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CLINICAL RESEARCH / KLİNİK ARAŞTIRMA

Investigation of the Effect of 4-Hydroxyphenylboronic Acid on Endothelial Damage due to LPS-Induced Endotoxemia in the HUVEC Line via Endocan

4-Hidroksifenilboronik Asitin HUVEC Hücre Hattında LPS Kaynaklı Endotoksemiye Bağlı Endotel Hasarına Etkisinin Endocan Yoluyla Araştırılması

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Abstract

Objective: In our study, the effect of 4-hydroxy phenylboronic acid (4OHFBA), a derivative of boronic acid, on lipopolysaccharide (LPS) induced endothelial damage was investigated in the human umbilical vein endothelial cell line (HUVEC). It has been shown that 4 hydroxy phenylboronic acid may be effective in LPS induced cell death and endothelial damage.

Methods: The study was conducted on the HUVEC line. The cells were treated with 10 µg/mL LPS for 12 h. They were treated with 4 different doses of 4OHFBA 1 h after LPS administration. Cell viability percentage and endocan levels were measured after 24th and 48th hours.

Results: When the endocan levels were examined at 24th and 48th hours, it was observed that the endocan level in the LPS group was significantly higher compared to the healthy group ($p<0.05$). The endocan levels were significantly lower in the groups treated with 31.25 µM, 62.5 µM, 125 µM, and 250 µM 4OHFBA compared to the LPS group ($p<0.05$). It is seen that this decrease is much greater in the 62.5 µM + 4OHFBA group and this is the group that is closest to the healthy group ($p<0.05$).

Conclusion: These findings show that 4OHFBA acid may be effective for LPS-induced cell death and endothelial damage.

Keywords: 4-hydroxy phenylboronic acid, HUVEC, LPS, endothelium, endocan

Öz

Amaç: Çalışmamızda boronik asit türevi olan 4-hidroksi fenilboronik asidin (4OHFBA), lipopolisakkarit (LPS) ile indüklenmiş endotel hasarındaki etkisi insan umblikal ven endotel hücre hattında (HUVEC) incelenmiştir. 4OHFBA'nın LPS'ye bağlı hücre ölümünde ve endotel hasarında etkili olabileceği endokan seviyeleri üzerinden göstermiştir.

Yöntem: Çalışma HUVEC hücre hattında yapılmıştır. Hücreler 10 µg/mL LPS ile 12 saat muamele edildi. LPS verildikten 1 saat sonra 4 farklı dozda 4OHFBA ile muamele edildi. Yirmi dördüncü ve 48. saatlerde hücre canlılık yüzdesi ve endokan seviyeleri ölçüldü.

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Öz

Bulgular: Endokan seviyeleri 24. ve 48. saatte incelendiğinde; LPS grubundaki endokan seviyesinin sağlıklı gruba göre anlamlı şekilde arttığı görülmüştür (p<0,05). 31,25 µM, 62,5 µM, 125 µM ve 250 µM 4OHFBA ile muamele edilen gruplarda endokan seviyelerinin LPS grubuna göre anlamlı derecede azaldığı gözlemlenmiştir (p<0,05). 62,5 µM + 4OHFBA grubunda bu azalmanın çok daha fazla olduğu ve sağlıklı gruba en yakın olan grup olduğunu görülmektedir (p<0,05).

Sonuç: Bu bulgular bize 4OHFBA'nın LPS'ye bağlı hücre ölümünde ve endotel hasarında etkili olabileceğini göstermiştir.

Anahtar Kelimeler: 4-hidroksi fenilboronik asit, HUVEC, LPS, endotel, endocan

Introduction

Sepsis is defined as multiple organ dysfunction caused by an irregular host response to infection. Shock and organ failure may develop due to sepsis. It is a systemic infectious disease that should be treated immediately as it can lead to death. Although new methods of treatment have been developed recently, sepsis still has a high mortality rate[1]. It is one of the main health problems worldwide. It is estimated that 31.5 million cases of sepsis and 19.4 million cases of severe sepsis are treated in hospitals worldwide each year, with up to 5.3 million deaths per year worldwide[2]. Sepsis is a complex pathophysiological process leading to low blood pressure, acid-base balance disorders, systemic inflammatory response, multiple organ failure and tissue damage, acute lung injury, or acute respiratory distress syndrome[3]. There are 3 main elements involved in the pathogenesis of sepsis: systemic inflammation, coagulation, and impaired fibrinolysis. Our humoral system is activated due to infection or traumatic damage to tissues that occurs for any reason. A large number and variety of cytokines are released into circulation. This common inflammatory response leads to violation of hemostatic balance. As a result, damage to many tissues and organs occurs[4]. To balance the inflammatory response completely affecting the organism that occurs in sepsis, molecules, mediators, and cytokines with counter-effects are released. Thus, an attempt is made to stabilize and regulate the inflammatory response. Interleukin 1 (IL-1) β, IL-6, IL-8, tumor necrosis factor-alpha (TNF-α), and platelet-activating factor are released into circulation. TNF-α and IL-1 β constitute the prototype of inflammatory cytokines. The prototype of anti-inflammatory cytokines is IL-10. TNF-α and IL-1 β are released shortly after the induction of sepsis and release secondary cytokines, lipid mediators, and reactive oxygen metabolites[5].

In recent years, endothelial dysfunction and neutrophil dysregulation have been identified as central events in the physiopathology of sepsis[6,7]. Normal vascular endothelium is formed by a layer of endothelial cells on a basal membrane with glycocalyx[8]. The vascular endothelium is a semi-permeable barrier that covers the inner surfaces of blood vessels and controls the exchange of fluids, leukocytes, and plasma proteins by coordinating the opening and closing of the cell connections that form its structure[9]. It prevents entry of microorganisms into tissues. At the same time, it acts as a natural anticoagulant that prevents uncontrolled activation of coagulation. Endothelial dysfunction promotes tissue edema, increases vascular permeability and disrupts the perfusion of vital organs. it plays a critical role in the pathogenesis of organ failure due to sepsis[10]. One of the molecules that indicate endothelial cell activation is endocan. Endocan was first cloned by Lassalle and colleagues in 1996 from a human umbilical vein endothelial cell line (HUVEC)[11]. This molecule was originally named endothelial cell-specific molecule 1 (ESM-1) because it was thought to have a limited distribution in vascular endothelial cells. However, as studies have progressed, it has been understood that ESM-1 belongs to the proteoglycan family and therefore was named It is a proteoglycan secreted and circulated by endothelial cells. Endocan expression has been detected mainly in lung tissue, cultured endothelial cells, vascular structures of the skin and adipose tissue, and coronary and pulmonary arteries[12,13]. It interacts with signaling molecules. It has also been found to regulate cell migration and proliferation in the event of illness. It is known that endocan is overexpressed in various vascular diseases in which endothelial function is compromised[14-16]. It has been found that endocan increases during sepsis and decreases when sepsis improves[17]. At the same time, it has been shown that there is a correlation between endocan levels and sepsis severity[18]. Numerous experimental and clinical studies are being conducted and new pharmacological agents are being tried to prevent or treat endothelial dysfunction of sepsis and ultimately reduce mortality[19,20].

Boron ore, of which 73% of the world's reserves are located in this country, is used in different fields of health[21].

The history of boron actually dates back to ancient times. There is historical evidence that it was used by the Babylonians 4000 years ago, and the Egyptians also used it for embalming, metallurgical, and medical purposes[22]. Boron is a non-metal found in nature bound to oxygen and has been shown to be necessary for the completion of the life cycle of organisms in the phylogenetic kingdom[23]. It has not been previously shown that boron has no beneficial effects on human health. In recent years, *in vitro* and *in vivo* studies have shown that a nutritional amount of boron can have beneficial effects on human health[24]. It has been shown in animal studies that the use of appropriate doses of boron is important for normal immune function and responses[25,26]. There are also studies that suggest that boron has effects on inducing antioxidant defense mechanisms that can destroy free oxygen radicals[27]. The lipoxygenase enzyme involved in the inflammation response in cultured human leukocytes has been inhibited by boron[28]. Boron also directly affects the activity of enzymes such as collagenase, elastase, and trypsin. With this effect, it regulates the extracellular matrix[29]. Gene expression levels of some extracellular matrix proteins related to bone tissue, such as type 1 collagen, osteopontin, osteocalcin, and bone sialoprotein, increase with boron therapy[30,31]. Boron is thought to improve wound healing by its effect on the extracellular matrix and by its antimicrobial effect[32,33].

Based on all the information we have mentioned about boron and its derivatives, we think that there may be beneficial effects of 4-OH-phenylboronic acid, a boron derivative, in the prevention of LPS-induced endothelial damage. There is not much information in the literature on 4-OH-phenylboronic acid. However, its pharmacological properties are similar and it is possible that it may have effects similar to those of other boronic acid derivatives in the same group. We believe that our study will make an important contribution to the literature by using boron and its derivatives to prevent endothelial damage. In our study, the protective effect of 4-OH-phenylboronic acid on LPS-induced endothelial damage in HUVECs was demonstrated by endocan level and cell viability tests showing endothelial damage.

Materials and Methods

Chemicals, Reagents, and Kits

LPS L2880 Sigma-Aldrich lipopolysaccharides from *Escherichia coli* purified by phenol extraction O55:B5 were supplied by Merck Industrial & Lab Chemicals. 4-hydroxy phenylboronic acid (4OHFBA) was provided by the National Boron Research Institute (TENMAK BOREN). Dulbecco's modified Eagle medium (DMEM), cell culture medium, and reagents such as fetal bovine serum and penicillin/streptomycin were obtained from Gibco (Invitrogen Inc., Grand Island, New York, USA). The methylthiazole tetrazolium (MTT) cell proliferation kit was obtained from Roche (Basel, Switzerland). The ELISA kit was obtained from Sunlong Biotech (China).

Cell Culture and Lipopolysaccharide Administration

The HUVEC line obtained from the American Type Culture Collection (ATCC, USA) was removed from the liquid nitrogen tank and planted in a T75 cm^2 flask with a DMEM feeding slot containing 10% FBS and 1% penicillin streptomycin and incubated in an incubator with 5% CO_2 at 37 °C and 90% humidity. The cells were successively subcultured. After the second subculture, the cell count was performed and cells were transplanted into each well of the 48-well plate (2×10^5 cell/well). The cells were incubated for 24 h for adhesion when they were about 80% confluent. Then, the cells were treated with 10 µg/mL LPS for 12 h[34]. 4OHFBA treatments were applied 1 h after LPS administration[35]. The 4OHFBA doses were 31.25 µM, 62.5 µM, 125 µM, and 250 µM.

Experimental Groups

The cells were divided into six groups as described below.

Healthy group: Group to which LPS was not administered and that was not treated with 4OHFBA.

The LPS group: Group treated with 10 µg/mL LPS for 12 h but not treated with 4OHFBA.

- LPS + 31.25 µM 4OHFBA groups: group treated with 10 µg/mL LPS for 12 h and received 31.25 µM 4OHFBA treatment.

- The LPS + 62.5 µM 4OHFBA group: group treated with 10 µg/mL LPS for 12 h and received 62.5 µM 4OHFBA treatment.

- The LPS + 125 µM 4OHFBA group: group treated with 10 µg/mL LPS for 12 h and received 125 µM 4OHFBA treatment.

- The LPS + 250 µM 4OHFBA groups: group treated with 10 µg/mL LPS for 12 h and received 250 µM 4OHFBA treatment[36].

MTT Assay

The HUVEC line, supplied by the ATCC, USA, was transplanted to each well of 3 separate 96-well plates (5×10^4 cell/well) after subculture and cell count; subsequently, the cells were exposed to 10 µg/mL LPS. The 4OHFBA treatment

with different concentrations (31.25 µM, 62.5 µM, 125 µM, and 250 µM) was given after 1 h of LPS administration. To study the effect of 4OHFBA against LPS on both cell proliferation and viability, the cells were incubated for 24 and 48 h. After incubation, 20 µl of MTT solution (5 mg/mL) was added to each well and the cells were incubated at 37 °C for 4 h. Subsequently, DMSO was added to dissolve the blue formazan product and cell viability was determined by measuring absorbance at a wavelength of 550 nm (Epoch Microplate Spectrophotometer, BioTek, USA)[37].

ELISA

Samples collected from cell lines were centrifuged with the cell culture supernatant that was in the kit manual for 20 min at 2 to 8 °C and 3000 rpm and then the supernatants obtained underwent an ESM-1 (endocan) assay using ELISA measured on the Epoch Spectrophotometer System and Take 3 Plate (BioTek) device at 24th and 48th hours. A standard curve was drawn and an equation was obtained from the absorbance of standards. Linear endocan concentrations expressed in ng/ml were calculated according to this equation. The ELISA was performed in accordance with the steps described in the kit protocol.

Statistical Analysis

For all statistical analyzes, SPSS-20 for Windows (IBM Corp., NY, Armonk, USA) was used The normality of the distribution of variables was determined by the Shapiro-Wilk test. Since the ELISA results showed a normal distribution, the comparisons of the groups were analyzed with the one-way ANOVA post hoc Duncan test the normality of the distribution of the MTT assay results was determined by the Shapiro-Wilk test. The results were analyzed using one-way ANOVA post hoc Tukey test. P values less than 0.05 were considered statistically significant[38].

Results

MTT Results

When the MTT results were examined at 24 h, it was observed that the percentage of cell viability in the LPS group decreased significantly compared to the healthy group ($p<0.001$). The percentage of cell viability was significantly higher in groups treated with LPS and subsequently treated with 31.25 µM, 62.5 µM, 125 µM, and 250 µM 4OHFBA compared to the LPS group ($p<0.001$). When the groups given 4OHFBA were compared to the LPS group, it was determined that the percentage of viability in the 62.5 µM + 4OHFBA group was significantly higher than that in the other groups, and this was the closest group to the healthy group ($p<0.001$) (Figure 1).

When the MTT results were examined at 48 h, it was observed that the percentage of cell viability in the LPS group decreased significantly compared to the healthy group ($p<0.001$). The percentage of cell viability was significantly higher in the groups treated with 31.25 µM, 62.5 µM, 125 µM, and 250 µM 4OHFBA compared to the LPS group ($p<0.001$). When the groups given 4OHFBA were compared to the LPS group, it was determined that the percentage of viability in the 62.5 µM + 4OHFBA group was significantly higher than that in the other groups, and this was the closest group to the healthy group ($p<0.001$) (Figure 1).

ELISA Results

When the endocan levels were examined at 24 h, it was observed that the endocan level in the LPS group was significantly higher compared to the healthy group ($p<0.05$). The endocan levels were significantly lower in the groups treated with 31.25 µM, 62.5 µM, 125 µM, and 250 µM 4OHFBA compared to the LPS group ($p<0.05$). It is seen that this decrease is much greater in the 62.5 µM + 4OHFBA group

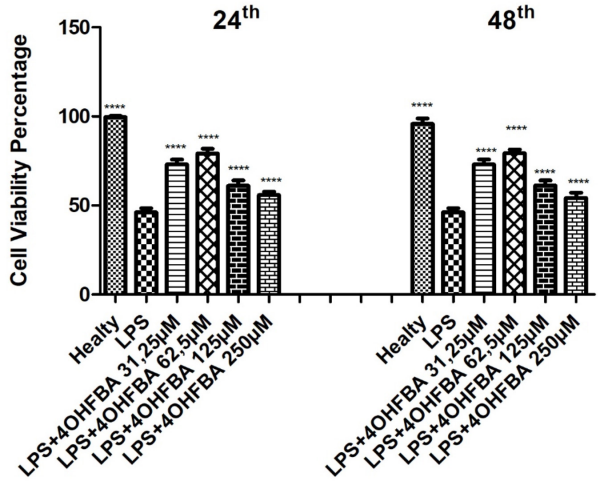

Figure 1. 24- and 48-h MTT measurement results for groups. Comparison of cell viability percentage. The results were expressed as percentage of cell viability compared to LPS group. According to the one-way ANOVA Tukey test, the difference between the groups was statistically significant in comparison between the LPS group and the other groups ($p<0.001$) (**** refers to $p<0.001$)

MTT: Methylthiazole tetrazolium, LPS: Lipopolysaccharide, 4OHFBA: 4-hydroxy phenylboronic acid

and this is the group that is closest to the healthy group ($p<0.05$) (Figure 2).

When the endocan levels were examined at 48 h, it was observed that the endocan level in the LPS group was significantly higher compared to the healthy group ($p<0.05$). The endocan levels were significantly lower in the groups treated with 31.25 µM, 62.5 µM, 125 µM, and 250 µM 4OHFBA compared to the LPS group ($p<0.05$). It is observed that this decrease is much greater in the 62.5 µM + 4OHFBA group and this is the group that is closest to the healthy group ($p<0.05$) (Figure 3).

Discussion

In the present study, the possible protective effect of 4-OH-phenylboronic acid, a derivative of boronic acid, on the LPS-induced endothelial damage model was studied *in vitro* in the HUVEC line. Examination of the results of the study revealed that the rate of cell viability of HUVEC lines endothelially damaged with LPS increased after treatment with 4-OH-phenylboronic acid. It has been shown that the increased amount of endocan, which is another finding of the study and indicates endothelial damage, is reduced by the administration of 4-OH-phenylboronic acid. These results revealed that 4-OH-phenylboronic acid is effective in preventing endothelial damage.

Endothelial cells line the lumen surface of blood vessels. Aligned side by side, they form the endothelial layer, which serves as a barrier between the vascular bed and the blood[39]. The surface of the endothelium is covered with a single layer of glycocalyx, which is formed by proteoglycans and glycoproteins. They consist of proteoglycans, proteins, and glycosaminoglycan side chains and are involved in the regulation of physiological and pathophysiological functions[40]. Maintaining vascular endothelial integrity is crucial for organ health. This integrity is preserved by maintaining the balance between the decay and repair of endothelial cells. If the balance is disturbed, endothelial dysfunction occurs[41]. Sepsis and inflammation are conditions associated with endothelial dysfunction, including vasodilation, edema, coagulopathy, ischemia, and multiorgan failure. Endothelial dysfunction also occurs in cases such as acute lung injury and anaphylaxis. In the case of dysfunction, the protective balance of the endothelium is disturbed and the permeability of the endothelial barrier changes.

Endocan, also called ESM-1, is a specific endothelial mediator[42]. It is a dermatan sulfate proteoglycan that is constantly secreted by endothelial cells and circulates in a soluble form in the blood[43]. Endocan is involved in the regulation of biological processes related to cell adhesion,

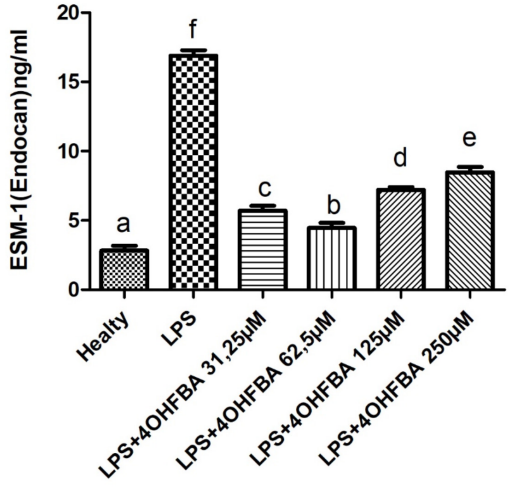

Figure 2. The amount of endocan at 24 h. (According to the one-way ANOVA Duncan test, there is no significant difference between columns marked with the same letter, while the difference between groups marked with different letters is statistically significant $p<0.05$)

ESM-1: Endothelial cell-specific molecule 1,
LPS: Lipopolysaccharide, 4OHFBA: 4-hydroxy phenylboronic acid

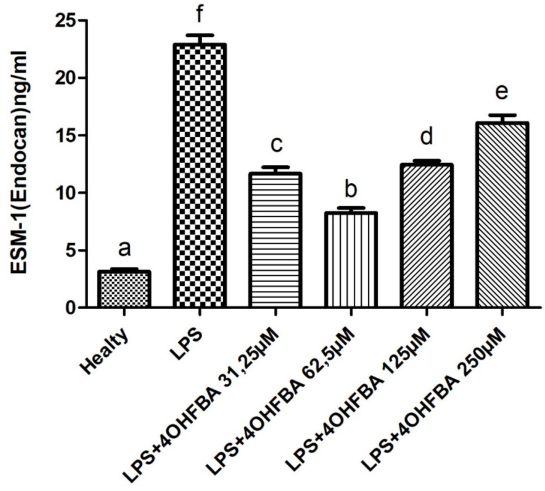

Figure 3. The amount of endocan at 48 h. (According to the one-way ANOVA Duncan test, there is no significant difference between columns marked with the same letter, while the difference between groups marked with different letters is statistically significant $p<0.05$)

ESM-1: Endothelial cell-specific molecule 1,
LPS: Lipopolysaccharide, 4OHFBA: 4-hydroxy phenylboronic acid

migration, proliferation, and neovascularization to interacting with various signaling molecules[44]. Endocan is a vasculoprotective molecule under normal physiological conditions and promotes vasodilation. It protects the endothelium from migration and proliferation of inflammatory cells. It plays a central role in the regulation of endothelial dysfunction due to inflammation[45]. It has been shown that endocan expression is induced under pathological conditions after disruption of endothelial function. Therefore, endocan can be considered an indicator of endothelial cell function and activity[46]. In addition to the well-defined role of endocan in endothelial cell function, recent studies suggest that it also plays a role in the regulation of inflammation[14,45]. The increased endocan expression has been reported in several inflammatory disease cases. For example, it has been shown that endocan levels increase in synovial tissues with arthritis[47]. Similarly, endocan levels were found to be elevated in various types of cancer. For this reason, it is a potential new endothelial cell marker for cancer and a new target for cancer treatment[48,49]. Circulating endocan levels, when examined in overweight and obese individuals, were found to be consistent with the idea that endocan may play an important role in obesity-related vascular diseases[50]. The amount of endocan in the blood indicates the presence and severity of inflammation but also reflects the response to treatment. In a recent study, endocan blood levels were found to be quite high in patients with sepsis, while the control subjects showed no endocan presence[51]. In another study, it was reported that the level of circulating endocan is associated with the severity of sepsis and indicates the clinical course of the patient[18]. It is thought that endocan may be a potential strategy for the treatment of sepsis in in vitro and in vivo studies[52].

The anti-inflammatory effects of boron and its derivatives have been demonstrated in various studies. It was observed that intensive inflammation after LPS in RAW 264.7 cells improved when bortezomib, a boron derivative, was administered as a pretreatment. It is thought that bortezomib pretreatment may be a new therapeutic target for the treatment of intense inflammation seen in severe sepsis[53]. Again, in a different cell culture study, it was found that the use of boric acid had an anti-inflammatory effect in the model of inflammation caused by LPS in THP-1 cells[54]. It has been shown that different doses of boric acid treatment can reduce renal inflammation and kidney damage[55]. It has been stated that the use of boric acid has an antioxidant and anti-inflammatory effect in the ovarian ischemia/reperfusion model[56]. Various studies have shown that boron and its derivatives, which have been shown to have potential positive effects on various inflammatory processes, have similar protective effects on endothelial damage. Bortezomib has been found to have a protective effect on endothelial damage against oxidative stress through Nrf2 modulation in human microvascular endothelial cells[57]. In support of these studies, we have shown that phenylboronic acid has significant effects on LPS induced cell death and endothelial damage.

Study Limitations

Endocan, syndecan-1, and heparin sulfate have been suggested as markers of glycocaly degradation. In future studies, it will be appropriate to determine syndecan-1 and heparin sulfate levels.

Conclusion

When we examined all the findings in our study together, we observed that cell death, which increased with the application of LPS, and increased endocan levels, which are markers of endothelial damage, were reduced by 4OHFBA treatment. These findings have shown that 4-hydroxyphenylboronic acid may be effective for LPS-induced cell death and endothelial damage. This study needs to be supported by further clinical studies.

Ethics

Ethics Committee Approval: Ethical approval is not required as the study was a full cell culture study.

Informed Consent: N/A

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: P.A., Concept: P.A., M.Ç., Design: P.A., M.Ç., Data Collection or Processing: P.A., Analysis or Interpretation: M.Ç., Literature Search: M.Ç.,Writing: P.A.

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