

Research Article

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The effect of specific therapeutic agents on inflammation in sepsis-induced neonatal rats

Sepsis oluşturulmuş neonatal sıçanlarda spesifik terapötik ajanların inflamasyon üzerindeki etkisi

<https://doi.org/10.1515/tjb-2018-0532>

Received December 20, 2018; accepted October 17, 2019

Abstract

Objective: The aim of this study was to investigate the effect of Thalidomide and Etanercept on inflammation parameters in a neonatal rat sepsis model induced with Lipopolysaccharide (LPS).

Materials and methods: Four-week-old male *Wistar Albino* rats were used in the experiment. LPS (5 mg/kg) was administered to rats as sepsis-inducing agent and two anti-inflammatory drugs, Thalidomide and Etanercept were given intraperitoneally as chemotherapeutic agents. The septic neonatal rats were treated with Thalidomide (0.5 mg/kg), Etanercept (1 mg/kg), and a combination of the two. All therapeutic agents were injected half an hour after injecting LPS. It took 24 h to perform the entire experiment. Whereupon, liver tissues of the animals were removed, presepsin of liver tissue and NF- κ B levels were measured by ELISA analysis and NF- κ B protein expression levels were determined by Western blotting.

Results: A significant increase was detected in presepsin and NF- κ B levels in LPS group compared to sham and treatment groups. In Western Blot evaluations, there was a significant decrease in the expression of NF- κ B protein in treatment groups compared to sepsis group.

Conclusions: It was observed that Thalidomide and Etanercept had potential effects on the treatment of neonatal sepsis.

Keywords: Sepsis; Presepsin; NF- κ B; Thalidomide; Etanercept.

Özet

Amaç: Bu çalışmanın amacı, Thalidomide ve Etanercept'in LPS ile indüklenmiş neonatal sıçan sepsis modelinde inflamasyon parametreleri üzerine etkisini araştırmaktır.

Yöntem: Çalışmada, dört haftalık erkek *Wistar Albino* cinsi sıçanlar kullanıldı. Sıçanlara sepsisi indükleyici ajan olarak LPS (5 mg/kg/gün/ip), kemoterapötik ajan olarak ise iki antiinflamatuvar ilaç olan Thalidomide ve Etanercept intraperitoneal olarak verildi. Septik sıçanlar, tek başına Thalidomide (0.5 mg/kg/gün/ip), tek başına Etanercept (1 mg/kg/gün/ip) ve iki ilacın kombinasyonu halinde tedavi edildi. Tüm terapötik ajan enjeksiyonları, LPS'nin enjeksiyonundan yarım saat sonra verildi ve toplam deney süresi, 24 saat içinde gerçekleştirildi. Deneysel aşamanın sonunda uygun koşullar altında hayvanlar kurban edildi ve karaciğer dokuları alındı. Karaciğer dokusundaki presepsin ve NF- κ B seviyeleri ELISA analizi ile ölçüldü, doku NF- κ B protein ekspresyon seviyeleri ise Western blot analizi ile ortaya konuldu.

Sonuç: Sham ve tedavi grupları ile karşılaştırıldığında LPS grubundaki presepsin ve NF- κ B düzeylerinde anlamlı bir artış saptanmıştır. Western Blot değerlendirmelerinde ise, tedavi gruplarındaki NF- κ B protein ekspresyon seviyelerinde sepsis grubuna göre kıyasla anlamlı bir azalma gözlenmiştir.

Tartışma: Thalidomide ve Etanercept'in neonatal sepsisin tedavisi üzerinde potansiyel etkileri olduğu gösterilmiştir.

Anahtar Kelimeler: Sepsis; Presepsin; NF- κ B; Thalidomide; Etanercept.

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Introduction

Affecting multiple bodily systems, neonatal sepsis is a serious condition with a high mortality rate and leads to hemodynamic changes as shock, organ dysfunction, and organ failure. The most discussed agents in the pathogenesis of sepsis are gram-negative bacteria [1, 2]. Lipopolysaccharide (LPS) is the main component of gram-negative bacteria's cell outer wall, and is responsible for biological processes that can lead to fever, septic shock, and even death [3]. When LPS is injected to the body, defense cells trigger a series of inflammatory responses, including forming a LPS-LPB-CD14 complex, which is made up of LPS, LPS-binding proteins (LPB), alongside forming CD14 glycoprotein, which serves as a specific receptor task for LPS and LPB. This complex activates the inflammatory cascade, upon which monocytes and macrophages cause the release of proinflammatory cytokines and interleukins. This leads to the fragmentation of CD14 from cell membranes and the hepatocyte cells and fragments are known as soluble-CD14 (sCD14) [4]. A subtype of sCD14 (sCD14-ST) results from plasma protease activity during inflammation and is an N-terminal fragment of sCD14 composed of 64 amino acids and found in blood circulation. This subtype contributes to the transmission of intracellular signals, thus playing an important role in the occurrence of septic shock [5, 6].

Presepsin is a polypeptide in sCD14-ST group. It weighs 13 kDa and is located on the N-18 terminal of CD14 [7]. The synthesis of presepsin in the bloodstream increases in the early stages of sepsis and during systemic bacterial infections. The release of presepsin, synthesized from the liver into the bloodstream, in turn rises. Sepsis studies have revealed that presepsin directly modulates both humoral and cellular immune responses by interacting with T and B cells. Moreover, researchers have found that presepsin significantly reduces the mortality rate resulting from the severity of endotoxemia and gram-negative bacterial infections, and thus have suggested that presepsin may serve as a potential biomarker for routine use [6, 8–12].

The Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF- κ B) is a dimeric transcription factor associated with regulating the genes responsible for many pathological events such as apoptosis and inflammation. It is believed that many pathogens and cytokines activate NF- κ B signaling pathways. In the presence of sepsis, NF- κ B is involved in two major components of the host response: (1) the stimulation of monocyte and macrophage activation resulting from rapid production of large amounts of inflammatory cytokines and (2) the intense activation of endothelial cells [13, 14].

Thalidomide is a glutamic acid derivative associated with bemegride (alpha-ethyl-alpha methyl glutarimide) and glutethimide (beta-ethyl beta-phenylglutarimide), albeit containing different pharmacological properties. For example, the use of Thalidomide in clinical practice as a therapeutic drug for multiple myeloma and erythema nodosum leprosum has been approved. It has a number of side effects, including teratogenicity, sedation, and redness [15]. Thalidomide inhibits NF- κ B activation, even in the presence of potent NF- κ B activators by inhibiting kappa-light-polypeptide gene enhancers during B-cell (I κ B) kinase activity. Additionally, NF- κ B prevents transcription events by inhibiting itself to DNA. Thus, the inhibited activation of NF- κ B results in the down-regulation of the target transcriptional genes. Tumor necrosis factor alpha (TNF- α) is another physiological activator of NF- κ B as well. The release of TNF- α during inflammatory responses up-regulates the expression of NF- κ B. Thalidomide specifically inhibits monocytes to release TNF- α as well as NF- κ B activation [16]. It is likely to consider Thalidomide as an alternative therapeutic agent for sepsis since it acts as anti-inflammatory activities by reducing TNF- α expression, inhibiting NF- κ B, and downregulating inflammatory events.

Etanercept is a fusion protein that is produced using recombinant DNA technology, and is obtained by combining the human tumor necrosis factor receptor (TNFR2/p75) with the human IgG1-Fc (immunoglobulin) protein. It competitively inhibits the binding of TNF- α to cell surface receptors [17]. Etanercept being an anti-TNF- α monoclonal antibody has been widely discussed in the literature as a therapeutic agent that can be used to treat the immune modulation of numerous systemic inflammatory and autoimmune diseases [18].

Therefore, we believe that if we can indirectly down-regulate NF- κ B expression using Etanercept, we then can develop an alternative treatment to improve sepsis. By considering the potential role that both Thalidomide and Etanercept play in inflammation, this study was conducted to investigate the possible protective effects of these drugs in LPS-induced rat sepsis model.

Materials and methods

Animal supply

The study was conducted using 29 four-week-old male Wistar-Albino rats having a body weight (bw) of 55 ± 10 g. All of the animals were kept at the Experimental Animal Center at Firat University at $25 \pm 3^\circ\text{C}$, 50–60% humidity,

under a 12 h/12 h light/dark cycle. The rats in all groups were fed standard rat food and cared for daily. The rats were divided randomly into five groups: (1) the sham group ($n=5$), (2) the Lipopolysaccharide (LPS) septic group ($n=6$), (3) the LPS + Thalidomide (LPS + Thal) group ($n=6$), (4) the LPS + Etanercept (LPS + Eta) group ($n=6$), and (5) LPS + Thalidomide + Etanercept (LPS + Thal + Eta) group ($n=6$). Firat University's Clinical Ethics Committee and the Animal Care Committee approved all experimental procedures (Meeting no. 17, Decision no. 156, July 23, 2014).

Drug treatments

All injections were freshly prepared on the day of treatment. The sham group received 1 mL intraperitoneal (ip) injection of a 0.9% saline solution. In the LPS group only *Escherichia coli* 0111:B4 strain (L2630-100M6, Sigma-Aldrich®) was administered at a dose of 5 mg/kg intraperitoneally once. The LPS was prepared in a 0.9% saline solution. In the LPS + Thal group, the rats were treated with LPS and Thalidomide (T144, Sigma-Aldrich®) within its therapeutic anti-inflammatory dose (ED50 for rats, 0.5 mg/kg/ip) once. In the LPS + Eta group, the rats were injected with Etanercept (ATC code: L04AB01) (ED50 for rats, 1 mg/kg) as ip in a single dose after LPS injection. We selected both design of sepsis model alongside all of the agents' doses according to İlhan et al. [19]. In the LPS + Thal + Eta group, the rats were administered with both Thalidomide and Etanercept at the above-mentioned doses. All therapeutic agents were injected half an hour after injection of LPS. It took 24 h to perform the entire experiment.

At the end of the experiment, we then anesthetized all the live rats with 75 mg/kg of ketamine (Ketalar®, Pfizer Pharma GMBH, Germany) and 10 mg/kg of xylazine (Alfazyne®, 2%, Alfasan International, 3440 AB, Woerden, The Netherlands) and then sacrificed them. Their liver tissues were excised.

Preparing tissue homogenates

We weighed and then homogenized the liver tissues in ice-cold phosphate buffered saline (PBS) (1/10; w/v, pH 7.4) using an automatic tissue homogenizer (Ultra Turrax Type T25-B, IKA Labortechnik, Germany) at 3000 rpm for 10 min at +4°C for ELISA analysis. We centrifuged the samples at 5000×g for 45 min at +4°C, removed the supernatants, and stored them in sterile tubes at -80°C. Their protein contents were measured by using the Lowry method [20].

Measurement of Tissue Presepsin and NF-κB levels by ELISA analysis

Tissue presepsin (ref. no: DZE201115366, lot no: 201502, catalog no: 201-11-5366, SunRed Biological Technology Co. Ltd., Shanghai, China) and NF-κB (ref. no: DZE201110288, lot no: 201502, catalog no: 201-11-0288, SunRed Biological Technology Co. Ltd., Shanghai, China) levels were measured using commercially available ELISA kits in accordance with manufacturer's instructions. For presepsin, the assay range of the kit was 5–1500 pg/mL, its sensitivity was 4.233 pg/mL, its inter assay CV was 12%, and its intra assay CV was 10%. For NF-κB, on the other hand, the assay range of the kit was 0.08–20 ng/mL, its sensitivity was 0.072 ng/mL, its inter assay CV was 12%, and its intra assay CV was 10%. We used an automated enzyme-linked immunosorbent assay reader (ELX 800, Bio-Tek® Instruments Inc., Winooski, VT, USA) to measure the color intensity of each well at 450 nm for all parameters. We then calculated the concentrations based on standard curves. The results were expressed in terms of pg/mg protein and ng/mg protein, respectively.

Measurement of NF-κB levels by Western blot analysis

We weighed and added the frozen liver tissues to RIPA lysis buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) with a cocktail protease inhibitor, and then homogenized the mixture with an automatic tissue homogenizer (Next Advanced Inc., Averill Park, NY, USA). Afterwards, we centrifuged the homogenized sample at 10,000×g for 10 min to obtain the supernatant, and then recentrifuged it to form a clear lysate. The amount of protein in each sample was identified using a fluorometer (Qubit® fluorometer, Invitrogen™, Carlsbad, CA, USA) as well as a Quant-iT™ protein assay kit (Invitrogen™, Carlsbad, CA, USA). We loaded twenty microliters of each sample on gels (NuPAGE 4–12% Bis-Tris Protein Gel; Invitrogen™, Carlsbad, CA, USA) for electrophoresis, transferred them to a PVDF membrane, and probed them with a rabbit polyclonal antibody against NF-κB-p105 (rabbit IgG, obtained from Arigo biolaboratories, Taiwan, China) at 1:500–1:1000 dilution. β-Actin was used as the reference protein (Boster Biological Technology Co. Ltd., Pleasanton, CA, USA) at a dilution of 1:200–1:400. We incubated the blot with a horseradish peroxidase-conjugated secondary antibody, and made the protein bands visible via chromogenic substrates using a WesternBreeze Chromogenic Immunodetection Kit (Invitrogen™, Carlsbad, CA, USA).

NF- κ B protein expression levels were assessed using Image J software (National Institute of Health, Bethesda, MD, USA) which is used to compare the density of bands on western blots.

Statistical analysis

The one-way analysis of variance (ANOVA) and the post-hoc analysis (Tukey HSD test) were used to compare the different groups. The value of $p < 0.05$ was accepted as significant. The data were expressed as mean \pm standard deviation (SD).

Results

Table 1 shows the levels of tissue Presepsin and NF- κ B for all groups. It was found that presepsin levels increased statistically significantly in the Lipopolysaccharide (LPS)-induced sepsis group (109.42 ± 27.33 pg/mg protein) compared to the sham group (41.08 ± 19.79 pg/mg protein) ($p < 0.001$). Administration of Thalidomide, Etanercept and Thalidomide + Etanercept along with LPS provided a significant reduction in presepsin levels (67.92 ± 15.16 , 69.59 ± 19.68 , and 59.67 ± 23.09 pg/mg protein, respectively) in the rats compared to the rats in LPS group ($p < 0.01$, $p < 0.01$, $p < 0.005$, respectively) (Table 1).

There was a statistically-significant increase in NF- κ B levels in the sepsis group compared to sham group ($p < 0.001$). NF- κ B levels increasing due to LPS exposure significantly decreased by the use of treatment agents ($p < 0.005$ for all treatment groups) (Table 1).

However, the expression of NF- κ B protein was higher in the LPS group than the sham group ($p < 0.001$). The administration of Thalidomide, Etanercept, and Thalidomide + Etanercept significantly decreased the expression levels of NF- κ B protein ($p = 0.001$ for all treatment groups) when compared to the LPS group (Figure 1).

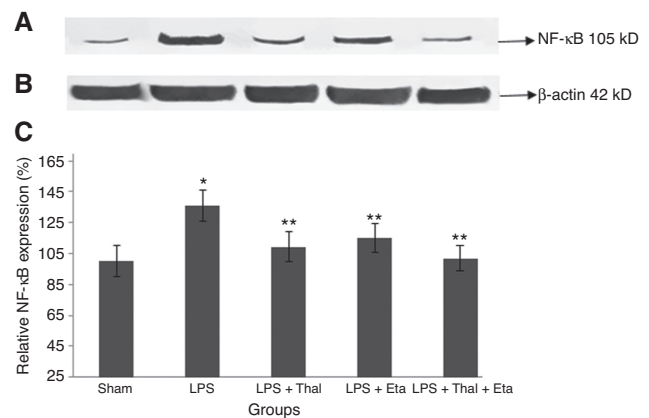


Figure 1: Western blot results for NF- κ B cleavage according to groups. The analysis showing the effect of LPS and co-administered Thal, Eta and Thal + Eta on NF- κ B protein expression (A). Western blotting was normalized to β -actin expression and expressed relative to the control sample (B). The corresponding densitometric analysis is also shown (C). Data are means \pm SD of three independent experiments. * $p < 0.001$, compared with the sham group; ** $p = 0.001$ compared with LPS alone (Tukey HSD).

Discussion

According to current sepsis hypotheses proposed by various experimental studies and theories, the immune system's excessive proinflammatory response following infection leads to multi-organ failure or further damage. Despite recent advances in antimicrobial therapies, better-defined pathophysiology, and technological developments with regards to diagnostic methods in recent years, sepsis continues to be defined as a systemic inflammatory response with serious clinical conditions.

Lipopolysaccharide (LPS)-[*E. coli*, 0111: B4] is easy and reliable to use; therefore, we selected this agent to induce experimental neonatal rat sepsis model [21]. This study investigated the effects of Thalidomide and Etanercept, which are known as TNF- α blockers in sepsis treatment, on inflammatory parameters such as presepsin and NF- κ B.

Table 1: The levels of liver tissue Presepsin and NF- κ B for all groups.

Groups	Presepsin (pg/mg protein)	p-Value	NF- κ B (ng/mg protein)	p-Value
Sham	41.08 ± 19.79		0.75 ± 0.19	
LPS	109.42 ± 27.33^a	0.0009	1.88 ± 0.49^a	0.0007
LPS + Thal	67.92 ± 15.16^b	0.0083	0.97 ± 0.43^c	0.0043
LPS + Eta	69.59 ± 19.68^b	0.0087	0.99 ± 0.22^c	0.0049
LPS + Thal + Eta	59.67 ± 23.09^c	0.0044	0.95 ± 0.38^c	0.0041

All the data were presented as mean \pm SD. While $^a p < 0.001$ shows statistically significance compared to sham group, $^b p < 0.01$, $^c p < 0.005$ shows statistically significance compared to LPS group.

Various studies have examined the diagnostic value of presepsin, and have revealed meaningful results. First, Okamura and Yokoi [22] investigated presepsin levels and the value of diagnosis in neonatal sepsis patients, and provided prospective ancillary diagnostic information. Afterwards, the studies have also revealed remarkable differences in presepsin levels in healthy people, in patients with sepsis who have local infections, and in severe sepsis patients who systemic inflammatory response syndrome (SIRS). Endo et al. [23] studied presepsin, procalcitonin, and IL-6 levels in patients suffering from suspected sepsis, and found an increase in presepsin concentrations in predominantly bacteremic patients. Presepsin is used to investigate adult and neonatal cases with sepsis as well as deaths due to sepsis [6, 7, 23, 24].

The present study showed that presepsin levels were higher in the LPS-induced sepsis group than both sham and treatment groups, which supported that presepsin could serve as a biomarker candidate for neonatal sepsis. In the light of these results, it has been observed that both Etanercept and Thalidomide may have beneficial effects in reducing presepsin levels, however it was thought that Thalidomide + Etanercept combination was more effective than individually Etanercept and Thalidomide treatments.

Although animal experiments and in vitro studies have supported the hypothesis that different inhibitors stop the sequence of events that causes sepsis by inhibiting the NF- κ B pathway, there is still no consensus on the mechanism involved. For example, in a study it was observed that the pyrrolidine dithiocarbamate (PDTC), being an antioxidant substrate, protected mice from septic shock without affecting lymphocyte infiltration, tissue interleukin-1 (IL-1), and IL-6 production against a lethal dose of LPS. Another in vivo study revealed that PDTC directly affected the translocation of NF- κ B into the nucleus (breaking), and inhibited the gene expression of proinflammatory proteins such as intercellular adhesion molecule-1 (ICAM-1) and TNF- α , cyclooxygenase-2 (COX-2), which both use the NF- κ B pathway during sepsis [25]. The NF- κ B-dependent synthesis mechanism of adhesion molecules during endothelial activation has also been studied extensively. Some in vitro studies have used protein kinase inhibitors and NF- κ B inhibitors, which block TNF- α -activated signal transduction. Moreover, there have been ongoing clinical trials that use neutralizing antibodies against adhesion molecules in order to reduce the damage that LPS causes to the body [26]. In their studies, both Von Moos et al. [27] and Teo [28], identified Thalidomide as an immunomodulatory agent that inhibits the release of cellular adhesion molecules, angiogenesis, and the production of various anti-inflammatory

mediators. Other studies have also reported that Thalidomide and Etanercept prevent NF- κ B and inhibit the production of various cytokines, thereby eliminating immune and inflammatory responses [18, 29]. In the present study, increased NF- κ B levels in the LPS group showed a statistically significant decrease in all the treatment groups and this suggested that anti-inflammatory functions of treatment agents were controlled by the transcriptional factor NF- κ B. This situation may be regarded to support that thalidomide and etanercept mentioned in our previous study are an efficient anti-inflammatory efficient drug [30].

NF- κ B is a generic term for a family of transcription factors consisting of various homo- and/or hetero-dimeric complexes belonging to the Rel family such as NF- κ B 1 (p50 and its precursor p105), NF- κ B 2 (p52 and its precursor p100), RelA (p65), RelB, and c-Rel [31, 32]. This transcription factor and its complexes have been investigated in various inflammation studies. For instance, NF- κ B p50 and its precursor p105 can be regarded as the master regulator of the inflammatory process [33]. Another study showed that p50-deficient mice heterozygous for RelA were extremely sensitive to LPS-induced shock and P50/105 subunits of NF- κ B having no transactivation domains repressed the expression of NF- κ B target genes and inhibited inflammation [34]. However, other gene knockout studies have revealed that the protein expression of NF- κ B can have both pro- and anti-inflammatory roles. However, other gene knockout studies have revealed that the protein expression of NF- κ B can have both pro- and anti-inflammatory roles.

In the present study, NF- κ B p-105 (the precursor of P50) expression levels in liver tissue homogenates were determined by using Western blot method. Relative protein expression values were similar to the ELISA results. NF- κ B expression was higher in the LPS group than the sham group. Similarly, a significant decrease was found in NF- κ B p-105 levels in all of the treated groups compared to LPS group. Despite the fact that there are a limited number of studies on tissue expression of NF- κ B levels, this present study supports the results of our previous study which also investigated neonatal sepsis [19]. Moreover, in their study, Yang et al., evaluated NF- κ B expression in cecal ligation puncture (CLP)-induced sepsis model in lung tissue. According to their results, the Liriodendrin treatment inhibited the sepsis-dependent inflammation by decreasing NF- κ B activation in the lung tissue [13]. These results were compatible with the results of the present study. Considered that the specific treatment agents applied in the present study prevented sepsis-induced inflammation by downregulating tissue NF- κ B expression levels and by inhibiting TNF- α

pathways. In other words, it can be asserted that blocking NF- κ B nuclear translocation and inhibiting NF- κ B expression may serve as another way to prevent neonatal sepsis development. However, we need to acquire more knowledge about these pathways in the presence of neonatal sepsis, which then can help us develop specific blocking drugs and use them in animal-based experiments in order to obtain better results.

It is thought that while increased NF- κ B levels and NF- κ B expression are both related to LPS-induced sepsis inflammation, evaluation of these parameters may be useful to estimate the inflammation in LPS-induced newborn sepsis. It is also suggested that agents like Etanercept and Thalidomide show anti-inflammatory activity by blocking this pathway directly and/or indirectly. Therefore, the results of the present study offer new perspectives on the effects of Thalidomide and Etanercept alone or in combination, which could be used to treat neonatal sepsis by suppressing the inflammatory response.

In conclusion, we can say that these results may lead us to an alternative treatment protocol for sepsis in the near future in the hope of significantly lowering both mortality and morbidity rates.

Ethical considerations: Firat University's Clinical Ethics Committee and the Animal Care Committee approved the experimental procedures (Meeting no. 17, Decision no. 156, July 23, 2014). There is no conflict of interest between authors.

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