α and IL-1β values in Group II were significantly higher than those in Group VI (p values p=0.004, p=0.004, p=0.004, p=0.004, p=0.004 and p=0.004, respectively)

Group III serum CRP and IL-1β values were significantly higher than those in Group VI (p values p=0.009 and p=0.012, respectively). However, no significant difference was determined in serum TNF-α levels. Group III CRP, TNF-α and IL-1β values in CSF were significantly higher than those in Group VI (p values p=0.004, p=0.004 and p=0.004, respectively).

Group IV CRP, TNF-α and IL-1β values in serum and CSF were significantly higher than those in Group VI (p values p=0.004, p=0.004, p=0.004, p=0.042, p=0.004 and p=0.004, respectively).

Group V CRP, TNF-α and IL-1β values in serum and CSF were significantly higher than those in Group VI (p values p=0.012, p=0.019, p=0.004, p=0.007, p=0.004 and p=0.004, respectively).

Group II serum CRP and TNF-α values were significantly higher than those in Group III (p values p=0.003 and p=0.002, respectively). However, there was significant difference between the two groups' serum IL-1β values. While CRP in CSF was significantly higher in Group II than in Group III (p=0.013), TNF-α and IL-1β were significantly higher in Group III than in Group II (p=0.03 and p=0.004, respectively).

Table 1. *Comparison of the group's serum CRP, TNF-α, IL-1β and leukocyte*

Serum	CRP (µg/L)	TNF-α (pg/mL)	IL-1β (pg/mL)	Leukocyte
Group I (n=7)	188.47 ± 35.605	201.22 ± 44.594	137.32 ± 17.821	12.514 ± 5.0697
Group II (n=7)	178.49 ± 17.324	209.24 ± 11.830	145.63 ± 17.370	12.457 ± 6.7982
Group III (n=7)	123.72 ± 15.403	112.22 ± 17.289	131.10 ± 30.700	10.314 ± 5.7736
Group IV (n=7)	165.17 ± 16.910	203.44 ± 16.945	141.69 ± 17.772	12.943 ± 5.8057
Group V (n=7)	136.96 ± 27.174	128.02 ± 29.729	132.33 ± 27.492	11.043 ± 2.6203
Group VI (n=5)	100.36 ± 7.034	98.21 ± 13.395	68.64 ± 17.931	6.120 ± 1.8913
p	**0.00**	**0.00**	**0.017**	**0.119**

Table 2. *Comparison of groups CSF CRP, TNF-α and IL-1β values*

CSF	CRP (µg/L)	TNF-α (pg/mL)	IL-1β (pg/mL)
Group I (n=7)	209.74 ± 32.014	276.69 ± 68.337	174.24 ± 23.868
Group II (n=7)	215.27 ± 34.213	257.98 ± 58.089	172.74 ± 25.945
Group III (n=7)	166.39 ± 18.629	363.54 ± 38.513	237.71 ± 30.431
Group IV (n=7)	183.41 ± 54.494	205.01 ± 28.031	186.59 ± 25.894
Group V (n=7)	157.72 ± 18.756	286.49 ± 36.340	232.17 ± 35.348
Group VI (n=5)	111.52 ± 21.096	82.38 ± 9.629	108.82 ± 10.440
p	**0.00**	**0.00**	**0.00**

Table 3. *Comparison of CRP, TNF-α and IL-1β Values in CSF in groups I and II*

CSF	CRP (µg/L)	TNF-α (pg/mL)	IL-1β (pg/mL)
Group I (n=7)	209.74 ± 32.014	276.69 ± 68.337	174.24 ± 23.868
Group II (n=7)	215.27 ± 34.213	257.98 ± 58.089	172.74 ± 25.945
p	**0.848**	**0.749**	**0.655**

No statistically significant difference was observed in serum CRP, TNF-α and IL-1β values between Group II and Group IV. Comparison of CSF values in these groups also revealed no statistically significant differences between CRP, TNF-α and IL-1β values in serum.

No statistically significant difference was observed between serum CRP, TNF-α and IL-1β values in Group III and those in Group V. Although no significant difference was determined in CRP and IL-1β values in CSF between groups III and V, TNF-α values in CSF in Group III were significantly higher than those in Group V (p=0.006).

Serum TNF-α in Group IV was significantly higher than in Group V (p=0.006). However, no significant difference was determined between the two groups in terms of serum CRP and IL-1β levels or of CRP in CSF. Group V CSF TNF-α vs. IL-1β values were significantly higher than those in Group IV (p values p=0.002 and p=0.035, respectively)

Discussion

Anti-inflammatory therapy has been shown to reduce development of meningeal inflammation, intracranial pressure, cerebral edema, tissue injury and neurological sequelae and mortality rates [13,14]. Dexamethasone therapy is the most widely approved anti-inflammatory and adjuvant treatment regimen in the literature. Since the anti-inflammatory efficacy of high-dose steroid is already known, low-dose dexamethasone therapy was used (0.25 mg/kg). EPO, whose primary function is maturation and differentiation of erythroid series cells, has recently been shown to act as a powerful anti-inflammatory in chronic inflammatory disorders and infectious diseases [15-19]. We determined that EPO applied at a dose of 1000 IU/kg at the 12th hour of pneumococcal meningitis induced in rabbits and dexamethasone at a dose of 0.25 mg/kg had no significant effects on IL-1β, TNF-α and CRP in serum and CSF. Since bacterial meningitis develops with neuronal injury, inflammation, reactive oxygen species and excitatory amino acid toxicity, EPO's antiapoptotic, antioxidative, anti-inflammatory and glutamate inhibitor activities suggest that EPO may be beneficial in bacterial meningitis [20-25].

Studies of bacterial and viral meningitis in children have investigated TNF-α, IL-1β, IL-6 and IL-8 levels in CSF and have shown higher levels of these cytokines in children with bacterial meningitis compared to meningitis developing due to other agents and control groups [26-28]. Comparison of groups with induced meningitis and the control group in this study revealed higher TNF-α and IL-1β levels in CSF in the meningitis groups than in the control group. This was compatible with studies emphasizing the importance of TNF-α and IL-1β in CSF in the diagnosis of meningeal infection [26,29]. We also investigated serum IL-1β and TNF-α values in this study. Serum IL-1β and TNF-α values were higher in the induced meningitis groups compared to the control group. Similarly, Ohga et al. [30] measured both cytokines in serum and CSF in their study of 11 patients with bacterial meningitis, 50 with aseptic meningitis and a control group. They determined higher IL-1β and TNF-α levels in the patients with bacterial meningitis compared to the control and aseptic meningitis groups. CSF IL-1β and TNF-α values were also higher than serum IL-1β and TNF-α levels. Similarly in our study, CSF IL-1β and TNF-α values were higher than serum IL-1β and TNF-α values. Odabaşı et al. [31] obtained similar results, with IL-1β and TNF-α in CSF being higher than in serum supporting the idea that these cytokines are produced locally in the CNS [32].

Long-term studies of children with bacterial meningitis have determined a significant decrease in morbidity and mortality with steroid use in the early stage [33-35]. In their *in vitro* study, Van Furth et al. [36] reported increased TNF-α and IL-1β in leukocytes stimulated by *S. pneumoniae* and that TNF-α and IL-1β production decreased with dexamethasone administration. Sáöz-Llorens et al. [37] investigated a *H. influenzae* model of meningitis in rabbits and reported significantly lower TNF-α in CSF and leukocyte levels in blood with administration of 1 mg/kg dexamethasone before antibiotic compared to the group receiving antibiotic alone. In a study performed using a rabbit model of pneumococcal meningitis, Lutsar et al. [38] reported that TNF-α and lactate concentrations in CSF induced with antibiotic decreased with administration of 1 mg/kg dexamethasone. In a similar study, Tuomanen et al. [39] also reported a decrease in TNF-α and lactate levels in CSF with dexamethasone.

Comparison of CSF and serum TNF-α and IL-1β levels in the meningitis group receiving dexamethasone and the meningitis only group revealed a significant decrease in TNF-α levels in CSF in the meningitis and dexamethasone group. This finding is compatible with studies reporting that dexamethasone reduces CSF TNF-α levels. However, we determined no change in IL-1β levels in CSF and TNF-α and IL-1β levels in serum. This may be due to the experimental animals receiving 0.25 mg/kg dexamethasone. Experimental studies have determined an anti-inflammatory effect of dexamethasone in bacterial meningitis models when administered at a dose of 1 mg/kg [38,40,41]. Since the effectiveness of high-dose steroid is already known from previous studies, our aim was to observe the effect of low-dose steroid. We thought that steroid-related side-effects might thus be reduced. When we compared the meningitis and dexamethasone group and the meningitis, dexamethasone and antibiotic treatment group, CSF TNF-α and IL-1β levels were higher in the group receiving concurrent antibiotic therapy. These findings may be attributed to antibiotics triggering anti-inflammatory response. Rapid sterilization in CSF takes place with antibiotic therapy in bacterial meningitis; however, clinical condition intensifies after the start of antibiotic therapy. This is due to secondary inflammation associated with bacteria being released inside the

subarachnoid space of the cell wall components. Bacterial products increase inflammatory response and cytokines [36]. The high increase in cytokines in CSF is correlated with morbidity and mortality. Therefore, dexamethasone can be used in higher doses when elevated bacterial products are identified in CSF.

EPO is a powerful anti-inflammatory cytokine in chronic inflammatory disorders and infectious diseases (16-19,42). Strunk et al. [7] determined a decrease in IL-2, IFN-γ, TNF-α, IL-4, IL-5, IL-6 and IL-10 synthesis following EPO administration after stimulation of child and adult leukocytes with LPD. In a mouse model of autoimmune encephalitis, Agnello et al. [23] observed a decrease in IL-6 and TNF-α synthesis in CSF with administration of EPO but reported no effect in mice with induced arthritis and concluded that the inflammatory effect is specific to the CNS. Sun et al. [42] determined a decrease in IL-1β levels and leukocyte infiltration into CSF in the group treated with EPO in a hypoxic-ischemic mouse model.

In the *S. pneumoniae*-induced rabbit meningitis model in our study, comparison of IL-1β and TNF-α values in CSF and serum at the 12th h after induction of meningitis and on the 12th hour after administration of 1000 IU/kg EPO with CSF and serum values in the group with induced meningitis alone revealed no significant difference. Comparison of serum IL-1β and TNF-α levels in the meningitis group receiving EPO and the meningitis group receiving EPO and antibiotic therapy revealed that these values were higher in the meningitis, EPO and antibiotic group. We attribute this to antibiotic therapy stimulating secondary inflammatory response. Serum TNF-α levels were higher in the meningitis, EPO and antibiotic therapy group. Comparison of the efficacy of EPO with that of dexamethasone revealed lower serum and CSF TNF-α and IL-1β values in the meningitis and dexamethasone only group compared to the meningitis and EPO only group. However, the difference was not statistically significant. We also determined no significant difference between the meningitis group receiving EPO concurrently with antibiotic therapy and the meningitis group receiving dexamethasone concurrently with antibiotic therapy. CSF TNF-α values in the meningitis group receiving concurrent antibiotic therapy and EPO were significantly higher than those in the meningitis group receiving concurrent antibiotic therapy and dexamethasone. These results show that even at low levels dexamethasone has a greater effect on cytokine levels than EPO. Our study findings show that EPO does not affect IL-1β and TNF-α in serum or CSF in rabbit pneumococcal meningitis. To the best of our knowledge, there are no previous studies of the anti-inflammatory efficacy of EPO in meningitis. Similar to our study, Spreer et al. [40] observed no systemic anti-inflammatory and neuroprotective effect, and none in CSF, with administration of 1000 IU/kg EPO at the 12th hour of meningitis in an *Escherichia coli* rabbit meningitis model. That study investigated pleocytosis

lactate and protein concentrations in CSF. Investigation of CRP levels in serum and CSF at the 24th hour of meningitis in pneumococcal meningitis in rabbits in this study in the meningitis and control groups revealed higher CRP levels in both serum and CSF in the meningitis groups. To the best of our knowledge, this is the first study to investigate the effect of EPO on serum CRP levels in meningitis. Comparison of CRP values in CSF and serum in the meningitis group receiving EPO and the group receiving concurrent antibiotic therapy and EPO revealed significantly higher serum and CSF CRP levels in the group receiving EPO to the group receiving concurrent EPO and antibiotic therapy. We determined no significant difference in CRP values in serum or CSF between the meningitis and EPO therapy group and the meningitis only group. Our study shows that EPO has no effect on CRP in serum and CSF.

There was no difference in CRP values in serum and CSF between the groups receiving dexamethasone and the meningitis only group. This study determined no effect on CRP of either dexamethasone or EPO. In conclusion, no anti-inflammatory effect was determined with use of 1000 IU/kg EPO in a rabbit model of pneumococcal meningitis. To summarize all these findings, since EPO has no effect on cytokines or CRP, and since it is also a costly drug, we do not recommend its use as an ancillary therapy in meningitis. However, we think that wider-ranging studies involving different doses are needed to confirm this.

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