Evaluation of the Effects of a High Dose of Erythropoietin-beta on Early Endotoxemia Using a Rat Model

Ocena działania dużych dawek erytropoetyny beta na wczesną endotoksemię na modelu szczurzym

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Abstract

Background. Endotoxins can cause serious organ damage and death by triggering the secretion of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β in bacterial infections.

Objectives. The goal of this study was to evaluate the effects of a high dose (3000 U/kg) of erythropoietin-beta (EPO) on inflammatory cytokine levels, renal function and histological changes during the early period of Lipopolysaccharide (LPS)-induced endotoxemia using a rat model.

Material and Methods. Male Sprague Dawley (350–400 g) rats were randomized into 3 groups: Control group (n = 7); LPS group (received 20 mcg/kg LPS through intraperitoneal (i.p.) injection (n = 7)); LPS+EPO group (received 3000 U/kg, ip 30 minutes before LPS administration (n = 7)). Four hours after the administration of LPS, kidney tissue and serum samples were collected. Kidney function parameters, TNF-α, IL-6, IL-1β, C reactive protein (CRP) and complete blood counts (CBC) were measured. The severity of renal tubular injury and caspase-9 immunoreactive cells was expressed as a percentage.

Results. Serum levels of urea, creatinine, TNF-α, IL-6 and IL-1β were significantly increased in the LPS group (p < 0.0001 – p = 0.04) and were lower in LPS+EPO group (p < 0.0001, p = 0.01, p = 0.02, p = 0.01 and p < 0.0001, respectively). Pretreatment with EPO significantly increased platelet counts (p = 0.00) and decreased white blood cell counts (p = 0.02). The renal tubular injury percentage was significantly higher in the LPS group than in the control and LPS+EPO groups (p = 0.002, p = 0.003, and p = 0.005, respectively) and caspase-9 expression was lower in the LPS+EPO and control groups than in the LPS group.


Key words: erythropoietin, lipopolysaccharide, endotoxemia, acute kidney injury.

Streszczenie

Wprowadzenie. Endotoksyny mogą powodować poważne uszkodzenia narządów i zgon, wywołując wydzielanie cytokin prozapalnych, takich jak TNF-α, IL-6 i IL-1β w zakażeniach bakteryjnych.

Cel pracy. Ocena wpływu dużej dawki (3000 U/kg) erytropoetyny beta (EPO) na stężenie cytokin zapalnych, czynność nerek i zmiany histologiczne podczas wczesnej endotoksemii wywołanej przez lipopolisacharyd (LPS) na modelu szczurzym.

Materiał i metody. Szczury szczepu Sprague Dawley (350–400 g) przydzielono losowo do 3 grup: grupy kontrolnej (n = 7); grupy LPS (zwierzętom podano 20 mcg / kg LPS dostrzewnowo (ip) (n = 7)); grupę LPS + EPO (zwierzętom podano 3000 U / kg, ip 30 minut przed podaniem LPS (n = 7)). 4 godziny po podaniu LPS pobrano tkankę nerek oraz próbki surowicy. Zmierzono wskaźniki czynności nerek, TNF-alfa, IL-6, IL-1β, białko C-reactywne (CRP) oraz morfologię krwi (CBC). Ciężkość uszkodzenia kanalików nerkowych i komórek immunoreaktywnej kaspazy-9 wyrażono w procentach.
Acute kidney injury (AKI) is a common and often serious clinical problem caused by several factors such as hypovolemia, nephrotoxins, drugs, infections. Endotoxins are a leading cause of AKI and can trigger devastating organ damage and death [1]. Lipopolysaccharides (LPS) are major cell wall constituents of Gram-negative bacteria and are responsible for well known systemic responses in cases of sepsis. Several inflammatory mediators cause these responses, but their mechanism of action is not fully understood [2]. Although cytokines such as TNFα, IL-1 and IL-6 are key mediators in experimental models of kidney, brain, lung, and reperfusion injury, cisplatin, cyclosporine and radiographic models of acute kidney injury [6–9]. ESAs have shown renoprotective effects in nephrotoxicity induced by ischemia-reperfusion injury, cisplatin, cyclosporine and radiocontrast models of acute kidney injury [10–15]. ESAs have also been studied in experimental models of LPS-induced endotoxia. The pathogenesis of endotoxemic AKI is not fully understood [16, 17]. During endotoxia, a broad array of humoral mediators, including cytokines, are released into the circulation [18]. Systemic hypotension and intrarenal vasoconstriction due to an imbalance of vasodilatory and vasoconstrictory substances results in renal ischemia and contributes to AKI [19, 20]. Furthermore, inflammatory cells infiltrate the kidney [21] and cause tubular damage through the release of oxygen radicals, proteases, and additional inflammatory cytokines. Most evidence suggests that, when produced locally in response to injury, EPO has a negative effect on this process. However, pro-inflammatory cytokines suppress the local production of EPO in a concentration-dependent manner. The amount of local EPO necessary for tissue-protective effects is much higher than that needed for hormonal effects. In addition, EPO expression can be delayed, reaching a peak 12 or more hours after injury [22]. Therefore, the use of recombinant human EPO may help to heal and restore function after an injury by producing anti-apoptotic proteins and suppressing pro-inflammatory cytokines.

In recent years, the effects of EPO in LPS-induced endotoxia have been investigated through studies that administered EPO at varying time points and doses. Low doses of EPO (300 U/kg, intravenously (i.v.)) before resuscitation were able to eliminate renal dysfunction in hemorrhagic shock but not in endotoxic shock induced by LPS (6 mg/kg) in rats [15]. However, higher doses of EPO (4000 U/kg, intraperitoneally (i.p.)) 30 minutes before the administration of a low dose of LPS (2.5 mg/kg, i.p.) ameliorated renal dysfunction during endotoxia in mice [23]. In this murine model of endotoxia, the benefits of EPO were due to both its antioxidant and anti-inflammatory properties, rather than by effects on renal blood flow or apoptosis. The subcutaneous administration of 1000 U/kg of EPO to mice improved survival when it was initiated at 30 min, 1 hour, or 2 hours after a lethal dose (10 mg/kg) of LPS. However, it had no rescuing effect if given 1 hour before or 3, 4, or 16 hours after LPS administration [24]. Survival with EPO was associated with attenuated apoptosis, reduced nitric oxide production and tissue hypoxia, but not with inflammatory changes. Moreover, intravenous EPO administration at low doses (300 U/kg) immediately before the injection of LPS (20 mg/kg, i.v.) increased the release of TNF-α, IL-6 and IL-1 1 hour later [25].

This study aims to show the effect of a high dose (3000 U/kg) of EPO-beta on inflammatory cytokine levels, renal function and histological changes during the early stage of LPS-induced endotoxia in rats.
Material and Methods

Animals

The Animal Ethics Review Committee of Yeditepe University (Istanbul, Turkey) approved the experimental protocol. Male Sprague Dawley rats (n = 21) reared at the Yeditepe University Animal Research Center were used in the study. The rats were housed at the Animal Center in a controlled environment at a temperature of 22 ± 1 °C with a 12-hour light/dark cycle. Food and water were provided ad libitum. Animal use was in accordance with the guidelines of European Council for animal care.

Endotoxemic Model

Endotoxemia was induced through the intraperitoneal injection 20 mg/kg of LPS (Echerichia coli 0128:B12, Sigma-Aldrich Chemical, St. Louis, MO, USA) dissolved in 0.5 ml normal saline. The selection of a 20 mg/kg dose was based on preliminary experiments using 0.6 mg/kg, 10 mg/kg, 15 mg/kg and 20 mg/kg of LPS, renal and liver functions were examined as indicators of organ damage. The most significant change in serum urea and alanine aminotransferase (ALT) values occurred at a dose of 20 mg/kg, and this dose was used to induce endotoxemia. EPO-beta (3000 U/kg) was administered through intraperitoneal injection 30 min before LPS administration. The EPO dose was determined on the basis of previous publications [15, 23–25]. After the administration of the endotoxin, the animals were continuously observed for 4 h and then sacrificed.

The rats were divided into three groups: the control group, was treated with saline (n = 7); the LPS group was treated with 20 mg/kg intraperitoneal LPS and the LPS + EPO group was treated with 3000 IU/kg EPO-beta (Roche, Turkey) 30 min before LPS administration.

Measurement of Biochemical Parameters

At the end of the experimental period, blood samples were collected via decapitation. The samples were centrifuged (4032 × g for 3 min) to separate the serum. All samples were analyzed for urea, creatinine (Cr), C-reactive protein (CRP) and complete blood count (CBC) within 6 hours following collection. The samples were analyzed using commercially available clinical assay kits with an autoanalyzer (COBAS Integra 400, Roche Diagnostic) according to the manufacturers’ instructions. Urea and Cr concentrations were used as indicators of renal function.

TNF-α, IL-6, and IL-1β Measurement

Serum levels of TNF-α, IL-6 and IL-1β were determined using enzyme-linked immunosorbent assay (ELISA) kits (Rat Interleukin1-Beta ELISA Kit, BioSource Europe, Belgium; Rat Interleukin 6 ELISA Kit, BenderMed Systems, UK; Tumor Necrosis Factor Alpha (TNF-α) ELISA Kit, Assaypro, UK) with a DTX 880 multimode detector (Becman Counter, Nyon I, Switzerland) reader (Roche) and according to the manufacturers’ instructions.

Histological Evaluation

The kidneys were removed immediately after the animals were sacrificed (4 h after LPS administration) and submitted on Pathology Laboratory, Yeditepe University. The tissues were fixed in 10% buffered formalin for 24 h, and after routine tissue processing, the tissues were embedded in paraffin. 3 serial sections of each kidney were stained with hematoxylin and eosin for histopathological evaluation. The severity of renal tubular injury was scored by estimating the percentage of renal cortical or outer medulla tubules—including both distal and proximal tubules – that showed epithelial necrosis, luminal necrotic debris, tubular dilatation, and hemorrhage. Neutrophil infiltration was evaluated by counting the number of interstitial and tubular neutrophils per each 10 high power field (number of neutrophil/10 hpf; 1 hpf = the area that is observed through 40 × 10 lens aperture of Olympus BX51 light microscope, GmbH-Germany).

Caspase-9 Immunohistochemical Staining and Evaluation

For immunohistochemical evaluation, sections were deparaffinized in xylene and dehydrated in grading concentrations of ethyl alcohol.

Following deparaffinization, the slides were boiled for 20 minutes in a 10 mM citrate buffer (pH 6.0), cooled at room temperature for 20 minutes, and rinsed with distilled water. The slides were immersed for 30 minutes in 0.3% hydrogen peroxide diluted in methanol for endogenous peroxide inactivation and were then washed three times in phosphate buffer saline (PBS, pH 7.4) at room temperature. Subsequently, PBS containing 1% goat serum and 1% bovine serum albumin was applied for 30 min to block non-specific binding.
Next, caspase-9 (10 µg/ml, rabbit monoclonal antibody, Thermo Fisher Scientific, Fremont, CA) was applied for 30 min at room temperature. After washing in PBS, peroxidase activity was localized with 3,3’-diaminobenzidine (DAB; DAKO Liquid DAB-Substrate-chromogen K-3466, CA, USA) and 0.03% hydrogen peroxide. Sections were counter-stained with hematoxylin, cleaned and mounted. Negative control studies were performed concurrently in the absence of the primary antibody. Positive control studies were also performed simultaneously in sections of human tonsil tissue. Brown staining in cytoplasm, regardless of intensity or extension was considered “positive” and no staining was considered “negative” for caspase-9. The number of both negative and positive cells was counted on the slides. For each slide, the “percent caspase-9 expression” was calculated by dividing the number of positive cells by the total number of cells and multiplying by 100.

### Statistical Analysis

Data is presented as mean ± SD. ANOVA and Bonferroni’s tests were used to compare the means of continuous variables that were normally distributed and had equal variances. Welch and Games-Howell tests were used to compare the means of continuous variables that were normally distributed and did not have equal variances. The Kruskal-Wallis and Mann-Whitney U tests were used to compare the medians of non-normally distributed continuous variables. Two-tailed p-values less than 0.05 were considered statistically significant. Statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, Ill., USA).

### Results

#### Effect of EPO-beta on Renal Functions After Endotoxemia

Endotoxemia increased plasma urea and creatinine at 4 h. In LPS group plasma levels of urea and creatinine were significantly higher when compared to control group (p < 0.0001 and p = 0.01, respectively). Pretreatment with 3000 U/kg of EPO-beta may prevent a rise in plasma urea and creatinine levels. When compared with LPS group, LPS+EPO group had significantly lower levels of urea and creatinine (95%Confidence Interval (CI): 28.51±63.15, p < 0.0001; 95%CI: 0.04±0.43, p = 0.01, respectively) (Table 1).

#### Effect of EPO-beta on CRP After Endotoxemia

There was no difference in serum CRP levels during the first 4 h of endotoxemia. Compared with the control group, no significant changes in serum CRP levels were observed in the LPS group (p = 0.78) (Table 1).

#### Effect of EPO-beta on Hemoglobin (Hb), White Blood Cells (WBC) and Platelets (PLT) After Endotoxemia

Pretreatment with 3000 IU/kg EPO-beta significantly increased platelet counts and decreased

| Table 1. Laboratory findings of study groups
| Tabela 1. Badania laboratoryjne w grupach |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Control group  | LPS group       | LPS + EPO group | p value         |
| (Grupa kontrolna) | (Grupa LPS)  | (Grupa LPS + EPO) | Istotność statystyczna |
| (n = 7)         | (n = 7)         | (n = 7)         | p*              | p**              |
| Urea (mmol/L)  | 103.21 ± 12.04  | 255.66 ± 3.46   | 126.42 ± 3.72   | < 0.0001         | < 0.0001         |
| Creatinine (µmol/L) | 29.04 ± 2.64 | 51.04 ± 15.84   | 29.92 ± 7.04    | 0.01             | 0.01             |
| CRP (mg/dl)    | 0.23 ± 0.07     | 0.26 ± 0.07     | 0.29 ± 0.04     | 0.78             | 0.67             |
| WBC (x 10^9/µL) | 9.78 ± 2.03    | 12.83 ± 3.63    | 7.94 ± 2.43     | 0.21             | 0.02             |
| Hb (g/dl)      | 13.24 ± 2.16    | 13.75 ± 1.57    | 14.78 ± 0.44    | 0.80             | 0.39             |
| PLT (x 10^9/µL) | 3.16 ± 1.12    | 5.16 ± 2.02     | 9.56 ± 2.43     | 0.25             | 0.005            |

Data is presented as mean ± SD. P*: between control and LPS group, p**: between LPS and LPS+EPO group, CRP: C reactive protein, WBC: white blood cells, Hb: Hemoglobin, PLT: Platelet.

Dane przedstawiono jako średnie ± SD. P*: między grupą kontrolną i LPS, p**: między LPS i LPS + EPO, CRP – białko C-reaktywne, WBC – krwinki białe, HB – hemoglobina, PLT – płytki krwi.
white blood cell counts. Compared with the LPS group, the LPS+EPO group had significantly decreased white blood cell counts (95%CI: 0.44÷9.33, \( p = 0.02 \)). Platelet counts were significantly higher in the EPO+LPS group than in the control or LPS groups (\( p < 0.0001 \) and \( p = 0.005 \), respectively). LPS and EPO application did not cause significant changes in hemoglobin levels during the first 4 h of endotoxemia (Table 1).

**Effect of EPO-beta on TNF-α, IL-6, and IL-1β Levels After Endotoxemia**

Plasma concentrations of TNF-α, IL-6, and IL-1β increased significantly 4 h after LPS administration (\( p = 0.001 \), \( p < 0.0001 \), and \( p < 0.0001 \), respectively). Pretreatment with 3000 U/kg of EPO-beta may prevent a rise in inflammatory cytokines. Compared with the LPS group, the LPS+EPO group had significantly decreased levels of TNF-α, IL-6, and IL-1β (95%CI: 0.46÷8.12, \( p = 0.02 \); 95%CI: 8.39÷19.50, \( p < 0.0001 \); 95%CI: 2.98÷9.35, \( p = 0.0001 \), respectively) (Fig. 1).

**Effect of EPO-beta on Tubular Injury After Endotoxemia**

Compared with the LPS group, the LPS+EPO group had significantly decreased tubular epithelial necrosis, tubular dilatation and hemorrhage in the kidney cortex or outer medulla during the early stages of LPS-induced endotoxemia (95%CI: 16.01÷55.66, \( p = 0.01 \)) (Fig. 2).

**Effect of EPO-beta on Neutrophil Infiltration of the Kidney After Endotoxemia**

In LPS administered group the number of neutrophils detected in the kidney was increase when compared with control group, but this increase was not statistically significant (\( p = 0.42 \)). Although the number of neutrophils was lower in LPS+EPO group than in the LPS group but the difference did not reach statistical significance (\( p = 0.11 \)).

![Changes in serum TNF-α, IL-6, IL-1β at 4 h after LPS administration](image)

**Fig. 1.** Changes in serum TNF-α, IL-6, IL-1β at 4 h after LPS administration

* LPS group vs. Control group: 95%CI: 3.02÷11.05, \( p = 0.001 \); 95%CI: 18.93÷30.58, \( p < 0.0001 \); 95%CI: 4.54÷11.21, \( p < 0.0001 \), respectively

# LPS group vs. LPS+EPO group: 95%CI: 0.46÷8.12, \( p = 0.02 \); 95%CI: 8.39÷19.50, \( p = 0.001 \); 95%CI: 2.98÷9.35, \( p < 0.0001 \), respectively

**Ryc. 1.** Zmiany TNF-alfa, IL-6, IL-1b w surowicy 4 godziny po podaniu LPS

* grupa LPS vs grupa kontrolna: 95% CI: 3.02÷11.05, \( p = 0.001 \); 95%CI: 18.93÷30.58, \( p < 0.0001 \); 95% CI: 4.54÷11.21, \( p < 0.0001 \), respectively

# grupa LPS vs grupa LPS + EPO: 95% CI: 0.46÷8.12, \( p = 0.02 \); 95%CI: 8.39÷19.50, \( p = 0.001 \); 95% CI: 2.98÷9.35, \( p < 0.0001 \)
Effect of EPO-beta on Immunohistochemical Expression of Caspase-9 as a Marker of Apoptosis After Endotoxemia

The expression of caspase-9 was increased in the kidney of rat with LPS-induced endotoxemia and was reduced the kidney of rat pretreated with EPO. Compared with the control group, the kidneys of rats in the LPS group showed a significantly higher caspase-9 positive cell percentage (95%CI: 3.29÷40.38, p = 0.01). Pretreatment with 3000 U/kg EPO-beta significantly decreased the proportion of caspase-9 positive cells (95%CI: 0.65÷36.02, p = 0.04) during the first 4 h of endotoxemia (Fig. 2).

Discussion

This study shows that a high dose of EPO-beta (3000 U/kg) may have protective effects on both the function and histological appearance of kidneys during the early stages of LPS-induced endotoxemia in rats. The beneficial effects of EPO-beta administration on renal function were measured by serum urea; the effects on tubular histological changes were assessed with hematoxylin and eosin staining. These protective effects were associated with a reduction in inflammatory cytokines such as TNF-α, IL-1β and IL-6 as well as prevention of activation of apoptotic pathways secondary to the reduction of caspase-9 activation.

There are several known properties of EPO that may be involved in the renoprotective effects observed in this study. The molecular mechanisms underlying the tissue protective effect of exogenous EPO are still not completely understood, and only a few components have been identified or common pathways proposed [22, 26]. The best known property of EPO is its ability to bind to the erythropoietin receptor (EPOR) that is expressed in the kidney by endothelial cells, mesangial cells, and the epithelial cells of the tubule and collecting duct [27]. When bound to the EPOR, EPO activates the receptors associated with Janus kinase 2 (JAK 2), leading to tyrosine phosphorylation of the EPOR [28]. Erythropoietin signals through the phosphatidylinositol 3-kinase (PI3K)/Akt signal transducer and activates transcription 5 (STAT5), mitogen-activated protein kinase (MAPK) and protein kinase C pathways; these pathways serve as antiapoptotic mediators of EPO-induced cytoprotection.

The most probable renoprotective effect of EPO is its ability to reduce or prevent cell death.
The present study showed that the renoprotective effects of single, high dose (3000 U/kg) of EPO-beta are probably related to its ability to reduce tubular epithelial necrosis, tubular dilatation, luminal necrotic debris and hemorrhage in the kidney cortex or outer medulla during the early stages of LPS-induced endotoxemia. Moreover, immunohistochemical expression of caspase-9 showed that the caspase cascade was inhibited by preventing the mitochondrial pathway; a significant decrease in the caspase-9 positive cell count was observed during the first 4 h of endotoxemia.

The protective effects of EPO have been demonstrated in several experimental models [7, 9, 13, 29]. Most notably, the ability of EPO to protect kidneys from ischemia-reperfusion injury (IRI) has been shown in various animal studies [11, 12]. Although recent studies [13, 20–22] did not show a beneficial effect of EPO on histological findings and apoptosis, in the present study the authors find a positive effect of EPO during endotoxic injury.

The different doses of EPO used in these experimental models may explain the observed discrepancies. Three earlier studies used much lower doses of EPO (up to 100 U/kg) than the amount used in this study [15, 24, 25]. In addition, one earlier study [15] that used 4000 U/kg EPO only detected TUNEL positive apoptotic cells and did not examine tubular injury after LPS application. Thus, the authors suggest that the renoprotective effects of EPO during early endotoxemia are dose-dependent.

The protective effects of EPO against endotoxemia may involve a reduction in pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β. Some models of renal ischemia-reperfusion injury (IRI) [11, 12] have shown that EPO reduces various cytokines, but models of endotoxemia have been unable to demonstrate this effect [15, 24, 25]. Earlier studies have shown that TNF-α is detectable in serum during the first 4 h of endotoxemia and peaks at 2 h [31]; other studies measured inflammatory cytokines at 1 h [25], 16 h [13] and an unknown time [24] after LPS administration. This study showed that plasma concentrations of TNF-α, IL-6, IL-1β increased significantly 4 h after LPS administration, and pretreatment with a high dose (3000 U/kg) of EPO-beta may reduce rise in inflammatory cytokines.

No difference was detected in serum CRP levels during the first 4 h of endotoxemia in present experiment. A recent study reported that CRP increased at 6 h of endotoxemia and that EPO administration further enhanced its rise during this acute phase [23]. This result is intriguing because another experiment of transgenic mice showed that CRP has a protective effect by increasing cytokine binding [31] during endotoxemia. Current results suggest that this mechanism of protection may be more effective in a later stage of endotoxemia.

In the present study, platelet counts were significantly higher in the EPO group, but there was no difference in hemoglobin levels or red blood cell counts among the groups. Therefore, the authors suggest that the renoprotective effect of EPO in early endotoxemia is not due to the mitigation of ischemia via the correction of anemia, increase in the red blood cell mass and subsequent increase in the oxygen-carrying capacity of the blood. Many clinical and experimental studies indicate that ESAs augment thrombopoiesis and that a high dose of EPO increases intracranial hemorrhage and mortality rates [32]. The fact that a high dose of EPO-beta, even when administered early, leads to an increased platelet count and a potential increase in the risk of thromboembolic events may limit its use as a renoprotective agent in cases of sepsis.

One limitation of this study is the high dose of LPS used in the experiment. This dose was determined based on preliminary studies and titrated to cause more significant increases in liver and kidney function markers. The selected model may reflect serious clinical conditions characterized by rapid deterioration of organ functions and the role of EPO as rescue therapy. A second limitation is the short observation time; nevertheless, the study was designed to examine the effects of EPO during the early stages of LPS-induced endotoxemia.

In summary, this study suggests that a high dose of EPO-beta may have protective effects during the early stages of LPS-induced endotoxemia. Further studies with varying doses and observation times can further establish the renoprotective effects of ESAs and define the range of possible clinical applications.

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