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Sulfasalazine treatment can cause a positive effect on LPS-induced endotoxic rats

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Abstract: The aim of this study, was to determine the effect of sulfasalazine for different periods of time reduces disseminated intravascular coagulation, inflammation and organ damages by inhibiting the nuclear factor kappa beta pathway. The study was performed with 30 Wistar albino rats and the groups were established as Control group, LPS group; endotoxemia was induced with LPS, SL5 group: sulfasalazine (300 mg/kg, single dose daily) was administered for 5 days before the LPS-induced endotoxemia, and LS group: sulfasalazine (300 mg/kg, single dose) was administered simltenously with LPS. Hemogram, biochemical, cytokine (IL-1 β , IL-6, IL-10, TNF- α) and acute phase proteins (HPT, SAA, PGE₂) analyzes and oxidative status values were measured from blood samples at 3 and 6 h after the last applications in the all groups. The rats were euthanized at 6 h and *mRNA* levels of *BCL2* and *BAX* genes were examined from liver and brain tissues. Sulfasalazine reduced the increased IL-1 β , IL-6, TNF- α and PGE₂ levels and significantly increased anti-inflammatory cytokine IL-10 levels. In addition, decreasing of ATIII level was prevented in the SL5 group, and decreasing of fibrinogen levels were prevented in the LS and SL5 groups within first 3 h. In LPS group, leukocyte and thrombocyte levels were decreased, however sulfasalazine application inhibited decreases of leukocyte levels in LS and SL5 groups. In addition, sulfasalazine inhibited the decrease of total antioxidant capacity and unchanged apoptosis in brain and liver. In conclusion, the use of sulfasalazine in different durations reduce the excessive inflammation of endotoxemia cases.

Key words: antioxidant capacity, cytokine, endotoxemia, oxidative status, sulfasalazine

Introduction

Lipopolysaccharide (LPS), outer membrane of Gram-negative bacteria, causes endotoxemia [29]. LPS applications are used to create experimental endotoxemia or septic shock models and clinically reflect endotoxemic / septic shock laboratory and clinical findings [46, 47]. When enough LPS passes the circulation, it combines with the LPS binding protein and binds to the toll-like

receptor on the cell surface and the inflammatory response induces [2]. Various stimuli such as LPS, tumor necrosis factor (TNF)- α and interleukin (IL)-1 initiate the inflammatory mechanism by the phosphorylation of I kappa B (I κ B) and seperate from I κ B to nuclear factor kappa B (NF- κ B). Active NF- κ B translocates to the nucleus and induces the expression of various genes [40]. Proinflammatory cytokines (TNF- α , IL-1 β , IL-6, etc.) release by this inflammatory condition [10]. On the

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other hand, LPS-stimulated phagocytes (neutrophils, monocytes and macrophages) produce excessive amounts of free oxygen radicals as well as inflammatory mediators. Hydrogen peroxide and superoxide radicals enable the activation of NF- κ B in the cell [8, 47].

Increased proinflammatory cytokines have been detected in endotoxemic patients and the experimental endocytosis model [19]. The proinflammatory cytokines cause hemodynamic changes due to vasodilatation in vessels, tissue damage and clotting in veins [17, 31]. It has been reported that experimental systemic TNF- α administration leads to different organ damage [41]. The reduced release of proinflammatory cytokines in septic shock can improve hemodynamic changes, organ damage and mortality [17, 19].

The production of proinflammatory cytokines are inhibited by the release of the anti-inflammatory cytokines such as IL-10, IL-4 and IL-13. Interleukin-10 administration reduces mortality in experimental endotoxemia models [30].

In endotoxemia, interactions among T cells, stimulation of monocytes and macrophages result in release of prostaglandin E₂ (PGE₂). In addition, IL-1 and TNF potently induce IL-6 and they are the basic regulators of hepatic synthesis of acute phase proteins [9, 18].

Acute phase proteins reflect crucial effectors of the innate immune system and provide tissue protection. Serum amyloid A (SAA) and haptoglobin (HPT) play a protective role against LPS induced endotoxemia. These molecules are regulated by the synergistic effect of IL-1 and IL-6 secreted from macrophages and monocytes. However, this mechanism is controlled by the signal transduction and transcription activator 3 (STAT3) genes and NF- κ B mediated signal transduction [9].

Cytokines-stimulated endothelial cells expand the intercellular space. The stimulated endothelium initiates the clotting cascade and transforms from prothrombin to fibrin. The fibrin filaments, platelets, blood cells and plasma create clots. This effect of cytokines can lead to disseminated intravascular coagulation (DIC) [31]. In this case, anticoagulant activity starts by the increase of antithrombin (AT) III level and thrombin is inhibited. However, if the coagulation increases, ATIII amount may decrease in the blood. These events result lack of blood in the tissues and irregularities in the heart function [31, 37].

Small coagulation occurs in vital organs with the development of endotoxemia, and it continues to be in

systemic circulation [32]. Cytokines, inflammatory mediators, endotoxin, immunoparalysis, apoptosis, mitochondrial dysfunction, decayed cell signaling and coagulation cascade trigger tissue and organ damages [32, 37]. Specific cell deaths in organs containing both parenchymal and microvascular endothelium, organ dysfunctions are associated with increased apoptosis [20]. For this purpose, proapoptotic *BAX* and antiapoptotic *BCL2* expression levels evaluate in cellular [16].

The undesirable effects of drugs or diseases in organs or systems can be determined by biochemical and hematological parameters [1, 13]. Free oxygen radicals produce by various agents such as LPS cause oxidative stress and cellular damage. Thiobarbituric acid reactive substances (TBARS) are detected in the cellular damage [13, 47]. The underlying cause of oxidative stress in endotoxemia is cell damage and excessive TNF- α levels [38]. Oxidative stress increases and antioxidant capacity decreases in endotoxemia. It is aimed to increase the antioxidant capacity by the applied therapies and remove the reactive oxygen products [38, 47]. Although the mechanism of sulfasalazine could not be precisely defined, it has immunomodulator or antiinflammatory properties, which inhibited NF- κ B pathway, T lymphocytes and especially proinflammatory cytokines [36].

Sulfasalazine is a drug commonly used in the treatment of rheumatoid arthritis, ulcerative colitis and Crohn's disease [27]. Sulfasalazine and metabolites inhibit the migration of inflammatory cells and I κ B kinase pathway [44]. In addition, it inhibits PGE₂ synthetase, phospholipase A₂ (PLA₂) pathway and migration of endothelial cells [36]. At the same time, it reverses the effects of LPS and TNF- α *in vitro* study [44]. It has been stated that increased neutrophil and lipid peroxidation in experimental septicemic rats can be prevented by sulfasalazine. However, it can not treat if its low dose administration and in excessive inflammatory case [14].

Nowadays, endotoxemia and septic shock cause high mortality and economic loss in human and veterinary medicine [3, 21]. Despite the increased research on endotoxemia in the last years, it has been stated that this complex situation can not be fully treated [29].

It has been hypothesised that the administration of sulfasalazine in different durations reduced acute inflammation in endotoxemia (cytokines, prostaglandin, intercellular adhesion molecules, etc.) and related complications (DIC, apoptosis etc.) by inhibiting the NF- κ B pathway and other anti-inflammatory properties.

The aim of this study was to determine the effects of sulfasalazine on some cytokines (TNF- α , IL-1 β , IL-6 and IL-10), acute phase proteins (SAA and HPT), oxidative status (TBARS and TAC) and liver and brain apoptosis markers (*BCL2* and *BAX*) in endotoxiemia.

Materials and Methods

Experimental design

The current research procedure was approved by Selcuk University Experimental Medical Practice and Research Center and 30 adult Wistar albino male rat (200–250 g) were used in the study. All animals were provided ad libitum feed and water during the experiment.

Sulfasalazine (sulfasalazine analytical standard, $\geq 98\%$, USA) was dissolved in 0.9% NaCl before administration to rats. Lyophilized LPS (*Escherichia coli* 0111: B4, Sigma-Aldrich Chemie, USA) was diluted with 0.9% saline for experimental endotoxemia model. The chemicals were used intraperitoneally (i.p) injection.

The rats were divided into 4 groups for experiment:

1. Group (C) (Control, n=6): Animals were used as healthy controls in this group. The animals received totally 1 ml 0.9% saline (rat / day).
2. Group (LS) (n=8): Animals were administered 300 mg/kg sulfasalazine was performed immediately after a single dose of 4 mg/kg LPS.
3. Group (LPS) (n=8): Experimental endotoxemia model was generated by intraperitoneal 4 mg/kg LPS [23].
4. Group (SL5) (n=8): Sulfasalazine (300 mg/kg) was administered for 5 days for the purpose of prophylactic treatment before the formation of the endotoxemia model and single dose of 4 mg/kg LPS (i.p.) was administered at the same time as the last sulfasalazine application.

Blood samples were collected under anesthesia [20 mg/kg intraperitoneal thiopental sodium (Pental 1 g; Ulagay, Istanbul, Turkey)] from the animals at 3 and 6 h after the last administration in all groups, and then brain and liver tissue samples were taken after euthanasia at 6 h.

Analysis of parameters

The blood samples were collected by anticoagulant and serum separator tubes and hematological parameters (erythrocyte, leukocyte, platelet, hemoglobin) were determined by hemocell counter (BC-2800 Auto Hematology Analyzer, Mindray Bio-Medical Electronics, Shen-

zhen, China). The biochemical parameters (albumin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, creatinine, total bilirubin, total protein) were analyzed by autoanalyzer (Cobas Integra[®] 400 plus, Roche Diagnostics, Rotkreuz, Switzerland).

TNF- α (Rat TNF- α Platinum ELISA, Catalogue no: BMS622, Affymetrix eBioscience, Vienna, Austria), IL-1 β (Rat IL-1 β Platinum ELISA, Catalogue no: BMS630, Affymetrix eBioscience, Vienna, Austria), IL-6 (Rat IL-6 Platinum ELISA, Catalogue no: BMS625, Affymetrix eBioscience, Vienna, Austria) and IL-10 (Rat IL-10 Platinum ELISA, Catalogue no: BMS629, Affymetrix eBioscience, Vienna, Austria), serum amiloid A (Rat SAA ELISA kit, Catalogue no: E-EL-0881, Elabscience, Bethesda, USA), haptoglobin (Rat Haptoglobin ELISA kit, Catalogue no: E-EL-R0473, Elabscience, Bethesda, USA), prostaglandin E₂ (PGE2 ELISA kit, Catalogue no: E-EL-0034, Elabscience, Bethesda, USA), thiobarbituric acid reactive substances (TBARS Assay Kit, Item no: 10009055, Cayman Chemical Co., USA) and total antioxidant capacity (TAC, Antioxidant Assay Kit, Item no: 709001, Cayman Chemical Co., USA) levels were measured according to the manufacturer's protocol by using an enzyme-linked immunosorbent assay (ELISA) reader (MWGt Lambda Scan 200, Bio-Tek Instruments, Winooski, VT, USA).

Clotting factors Antithrombin III (Dialab GmbH) and fibrinogen (Dialab GmbH) were determined by coagulometer (Pacific Hemostasis ThromboScreen[®] 400, Fisher Diagnostics, Auburn, Australia).

Total RNA isolation

Brain and liver tissue samples were snap frozen immediately in liquid nitrogen and stored at -70°C until RNA isolation. Brain and liver tissue samples were homogenized in TRIzol[®] reagent (Invitrogen, MA, USA), and total RNA extraction was performed according to the manufacturer's instruction. The RNA integrity was controlled using 1% agarose gel electrophoresis and the determination of the A260:A280 ratio. For cleaning Deoxyribose Nucleic Acid (DNA) contamination Ribo Nucleic Acid (RNA) samples (2 mg) were treated with DNase I (ThermoFisher Scientific, MA, USA). cDNAs were made by using the iScript complementary DNA (cDNA) (Bio-Rad, California, USA) according to the manufacturer's protocol. cDNA was stored at -20°C for qPCR [5].

Table 1. Sequences of oligonucleotide primers and base pair

Gen	Sequences of oligonucleotide primers (5'-3')	Base Pair	Annealing Temperature (°C)	Accession
BAX_F	GAGAGGTCTTCTCCGTGTG	133	58	NM_017059.2
BAX_R	ATCAGCTCGGGCACTTTAG			
BCL2_F	TGGTACCTGCAGCTTCTTTC	131	58.3	NM_016993.1
BCL2_R	ATCTCCAGTATCCCACTCGTAG			
YWHAZ_F	TTGTAGGAGCCCGTAGGTCA	243	59.1	NM_013011.3
YWHAZ_R	CCTCAGCCAAGTAGCGGTAG			

Table 2. The effects of sulfasalazine (300 mg/kg, I.P.) for prophylactic and therapeutic purposes on the cytokines and acute phase proteins in LPS-induced endotoxemic rats (mean ± SEM).

Parameters	Sampling Time	Control	LS	LPS	SL5
TNF- α (pg/L)	3 h	30.05 ± 30.1 ^b	166.78 ± 41.8 ^b	1,156.78 ± 361.1 ^a	1,045.85 ± 286.1 ^a
	6 h	51.42 ± 21.7 ^b	60.20 ± 24.0 ^b	178.33 ± 34.4 ^a	37.85 ± 14.9 ^b
IL-1 β (pg/L)	3 h	0 ^b	35.77 ± 8.5 ^b	165.22 ± 48.1 ^a	59.69 ± 30.6 ^b
	6 h	0 ^b	135.85 ± 28.6 ^{ab}	303.41 ± 95.7 ^a	134.95 ± 41.3 ^{ab}
IL-6 (pg/L)	3 h	3.11 ± 1.5 ^b	63.16 ± 13.1 ^b	150.79 ± 44.5 ^a	27.16 ± 14.6 ^b
	6 h	9.46 ± 9.5 ^b	83.99 ± 32.4 ^b	298.04 ± 60.4 ^a	178.20 ± 111.8 ^{ab}
IL-10 (pg/L)	3 h	7.24 ± 2.6 ^b	454.89 ± 84.8 ^a	160.65 ± 29.9 ^b	386.64 ± 81.8 ^a
	6 h	10.43 ± 4.2 ^b	942.17 ± 209.3 ^a	529.56 ± 167.9 ^a	921.42 ± 218.6 ^a
HPT (ng/L)	3 h	2.81 ± 2.4 ^a	14.64 ± 5.0 ^a	32.39 ± 7.0 ^a	27.67 ± 14.5 ^a
	6 h	3.25 ± 1.8 ^b	27.86 ± 5.7 ^a	30.88 ± 8.1 ^a	22.72 ± 9.4 ^{ab}
SAA (μ g/L)	3 h	6.68 ± 0.6 ^b	13.03 ± 0.3 ^{ab}	12.98 ± 0.2 ^{ab}	12.42 ± 0.5 ^a
	6 h	6.21 ± 1.5 ^b	12.93 ± 0.6 ^a	13.01 ± 0.5 ^a	11.52 ± 1.0 ^a
PGE2 (pg/L)	3 h	85.61 ± 6.0 ^c	108.92 ± 16.6 ^{bc}	197.53 ± 37.3 ^a	159.20 ± 10.6 ^{ab}
	6 h	63.35 ± 4.5 ^a	75.76 ± 10.8 ^a	97.99 ± 11.6 ^a	82.88 ± 13.9 ^a

LS: sulfasalazine + lipopolysaccharide simultaneously, LPS: Lipopolysaccharide, SL5: Prophylactic sulfasalazine + lipopolysaccharide. TNF- α : Tumor necrosis- α , IL-1 β : Interleukin-1 β , IL-6: Interleukin-6, IL-10: Interleukin-10, HPT: Haptoglobuline, SAA: Serum Amyloid A, PGE2: Prostaglandin E2. ^{a, b, c}: Different letters in the same line are statistically different ($P < 0.05$).

Expression analysis by real-time PCR

Primers for target genes (*BCL2*, *BAX*) and housekeeping gene (*YWHAZ*) were designed with Oligo7 (<http://www.oligo.net/>) primer design programs based on sequences retrieved from the NCBI (<http://www.ncbi.nlm.nih.gov/>) (Table 1). Gene expression measurements were assessed by RT-qPCR using SsoAdvanced™ Universal SYBR® Green Supermix (Biorad, California, USA) and monitored in real-time by an iQ™-5 (Bio-Rad). The thermal cyclic conditions were initial denaturation and polymerase activation at 95°C for 30 sec, followed by 35 cycles of denaturation, annealing and amplification (30 sec at 95°C, 30 sec at 58–59°C, 45 sec at 72°C). The melting curve analysis was performed at every 0.5°C increment between 65°C and 95°C using the LightCycler® Nano qPCR System (Roche Diagnostics, Mannheim, Germany). Non-template controls were used in each experiment [5].

Statistical analysis

For determination of the efficiencies of amplification of target genes and housekeeping, serial dilutions of pooled cDNA were amplified by using real-time PCR. Amplification efficiencies of genes have been found approximately equal. For statistical analysis, data normalization process was performed according to Livak and Schmittgen via $\Delta\Delta C_t$ method [28].

All values are defined as mean ± standard error of the mean (SEM). The data were analyzed using ANOVA and Duncan test as a post hoc test (SPSS 22.0). In all parameters, $P < 0.05$ was the criterion for statistical significance.

Results

The effects of sulfasalazine on cytokines and acute phase proteins at different times for prophylaxis and treatment in experimental endotoxemic rats were presented in Table 2. TNF- α levels were highest in the LPS

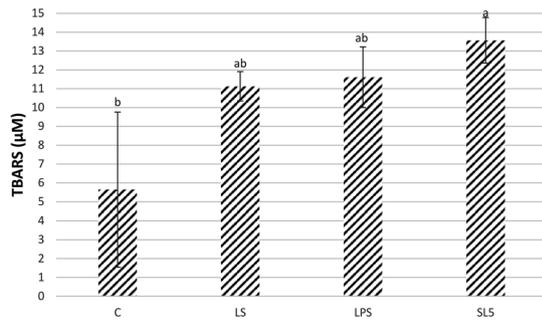


Fig. 1. The effects of sulfasalazine (300 mg/kg, I.P.) for prophylactic and therapeutic purposes on TBARS concentrations in LPS-induced endotoxemic rats (mean \pm SEM). C: Control, LS: sulfasalazine + lipopolysaccharide simultaneously, LPS: Lipopolysaccharide, SL5: Prophylactic sulfasalazine + lipopolysaccharide. ^{a, b}: Different letters in the same line are statistically different ($P < 0.05$).

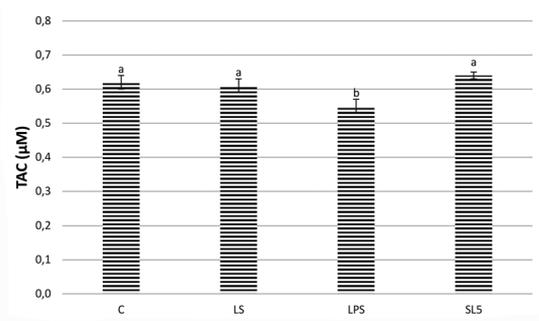


Fig. 2. The effects of sulfasalazine (300 mg/kg, I.P.) for prophylactic and therapeutic purposes on Total Antioxidant Capacity (TAC) concentrations in LPS-induced endotoxemic rats (mean \pm SEM). C: Control, LS: sulfasalazine + lipopolysaccharide simultaneously, LPS: Lipopolysaccharide, SL5: Prophylactic sulfasalazine + lipopolysaccharide. ^{a, b}: Different letters in the same line are statistically different ($P < 0.05$).

Table 3. The effects of sulfasalazine (300 mg/kg, I.P.) for prophylactic and therapeutic purposes on the clotting factors in LPS-induced endotoxemic rats (mean \pm SEM)

Parameters	Sampling Time	Control	LS	LPS	SL5
Antithrombin III (%)	3 h	129.5 \pm 10.2 ^a	95.4 \pm 3.0 ^b	116.3 \pm 6.3 ^{ab}	125.0 \pm 9.2 ^a
	6 h	112.3 \pm 17.0 ^a	61.8 \pm 4.5 ^{bc}	57.5 \pm 1.4 ^c	77.6 \pm 15.0 ^{bc}
Fibrinogen (mg/dL)	3 h	540.9 \pm 57.1 ^a	517.9 \pm 28.0 ^a	365.5 \pm 54.9 ^b	557.0 \pm 48.7 ^a
	6 h	174.0 \pm 21.0 ^a	219.7 \pm 27.7 ^a	149.7 \pm 9.9 ^a	174.8 \pm 30.6 ^a

LS: sulfasalazine + lipopolysaccharide simultaneously, LPS: Lipopolysaccharide, SL5: Prophylactic sulfasalazine + lipopolysaccharide. ^{a, b, c}: Different letters in the same line are statistically different ($P < 0.05$).

and SL5 groups at 3 h, and only in the LPS group at 6 h ($P < 0.05$). The levels of TNF- α were significantly lower in the LS group at 3 and 6 h while only in the prophylactic treatment of sulfasalazine (SL5) at 6 h compared to LPS group. IL-1 β level was at the peak level in the LPS group at 3 and 6 h and decreased statistically in the LS and SL5 groups at 3 h. However, the level of IL-1 β partially reduced in these groups at 6 h ($P > 0.05$). Although, IL-6 statistically increased in the LPS group, it was significantly inhibited in the SL5 and LS groups at 3 h and in the LS group at 6 h. Antiinflammatory cytokine IL-10 significantly increased in SL5 and LS groups compared to LPS group at 3 h.

HPT was the highest level in the LPS group and partially decreased in the SL5 group at 6 h ($P > 0.05$). SAA was higher in the endotoxemia model groups (LPS, LS, SL5) than the control group at 3 and 6 h ($P < 0.05$). PGE₂ levels were statistically increased in the LPS and SL5 groups compared to the control group however the increase was prevented in the LS group at the 3 h.

Oxidative stress marker TBARS was not statistically different between endotoxemic groups. However, it was

higher than the control group ($P < 0.05$, Fig. 1).

Antioxidant capacity was statistically lower in the LPS group than the control group. The application of sulfasalazine for the prophylactic (SL5) and treatment (LS) was observed to positively effect on the antioxidant capacity ($P < 0.05$, Fig. 2).

The level of ATIII reduced in the LS group at the 3 h ($P < 0.05$). However, the levels of ATIII in endotoxemic groups were lower than the control group at the 6 h ($P < 0.05$). Fibrinogen level decreased statistically in the LPS group at 3 h and this decrease was significantly inhibited in the SL5 and LS groups (Table 3).

Changes in the biochemical values (ALB, ALT, AST, ALP, CREA, BUN, T-BIL and T-Prot) were presented Table 4 [34]. The statistically changes of treatment groups in the biochemical parameters compared with control group. ALT levels decreased in LS and SL5 at 3 h ($P < 0.05$). AST levels decreased in LPS and SL5 at 3 h ($P < 0.05$). ALP levels increased in SL5 at 3 h and LPS and SL5 at 6 h ($P < 0.05$). CREA levels increased in LS and SL5 at 6 h ($P < 0.05$). BUN levels increased all endotoxemic groups at 3 and 6 h ($P < 0.05$). Although T-BIL

Table 4. The effects of sulfasalazine (300 mg/kg, I.P.) for prophylactic and therapeutic purposes on the biochemical parameters in LPS-induced endotoxemic rats (mean \pm SEM)

Parameters	Sampling Time	Control	LS	LPS	SL5
ALB (g/dL)	3 h	2.72 \pm 0.1 ^a	2.78 \pm 0.1 ^a	2.88 \pm 0.1 ^a	2.48 \pm 0.3 ^a
	6 h	2.41 \pm 0.1 ^{bc}	2.43 \pm 0.0 ^{bc}	2.60 \pm 0.1 ^b	2.02 \pm 0.1 ^d
ALT (U/L)	3 h	94.4 \pm 4.9 ^a	58.4 \pm 12.0 ^b	78.4 \pm 6.6 ^{ab}	29.2 \pm 5.7 ^c
	6 h	91.6 \pm 6.1 ^{ab}	131.2 \pm 16.5 ^a	106.8 \pm 15.4 ^{ab}	73.6 \pm 11.9 ^b
AST (U/L)	3 h	221.6 \pm 19.0 ^a	264.4 \pm 28.0 ^a	149.2 \pm 10.7 ^b	141.2 \pm 28.7 ^b
	6 h	282.2 \pm 35.25 ^a	351.2 \pm 23.6 ^a	197.3 \pm 20.5 ^a	301.6 \pm 70.9 ^a
ALP (U/L)	3 h	195.4 \pm 21.4 ^b	223.8 \pm 21.2 ^b	322.4 \pm 65.6 ^{ab}	412.8 \pm 92.6 ^a
	6 h	166.2 \pm 11.4 ^c	221.4 \pm 18.2 ^c	287.0 \pm 40.7 ^{ab}	351.6 \pm 67.9 ^a
CREA (mg/dL)	3 h	0.49 \pm 0.02 ^a	0.51 \pm 0.03 ^a	0.53 \pm 0.01 ^a	0.52 \pm 0.03 ^a
	6 h	0.45 \pm 0.0 ^c	0.62 \pm 0.0 ^{ab}	0.51 \pm 0.0 ^{bc}	0.66 \pm 0.0 ^a
BUN (mg/dL)	3 h	41.8 \pm 2.37 ^c	52.8 \pm 4.64 ^b	74.0 \pm 2.17 ^a	66.4 \pm 4.54 ^a
	6 h	37.3 \pm 1.2 ^b	90.2 \pm 4.9 ^a	101.2 \pm 18.5 ^a	104.2 \pm 10.5 ^a
T-BIL (mg/dL)	3 h	0.11 \pm 0.0 ^b	0.27 \pm 0.0 ^a	0.18 \pm 0.0 ^{ab}	0.28 \pm 0.1 ^a
	6 h	0.10 \pm 0.0 ^b	0.12 \pm 0.0 ^{ab}	0.16 \pm 0.0 ^{ab}	0.18 \pm 0.0 ^a
T-Prot (g/dL)	3 h	5.85 \pm 0.2 ^a	5.38 \pm 0.3 ^{ab}	5.56 \pm 0.3 ^{ab}	5.02 \pm 0.1 ^b
	6 h	5.05 \pm 0.1 ^a	4.76 \pm 0.1 ^a	4.92 \pm 0.1 ^a	4.82 \pm 0.2 ^a

LS: sulfasalazine + lipopolysacchride simultaneously, LPS: Lipopolysaccharide, SL5: Prophylactic sulfasalazine + lipopolysaccharide. ALB: Albumin, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, CREA: Creatinine, BUN: Blood urea nitrogen, T-BIL: Total Bilirubin, TP: Total protein. ^{a, b, c}: Different letters in the same line are statistically different ($P < 0.05$).

Table 5. The effects of sulfasalazine (300 mg/kg, I.P.) for prophylactic and therapeutic purposes on the hematology parameters in LPS-induced endotoxemic rats (mean \pm SEM)

Parameters	Sampling Time	Control	LS	LPS	SL5
WBC ($\times 10^9/L$)	3 h	8.81 \pm 0.9 ^a	7.79 \pm 1.9 ^a	2.39 \pm 0.3 ^b	9.90 \pm 1.5 ^a
	6 h	11.29 \pm 0.7 ^a	6.52 \pm 0.8 ^b	1.67 \pm 0.4 ^c	7.36 \pm 1.1 ^b
RBC ($\times 10^{12}/L$)	3 h	7.87 \pm 0.2 ^a	8.24 \pm 0.3 ^a	7.56 \pm 0.2 ^a	7.74 \pm 0.5 ^a
	6 h	8.19 \pm 0.2 ^a	8.87 \pm 0.3 ^a	7.98 \pm 0.4 ^a	8.92 \pm 0.5 ^a
PLT ($\times 10^9/L$)	3 h	896 \pm 61.0 ^a	281 \pm 45.3 ^b	499 \pm 98.7 ^b	425 \pm 123.2 ^b
	6 h	628 \pm 115.5 ^a	376 \pm 96.87 ^a	339 \pm 73.41 ^a	465 \pm 131.9 ^a
HGB (g/dL)	3 h	14.74 \pm 0.6 ^a	15.35 \pm 0.5 ^a	13.92 \pm 0.5 ^a	14.28 \pm 0.9 ^a
	6 h	10.80 \pm 0.5 ^a	11.78 \pm 0.5 ^a	10.58 \pm 0.4 ^a	11.92 \pm 0.6 ^a

LS: sulfasalazine + lipopolysacchride simultaneously, LPS: Lipopolysaccharide, SL5: Prophylactic sulfasalazine + lipopolysaccharide. WBC: White blood cell, RBC: Red blood cell, PLT: Platelet, HGB: Hemoglobin. ^{a, b}: Different letters in the same line are statistically different ($P < 0.05$).

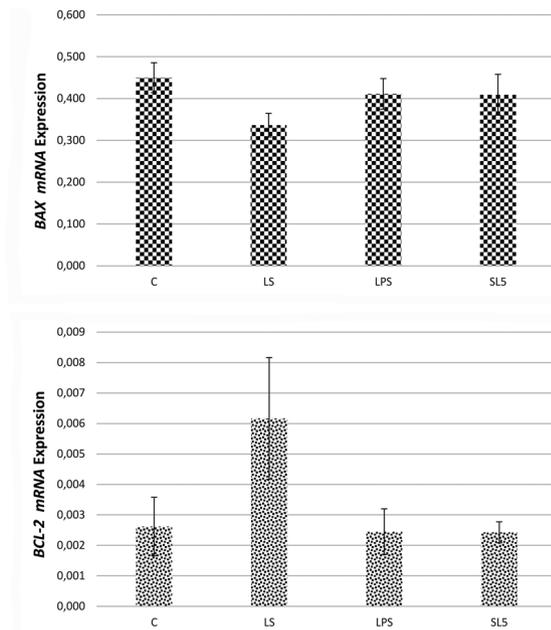
levels increased in LS and SL5 at 3 h, this level is higher only in SL5 group at 6 h ($P < 0.05$). T-prot levels decreased in SL5 at 3 h ($P < 0.05$). Leukocyte level decreased at 3 and 6 h in the LPS group compared to control group, and leukocyte levels of prophylactic (SL5) and treatment (LS) sulfasalazine groups were different LPS group at 3 and 6 h. Platelet counts decreased all endotoxemic groups at 3 h ($P < 0.05$, Table 5) compared to control group.

Molecular analyzes of the genes of the target (*BCL-2* and *BAX*) in liver and brain tissue were shown in Figs. 3 and 4. There were no statistical differences between *BCL-2* and *BAX* parameters in the liver and brain.

Discussion

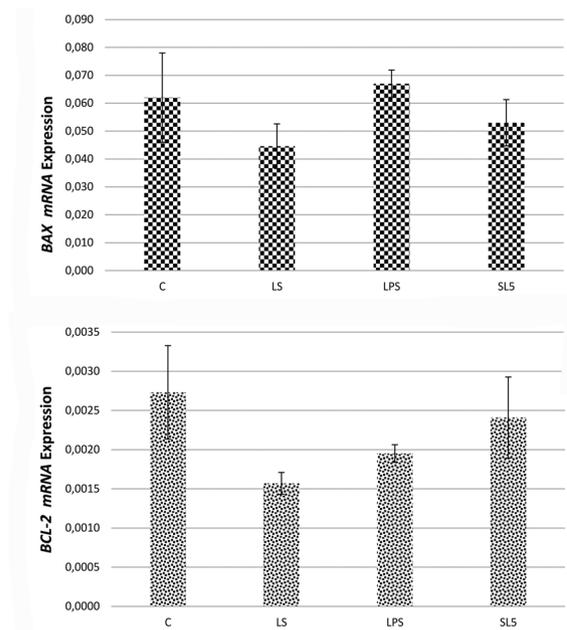
LPS leads to endotoxemia by binding to different plasma proteins in the bloodstream and inducing immunological cells. The stimulation and excessive inflammation causes pro-inflammatory cytokines such as TNF, IL-1 and IL-6 release and fatal clinical symptoms [10]. Many treatment protocols have been tried to increase survival [29].

The proinflammatory cytokines (TNF- α , IL-6 and IL-1) synthesis was suppressed and antiinflammatory cytokine IL-10 was induced by prophylactic (SL5) and treat-



C: Control, LS: sulfasalazine + lipopolysaccharide simultaneously, LPS: Lipopolysaccharide, SL5: Prophylactic sulfasalazine + lipopolysaccharide. There is no statistical difference between the groups ($P > 0.05$).

Fig. 3. The effects of sulfasalazine (300 mg/kg, I.P.) for prophylactic and therapeutic purposes in liver *BAX* and *BCL-2* expressions in LPS-induced endotoxemic rats (mean ± SEM).



C: Control, LS: sulfasalazine + lipopolysaccharide simultaneously, LPS: Lipopolysaccharide, SL5: Prophylactic sulfasalazine + lipopolysaccharide. There is no statistical difference between the groups ($P > 0.05$).

Fig. 4. The effects of sulfasalazine (300 mg/kg, I.P.) for prophylactic and therapeutic purposes in brain *BAX* and *BCL-2* expressions in LPS-induced endotoxemic rats (mean ± SEM).

ment (LS) sulfasalazine administrations within in the first 6 h in the endotoxemia model. *In vitro*, sulfasalazine blocks NF- κ B activation by inhibiting I κ B phosphorylation in the LPS and TNF- α -induced colon cells. Also, sulfasalazine suppresses proinflammatory cytokines and reduces oxidative stress [14, 44]. *In vivo* sulfasalazine is similar effect with *in vitro* results and the effect increases as parallel by the dose increase [12, 25]. In addition, administration of sulfasalazine (360 mg/kg) for 2 weeks reduces TNF- α and oxidative stress (MDA) in experimental bowel disease-induced rats [11]. Sulfasalazine at a dose of 100 mg/kg in experimental sepsis model with LPS (serotype 0127: B8) reduces NF- κ B expression, neutrophil and lipid peroxidation levels but liver damage doesn't fully treat [14]. In the current study, sulfasalazine may have generally reduced the levels of TNF- α , IL-1 β , IL-6 and increased IL-10 levels via inhibiting NF- κ B activation. Also, sulfasalazine may increase antioxidant capacity by reducing inflammation and induce antioxidant enzymes.

Sulfasalazine increases oxidative stress in the liver and kidney and decreases in antioxidant enzyme levels [27]. However, it has antioxidant activity by inhibiting inflammation [14]. The current research suggests that

anti-inflammatory properties of sulphasalazine may have antioxidant effects, because sulfasalazine application increased total antioxidant capacity. Prophylactic and therapeutic sulphasalazine may have inhibited NF- κ B or stabilize I κ B/NF- κ B inactive structure due to reduce inflammation in experimental endotoxemia, thereby showing antioxidant activity (Figs. 1 and 2).

Acute phase proteins, HPT and SAA levels, increased in experimental endotoxemia and the increase could not be prevented in the groups of prophylactic (SL5) and treatment (LS) sulfasalazine groups. Also, PGE₂ level was increased by the inflammation and it was prevented by sulfasalazine treatment (LS) at 3 h. Generally, sulfasalazine had no distinct effect on acute phase proteins within the first 6 h (Table 2). LPS is identified as the potential stimulator of the inflammatory response and acute phase proteins. Cytokines peak in the first few hours and acute phase proteins such as SAA reach maximum levels after about 24 h in endotoxemia [35]. HPT level above basal level by LPS induced endotoxemia in swine after 2 days, while it reaches the highest level in calves at 18 h [26, 35]. HPT levels in experimental endotoxemic dogs increase at 24th hour and reach peak level at 48th hour [43]. In the current study, acute phase

proteins can be partially induced because the research period is limited to 6 h. The effects of sulfasalazine on acute phase proteins may have limited, because acute phase proteins reach maximum levels for longer periods of time (Table 2). LPS effects PGE₂ levels at different doses and times [6]. Sulfasalazine inhibits cyclooxygenase (COX)-2 enzyme activity and has an anti-inflammatory effect [12]. In the current study, PGE₂ may not have induced long-term and sulfasalazine treatment may have restricted the activity of COX enzyme within the first 3 h.

Coagulation parameters ATIII and fibrinogen decreased in experimental endotoxemia. The decreased fibrinogen could be inhibited by prophylactic and treatment sulfasalazine application at first 3 h (Table 3). Activation of the endothelial NF-κB pathway in experimental endotoxemic mice results impaired anticoagulant mechanism, reduces fibrinogen levels, and occurs intravascular coagulation. Whereas, fibrinogen can inhibit by inactivation of the NF-κB pathway [42] and the decrease of fibrinogen levels can be caused by decreased synthesis, use in clotting, and increased vascular permeability [33, 45]. However, in some studies plasma fibrinogen and ATIII levels did not change in septic shock animals [4, 7]. In the current study, LPS administration at a dose of 4 mg/kg may have partially induced the intravascular coagulation mechanism. Also, sulfasalazine could be exhibited poor anticoagulant activity by inhibiting PGE₂ synthetase and PLA₂ enzymes [36]

Hematologic and biochemical values can change in infections and medical treatments [22]. All measured biochemical parameters in the present study are between the reference values. After induction of endotoxemia, leucocyte levels were reduced in the LPS group, sulfasalazine administration inhibited decrease of leucocyte levels in the prophylactic (SL5) and treatment (LS) groups. Platelet levels decreased all endotoxemic groups at 3 h (Table 5). Leukocyte and platelet levels are low in endotoxemic rat model up to 48 h [39]. Leukopenia occurs in the early stages of endotoxemia, because the defence cells move from blood to tissue gap. In addition, thrombocytes are used for clotting in endotoxemia and cause thrombocytopenia [48]. In the current study, sulfasalazine can inhibit the systemic inflammation and improve the endotoxemic hematological indication and effectively block the leukopenia.

NF-κB regulates pro and anti-apoptotic pathways and varies apoptosis level in tissues with endotoxemia [15,

24]. Apoptosis is more prominent in the late period of endotoxemia [24]. In the current study, the expression of liver and brain *BCL-2* and *BAX* expression did not change. The absence of severe endotoxemia and the short duration of the experiment may indicate that apoptosis in tissues are not sufficiently induced.

In conclusion, application of sulfasalazine for prophylaxis and treatment in LPS-induced experimental endotoxemia model prevent excessive inflammation and clotting. Especially, prophylactic and therapeutic sulphasalazine application may show similar effect in endotoxemia. Although prophylactic treatment is not clinically practical, it is thought to be helpful the radical treatment in cases of endotoxemia for any reason in patients with ulcerative colitis in rheumatoid arthritis. As a result, sulphasalazine may be an alternative treatment for endotoxemia treatment.

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