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RESEARCH

CAPE ameliorates vascular damage caused by sepsis

KAFE sepsisin neden olduğu vasküler hasarı iyileştirir

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Abstract

Purpose: In this study, we aimed to investigate the parameters of vascular and oxidative damage caused by sepsis and to evaluated the effects of caffeic acid phenethyl ester (CAPE) on these damages.

Materials and Methods: Wistar-Albino male rats were used for this study. Rats were divided into 4 groups (n = 10). Group 1 animals were intraperitoneally (i.p) injected with sterile saline (Control Group). Group 2 animals were i.p injected with lipopolysaccharide (LPS), 20 mg / kg-weight dose (Sepsis Group). Group 3 animals were i.p injected with lipopolysaccharide, 20 mg / kg-weight dose. Immediately after LPS injection, CAPE was i.p injected at single dose, 10 µmol / kg-body weight (Treatment Group). A single dose of CAPE, 10 µmol / kg-body weight / day, was injected i.p to Group 4 animals for 5 days. After 5th day CAPE injection, a single dose of LPS 20 mg / kg-weight was injected (Protective Group). At the 6th hour after the injections applied to all groups, blood sample were taken intracardiac and their serum were separated for the studies. Homocysteine (Hcy), asymmetric dimethyl arginine (ADMA), endothelin-1 (ET-1) and vascular cellular adhesion molecule-1 (VCAM-1) were measured by enzyme-linked immunosorbent assay (ELISA). In addition, the protective and therapeutic effects of CAPE on these parameters was investigated.

Results: Control group Hcy, ADMA, ET-1 and VCAM-1 levels were found to be 4.987 ± 0.096 µmol/l, 0.803 ± 0.020 nmol/ml, 21.123 ± 2.575 ng/l, 3.155 ± 0.078 ng/ml, respectively. Sepsis group Hcy, ADMA, ET-1 and VCAM-1 levels were found to be 8.975 ± 0.160 µmol/l, 3.953 ± 0.678 nmol/ml, 52.446 ± 2.546 ng/l, 10.783 ± 1.068 ng/ml, respectively. Treatment group Hcy, ADMA, ET-1 and VCAM-1 levels were found to be 5.286 ± 0.037 µmol/l, 1.304 ± 0.040 nmol/ml, 27.995 ± 1.299 ng/l, 3.72 ± 0.073 ng/ml, respectively. Protective group Hcy, ADMA, ET-1 and VCAM-1 levels were found to be 5.401

Öz

Amaç: Bu çalışmada sepsisin neden olduğu vasküler ve oksidatif hasar parametrelerindeki değişikliği araştırmayı ve kafeik asit fenetil esterin (KAFE) bu hasarlar üzerindeki etkilerini değerlendirmeyi amaçladık.

Gereç ve Yöntem: Bu çalışma için Wistar-Albino erkek ratlar kullanıldı. Ratlar 4 gruba ayrıldı (n=10). Grup 1'deki hayvanlara intraperitoneal (i.p) olarak steril salin enjekte edildi (Kontrol Grubu). Grup 2'deki hayvanlara 20 mg/kg ağırlık dozunda lipopolisakkarit (LPS) i.p olarak enjekte edildi (Sepsis Grubu). Grup 3'teki hayvanlara 20 mg/kg ağırlık dozunda lipopolisakkarit i.p olarak enjekte edildi. LPS enjeksiyonundan hemen sonra KAFE i.p olarak tek doz 10 µmol/kg vücut ağırlığı dozunda enjekte edildi (Tedavi Grubu). Grup 4'teki hayvanlara 5 gün boyunca 10 µmol/kg-vücut ağırlığı/gün olacak şekilde tek doz KAFE i.p olarak enjekte edildi. 5. gün KAFE enjeksiyonundan sonra tek doz 20 mg/kg-ağırlık LPS enjekte edildi (Koruyucu Grup). Tüm gruplara uygulanan enjeksiyonlardan sonraki 6. saatte intrakardiyak kan örnekleri alındı ve serumları çalışmalar için ayrıldı. Homosistein (Hcy), asimetrik dimetil arginin (ADMA), endotelin-1 (ET-1) ve vasküler hücresel adezyon molekülü-1 (VCAM-1) enzime bağlı immünosorbent yöntem (ELISA) ile ölçüldü. Ayrıca KAFE'nin bu parametreler üzerindeki koruyucu ve tedavi edici etkileri araştırıldı.

Bulgular: Kontrol grubunda Hcy, ADMA, ET-1 ve VCAM-1 düzeyleri sırasıyla 4,987 ± 0,096 µmol/l, 0,803 ± 0,020 nmol/ml, 21,123 ± 2,575 ng/l, 3,155 ± 0,078 ng/ml olarak bulundu. Sepsis grubunda Hcy, ADMA, ET-1 ve VCAM-1 düzeyleri sırasıyla 8,975 ± 0,160 µmol/l, 3,953 ± 0,678 nmol/ml, 52,446 ± 2,546 ng/l, 10,783 ± 1,068 ng/ml olarak bulundu. Tedavi grubunda Hcy, ADMA, ET-1 ve VCAM-1 düzeyleri sırasıyla 5,286 ± 0,037 µmol/l, 1,304 ± 0,040 nmol/ml, 27,995 ± 1,299 ng/l, 3,72 ± 0,073 ng/ml olarak bulundu. Koruyucu grup Hcy, ADMA, ET-

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± 0.042 μmol/l, 1.431 ± 0.056 nmol/ml, 32.708 ± 1.326 ng/l, 4.058 ± 0.069 ng/ml, respectively. It was observed that the Hcy, ADMA, ET-1 and VCAM-1 levels of the sepsis group increased significantly compared to the control group ($p<0.05$). It was observed that CAPE treatment significantly decreased these parameters levels. However, the use of CAPE as a protective was not as effective as its treatment effect.

Conclusion: Our results demonstrated that sepsis resulted in increase Hcy, ADMA, ET-1, VCAM-1 levels. All these changes indicate that sepsis-mediated vascular damage is increased. Our results demonstrated that CAPE is more effective in preventing sepsis-mediated damages when given as a treatment.

Keywords:. Caffeic acid phenethyl ester, sepsis, homocysteine, asymmetric dimethyl arginine, endothelin-1, vascular cellular adhesion molecule-1.

1 ve VCAM-1 düzeyleri sırasıyla 5,401 ± 0,042 μmol/l, 1,431 ± 0,056 nmol/ml, 32,708 ± 1,326 ng/l, 4,058 ± 0,069 ng/ml olarak bulundu. Sepsis grubunun Hcy, ADMA, ET-1 ve VCAM-1 seviyelerinin kontrol grubuna göre anlamlı düzeyde arttığı görüldü ($p<0,05$). KAFE tedavisinin bu parametre seviyelerini önemli derecede azalttığı görüldü. Ancak KAFE'nin koruyucu olarak kullanımı tedavi edici etkisi kadar etkili değildi.

Sonuç: Sonuçlarımız sepsisin Hcy, ADMA, ET-1, VCAM-1 seviyelerinde artışa neden olduğunu gösterdi. Bütün bu değişiklikler sepsis kaynaklı vasküler hasarının arttığını göstermektedir. Sonuçlarımız KAFE'nin tedavi edici olarak verildiğinde sepsis kaynaklı hasarları önlemede daha etkili olduğunu gösterdi.

Anahtar kelimeler: Kafeik asit fenetil ester, sepsis, homosistein, asimetrik dimetil arjinin, endotelin-1, vasküler hücresel adezyon molekülü-1.

INTRODUCTION

Sepsis is a response to infection characterized by the release of highly reactive oxygen and nitrogen intermediates and the production of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β) and interferon gamma (IFN-γ). Many important inflammatory mediators such as TNF-α, IFN-γ, inducible nitric oxide synthase (iNOS) and intracellular adhesion molecule 1 (ICAM-1), are expressed by hepatic cells in sepsis with lipopolysaccharide (LPS) or bacterial infection[1]. Microvascular and endothelial dysfunction play an important role in the pathogenesis of multiple organ failure and deaths in sepsis. The mechanism of vascular dysfunction in sepsis is not clearly known[2].

Caffeic Acid Phenethyl Ester (CAPE), which is one of the active components of propolis, has antiviral, anti-inflammatory, immunomodulatory and antioxidant properties due to its potent and specific inhibitory effects on nuclear factor kappa B (NF-κB) activation, lipid peroxidation, lipoxygenase, cyclooxygenase activities, protein tyrosine kinase and ornithine decarboxylase[3,4].

Increased homocysteine (Hcy) and asymmetric dimethyl arginine (ADMA) levels have recently been considered as cardiovascular risk factors. Increases in Hcy levels also lead to increased ADMA and associated increased risk of cardiovascular disease with direct inhibition of dimethylarginine dimethyl aminohydrolase (DDAH). In addition to this direct effect, Hcy and ADMA have metabolic connection. S-adenosyl methionine (SAM) in Hcy metabolism turns the methyl group into arginine to S-adenosyl homocysteine (SAH) and this molecule eventually turns into Hcy. Therefore, increased Hcy levels contribute to the increase in cardiovascular risk by causing an increase in ADMA. Recent studies suggest that increased plasma ADMA levels and hyperhomocysteinemia cause endothelial cell dysfunction depend on oxidative damage[5-7]. The relationship between multiple organ failure and adhesion molecule levels in patients with severe sepsis has been investigated and it has been shown that VCAM-1 serum levels increase in multiple organ failure. Different biomarkers such as cell adhesion molecules have been suggested to evaluate vascular endothelial damage in patients with sepsis developed multiple organ failure[8]. Vascular cell adhesion molecule-1 (VCAM-1), a member of the immunoglobulin (Ig) superfamily, has been described to be upregulated in endothelial cells by inflammatory mediators such as cytokines and bacterial lipopolysaccharides. Clinical studies have shown that increasing plasma concentrations of VCAM-1 can be used in the prediction of multi organ dysfunction syndrome and related deaths in neonatal and adult sepsis[9]. Endothelin-1 (ET-1), a member of the endothelin family, has been identified as a potent vasoconstrictor in the pathogenesis of sepsis induced multi organ dysfunction syndrome and sepsis. ET-1 is known as the most potent vasoconstrictor endothelin that has been studied[10]. ET-1 causes platelet aggregation and plays a role in increasing the expression of leukocyte adhesion molecules that synthesize inflammatory mediators that cause vascular dysfunction. The level of ET-1 in the blood increases in the case of sepsis and acts as a biomarker in understanding sepsis severity[11].

There may be in question endothelial damage due to oxidative stress in LPS induced experimental sepsis models. The aim of our study is to investigate the levels of ADMA, Hcy, ET-1, VCAM-1, which are indicators of vascular damage in LPS induced experimental sepsis model. In addition, as a result of the literature researches, the effects of CAPE on sepsis, whose antiviral, anti-inflammatory, immunomodulator, anticarcinogen and antioxidant properties have been studied in recent years, have not been investigated in line with the parameters we use. Therefore, the positive / negative effects of CAPE on vascular marker levels such as ADMA, Hcy, VCAM-1, ET-1 in sepsis were investigated in line with these parameters. As a result of this study, the effects of CAPE on vascular markers in sepsis were tried to enlighten as much as possible.

MATERIALS AND METHODS

Animals experimental design

The study was conducted in the laboratories of Erciyes University, Faculty of Medicine, Department of Medical Biochemistry and Erciyes University Experimental Researches and Application Center. The experimental procedure was followed by researchers who have the certificate of Experimental Animal Use. This study was approved by the animal experiments local ethical committee of Erciyes University (date: 08/10/2014, number: 14/131). The experimental procedures were performed in accordance with the national guidelines and protocols for care and use of laboratory animals. Wistar-Albino male rats, obtained from Erciyes University Experimental Research and Application Center, were used for this study. Animals were placed in standard cages and kept on a 12 h light and dark cycle conditions and were fed with standard laboratory food and water ad libitum.

Rats (250-300 g) were randomly divided into 4 groups (n = 10). Group 1 animals were i.p injected with sterile saline (Control Group). Group 2 animals were i.p injected with lipopolysaccharide, 20 mg / kg-weight dose (Sepsis Group)[12]. Group 3 animals were i.p injected with lipopolysaccharide, 20 mg / kg-weight dose [12]. Immediately after LPS injection, CAPE was i.p injected at single dose, 10 μmol / kg-body weight (Treatment Group) [13-19]. A single dose of CAPE, 10 μmol / kg-body weight / day, was injected i.p to Group 4 animals for 5 days[13-21]. After 5th day CAPE injection, a single dose of LPS 20 mg / kg-weight was injected (Protective Group)[12, 19]. At the 6th hour[22,23] after the injections applied to all groups, it is were taken blood intracardiacly from the animals under ketamine / xylazine (60-10 mg / kg i.p single dose) anesthesia. The bloods were centrifuged at 3000 rpm at +4 ^{0}C for 15 minutes and their serum were separated for the studies.

Biochemical analysis

Rat ADMA ELISA Kit (Cat.No : YHB0130Ra), Rat Hcy ELISA Kit (Cat No: YHB0560Ra), Rat ET-1 ELISA Kit (Cat No: YHB0380Ra), Rat VCAM-1 ELISA Kit (Cat No: YHB1126Ra) commercially available from Shanghai Yehuda Biological Technology Co. Ltd. was used for analysis. These ELISA kits that we used for analysis make assay based on the "biotin double antibody sandwich" technology. According to this method, the substance to be analyzed is added to the wells previously coated with the monoclonal antibody of the analyzed substance and incubated. After incubation, add anti-substance to be analyzed antibodies labeled with biotin to unite with streptavidin-HRP, which forms the immune complex. After incubation and washing, unbound enzymes are removed, then substrates A and B are added. The solution turns blue and changes to yellow with the effect of acid. The shades of solution and the concentration of analyzed substance are positively correlated.

Statistical analysis

The data were evaluated in IBM SPSS Statistics 25.0 (IBM Corp., Armonk, New York, USA) statistical package program. Unit number (n), percentage (%), mean ± standard deviation, minimum value, maximum value, median (M), 25th percentile ($Ç_1$) and 75th percentile ($Ç_3$) values as descriptive statistics was given. The normal distribution of the data of numerical variables was evaluated by Shapiro Wilk normality test. Because it was observed that the variables (ADMA, HCY, ET-1, VCAM-1) according to the groups followed a normal distribution, comparisons between groups were made using One-Way Analysis of Variance. In case of being difference as a result of comparisons between groups, parametric or nonparametric Student-Newman-Keuls tests were used as multiple comparison tests. A value of $p<0.05$ was considered statistically significant.

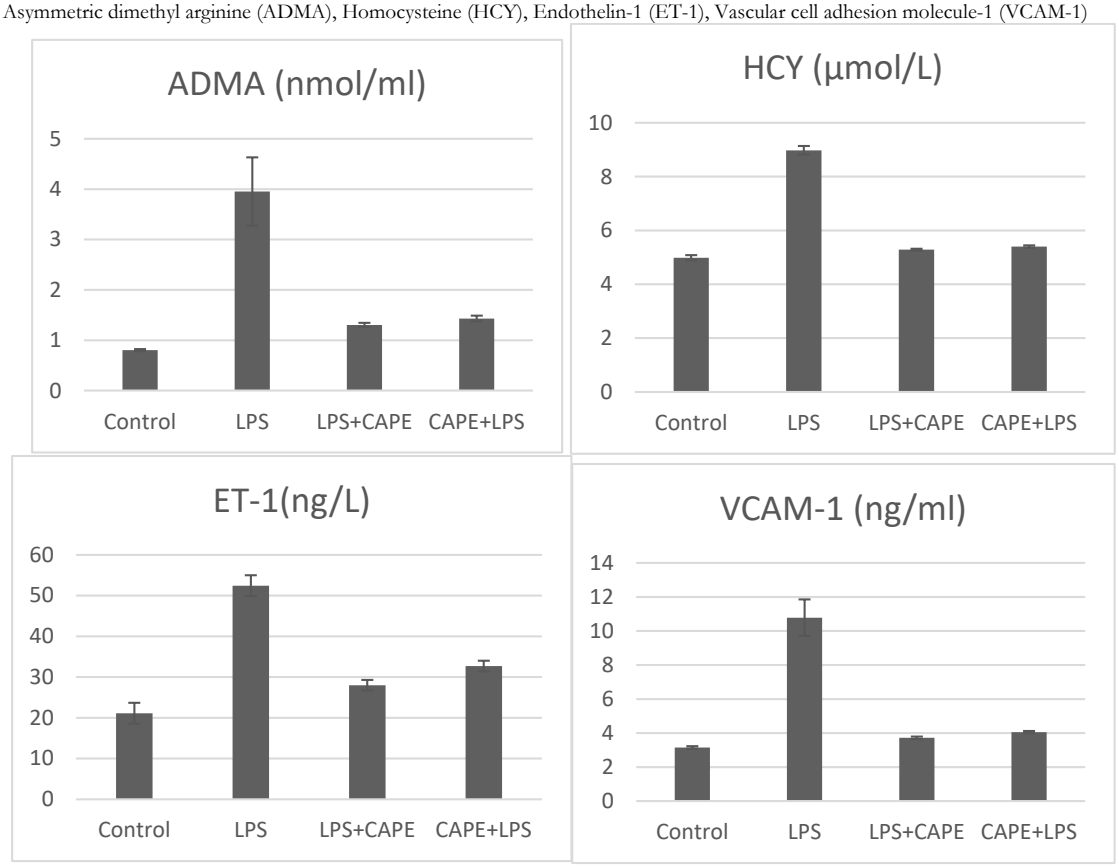
RESULTS

Mean values of the biochemical parameters of all groups obtained from the studies are given in Table 1 collectively and biochemical parameters of each group are given in the Figure 1. Compared ADMA, Hcy, ET-1, VCAM-1 levels, a statistically significant difference was found between the control group and the sepsis group ($p<0.05$). Sepsis significantly increases ADMA, Hcy, ET-1 and VCAM-1 levels. When ADMA, Hcy, ET-1, VCAM-1 levels were compared between sepsis and treatment group, a statistically significant difference was found ($p<0.05$) and the treatment group values of these parameters were found to be close to the control group values. When ADMA, Hcy, ET-1, VCAM-1 levels were compared between sepsis and protective group, a statistically significant difference was found ($p<0.05$) and protective group levels of these parameters approached the control group values (Fig 1, Table 1).

Table 1. Mean values of the biochemical parameters of all groups (X±SD).

Groups	ADMA (nmol/ml)	HCY (µmol/l)	ET-1 (ng/l)	VCAM-1 (ng/ml)
Control	0.803 ± 0.020	4.987 ± 0.096	21.123 ± 2.575	3.155 ± 0.078
Sepsis (LPS)	3.953 ± 0.678[a]	8.975 ± 0.160[a]	52.446 ± 2.546[a]	10.783 ± 1.068[a]
Treatment (LPS+CAPE)	1.304 ± 0.040[b]	5.286 ± 0.037[b]	27.995 ± 1.299[b]	3.72 ± 0.073[b]
Protective (CAPE+LPS)	1.431 ± 0.056[b]	5.401 ± 0.042[b]	32.708 ± 1.326[b]	4.058 ± 0.069[b]

[a]($p<0.05$) Compared to control group
[b]($p<0.05$) Compared to sepsis group
Asymmetric dimethyl arginine (ADMA), Homocysteine (HCY), Endothelin-1 (ET-1), Vascular cell adhesion molecule-1 (VCAM-1)



Sepsis group (LPS), Treatment group (LPS+CAPE), Protective group (CAPE+LPS), Asymmetric dimethyl arginine (ADMA), Homocysteine (HCY), Endothelin-1 (ET-1),Vascular cell adhesion molecule-1 (VCAM-1)

Figure 1. ADMA, HCY, ET-1 and VCAM-1 levels of all groups.

Compared with the control group; treatment group ADMA, Hcy, VCAM-1, ET-1 levels were found close to the control group levels. CAPE given protectively, significantly decreased ADMA, Hcy, ET-1 and VCAM-1 levels in the sepsis group and brought them closer to the control levels. However, the use of CAPE as a protective was not as much as its treatment effect. In other words, the treatmently use of CAPE was found to be more effective than its protectively use.

As can be seen from these results, the therapeutically administered CAPE significantly decreased the ADMA, Hcy, ET-1 and VCAM-1 levels in the sepsis group and approached them to the control levels. When CAPE was given as protectively, significantly decreased sepsis ADMA, Hcy, ET-1 and VCAM-1 values, but did not demonstrate as much effect as CAPE given as therapeutic.

DISCUSSION

Alici O et al.[17] reported that CAPE given to rats with sepsis reduced the levels of ET-1, TNF-α and malondialdehyde (MDA). According to these results, the researchers emphasized that CAPE has positive effects on ET-1, TNF-α levels and oxidative stress parameters in the experimental sepsis model in rats and that CAPE can be used to prevent the harmful effects of sepsis. In our study, an increase in ET-1 levels was observed in the group with sepsis, as in study of Alici O et al. In addition to the parameters of the study, other vascular parameters we studied were also found to be high in the sepsis group. In our study, the therapeutically and protectively use of CAPE significantly improved the vascular parameters. It has been observed that the therapeutic use of CAPE is more effective.

In our studies, rats were sacrificed 6 hours after LPS injection. Because other researchers working on this subject have shown that iNOS induction in tissues reached maximum at the 6th hour after LPS injection, fell rapidly after 12 hours, and reached control values at 18 and 24 hours[24-26]. Some researchers observed in their studies that plasma ADMA levels increased significantly 6 hours after LPS administration. These findings of the researchers seem to be consistent with our results. Because we also found our ADMA levels high in the LPS group. As in our study, these researchers also sacrificed animals at the 6th hour of LPS administration, when iNOS activity was maximum[27-31].

ET-1 is known as the most potent vasoconstrictor endothelin that has ever been studied. There are studies showing that ET-1 is upregulated at the 6th hour of LPS-induced sepsis / septic shock in animal models[10, 32]. Similarly, we also detected high ET-1 levels in samples taken at the 6th hour of sepsis administration in our study. Seki Y et al.[10] in a study in which they investigated whether landiolol hydrochloride corrected LPS-mediated changes in cardiac ET systems of septic rats, it was observed that expression of various components of the cardiac ET-1 system was induced in the LPS group compared to the control. It has been reported that landiolol administration significantly normalizes various components of the cardiac ET-1 signal in septic rats. Ogura Y et al.[33] administered LPS to one group of 8-week-old rats and following the LPS administration applied landiolol to the other group. It has been shown that renal expression of ET-1 is significantly induced in the LPS group compared to controls. Landiolol given to rats treated with LPS brought serum markers of renal damage to normal levels. Similar to these studies, in our study, ET-1 levels were found to be increased in rats with sepsis. Increased ET-1 levels in groups with sepsis were corrected by CAPE. In this respect, we can say that CAPE has a landiolol-like effect on sepsis.

Jesmin S et al. conducted a study to investigate whether ET has a role in sepsis-mediated acute lung injury and whether ET expression is down-regulated by TNF-α formed in the septic lung. In the LPS administered group, it was observed that the time-dependent expression of ETA receptor and ET-1 in the lung was induced by LPS, and it peaked by 3-fold increase 6 hours after endotoxin induction. ETB receptor levels, which have a vasodilator effect, have been down-regulated over time. These results show that the time-dependent increase in ETA receptor and ET-1 and down-regulation of ETB receptor may have an important role in the pathogenesis of acute lung injury resulting from endotoxemia[34]. As seen in the present study, inflammation conditions trigger the formation and release of ET-1. ET-1 release and concentration were also increased in inflammation induced by sepsis in our study.

Leng W. et al.[35] demonstrated that there is LPS-mediated VCAM-1 expression by MT2A (metallothionein 2A, an anti-inflammatory, antiendotoxin and tumor-inhibiting protein) in ECV304 (human umblical vein endothelial cells) cells and this VCAM-1 generation is inhibited by EOLA1

(Endothelial-overexpressed lipopolysaccharide-associated factor 1). Similarly, in our study, LPS-mediated expression of VCAM-1 increased and consequently serum VCAM-1 levels were found to be high.

Su CM et al.[36] found that serum VCAM-1 levels were increased in patients with severe sepsis and septic encephalopathy. In our study, we found that serum VCAM-1 levels were high in the sepsis group. Laudes IJ et al. [9] found that VCAM-1 and ICAM-1 levels and mRNA upregulations were significantly increased in total lung homogenates in sepsis created by cecal ligation and puncture method in mice. In the light of these results, they reported that sepsis impairs endothelial homeostasis and significantly promotes neutrophil adhesion in the lung microvascular system, thus increasing the risk of lung damage. Amalakuhan B et al.[8] investigated the relationship between multiple organ failure and the levels of adhesion molecules in patients with severe sepsis, and as a result; They observed that the serum levels of ICAM-1 and VCAM-1 increased in multi-organ failure. Whalen MJ et al.[37] reported that plasma levels of VCAM-1 were significantly higher in children who developed sepsis induced multi organ failure compared to the control group. As can be seen from the these studies, in all cases of sepsis or severe inflammation, VCAM-1 is expressed and its levels increase in response to inflammation. In other words, we can call the sepsis response mediator for VCAM-1. Similarly these studies, we detected high VCAM-1 levels in response to the experimental sepsis we created in our study. However, when the CAPE we used was used both as therapeutic and protectively, it prevented VCAM-1 expression.

Aydemir O et al.[2] conducted a study aim to investigate ADMA and L-arginine levels and their relationship with the severity of the disease in neonatal sepsis. They found higher L-arginine and ADMA levels in newborns with sepsis compared to controls. As a result, they showed that L-arginine and ADMA levels increased in neonatal sepsis. As can be seen from the above study and the studies we have mentioned before, in inflammatory conditions such as sepsis, plasma ADMA levels increase in response to sepsis. In our study, serum ADMA level was found to be high in response to inflammation. The level of ADMA, an endogenous inhibitor of nitric oxide synthase, is associated with increased endothelial dysfunction and atherosclerosis. ADMA level can be increased by Hcy. Hcy increases ADMA levels by inhibiting of DDAH enzyme activity[5]. Yücel H et al.[5] investigated the relationship between ADMA, $NO^{\cdot}$ and Hcy in patients with slow coronary flow (SCF). With this study, investigators showed that plasma $NO^{\cdot}$ concentrations decrease in SCF and that plasma ADMA and Hcy concentrations increase in SCF and that they are also independent predictors of SCF. Sreckovic B et al. [38] showed that abdominal obesity, hypertension, hypertriglyceridemia, inflammatory factors, homeostatic model assesment insulin resistance (HOMA-IR), microalbuminary and Hcy, which are markers of endothelial dysfunction, increased in patients with metabolic syndrome. Based on these results; they stated that there is a relationship between Hcy, hypertension and hyperlipoproteinemia and that Hcy is a potential marker in the atherosclerosis process. In our study, we found that ADMA and Hcy levels were high in sepsis. Our findings show that sepsis causes both SCF and endothelial dysfunction as in this study.

The limitation of the study is that the study could not be supported by molecular analyses, but supporting our data with molecular analysis in further studies will contribute to sepsis treatment approaches.

Our study show that vascular damage markers increase in sepsis conditions. CAPE prevents the formation of increased vascular damage markers in sepsis conditions. CAPE brought ADMA, Hcy, ET-1 and VCAM-1 levels closer to the control values, which increased significantly as a result of sepsis compared to controls. The results of our study show that CAPE is more effective therapeutically. Therefore, we concluded that CAPE can be used therapeutically to prevent sepsis mediated vascular damage.

Author Contributions: Concept/Design : LC, AC, FE; Data acquisition: LC, FE; Data analysis and interpretation: LC, FE; Drafting manuscript: LC; Critical revision of manuscript: LC, AC, FE; Final approval and accountability: LC, AC, FE; Technical or material support: LC, AC; Supervision: LC, AC; Securing funding (if available): n/a.
Ethical Approval: This study was approved by the animal experiments local ethical committee of Erciyes University (date: 08/10/2014, number: 14/131).
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