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Comparison of endothelin and nitric oxide synthase blockers on hemorheological parameters in endotoxemic rats

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Background/aim: Septic shock is an important health problem that vastly alters cardiovascular and hemodynamic status. Increased production of nitric oxide (NO) and endothelin is a counterpart of this endotoxemic state. This study was conducted to test the hypothesis that nonselective NO synthesis blocker (L-NAME), inducible NO synthesis blocker (L-canavanine), or endothelin receptor antagonist (bosentan) will reverse the effects of sepsis on hemorheological parameters.

Materials and methods: Forty-eight male Sprague-Dawley rats were used in 8 groups: saline (control), endotoxin, bosentan, L-NAME, L-canavanine, endotoxin + bosentan, endotoxin + L-NAME, and endotoxin + L-canavanine. Blood was withdrawn at the 4th hour of endotoxemic state. Erythrocyte deformability and erythrocyte aggregation were determined by laser-assisted optical rotational cell analyzer at 37 °C. Plasma viscosity (mPa.s) was measured by a cone-plate viscometer with 0.5 mL of plasma.

Results: Endotoxin administration significantly increased aggregation half-time and lowered erythrocyte aggregation amplitude and aggregation index compared to the control, indicating a slower and weaker aggregation pattern. L-NAME and L-canavanine alleviated the effects of endotoxin on erythrocyte aggregation without altering the values in the control animals. However, bosentan did not perform such a restoration.

Conclusion: This finding suggests that these restoration effects of the blockers occur via their modulation of nitric oxide synthesis rather than through the endothelin pathway.

Key words: Hemorheology, plasma viscosity, erythrocyte deformability, erythrocyte aggregation, sepsis, endotoxemia

1. Introduction

Sepsis alters the cardiovascular and hemodynamic status vastly and constitutes a major health problem. Mortality rate is high with this complex, life-threatening clinical condition. Animal experiments have shown altered cardiovascular properties during sepsis. Low blood pressure, multiple organ damage, irresponsiveness of vascular beds to constrictor agents, and other severe pathologies have been observed. In experimental sepsis models, lipopolysaccharides (LPS) extracted from certain microorganisms are administered to laboratory animals to induce septic shock. LPS released from the cell wall of the bacteria cause systemic inflammation, which is followed by hypotension, disseminated intraarterial coagulation, and attenuation of blood flow to the vital organs, followed by multiple organ failure and death (1). Increase in the production of endothelium-derived substances, such as nitric oxide (NO) and endothelin, is a component of this endotoxemic state. To reverse this situation, blockers of such substances have been tested in animal experiments.

Changes in hemorheological parameters have been claimed to be a cause of microcirculatory disturbances in sepsis. It has been reported that sepsis and septic shock decrease erythrocyte deformability and increase erythrocyte aggregation, both in human subjects and in experimental animals (2–11). Oxidative stress, LPS and related inflammatory mediators, decreased ATP reserves, and changes in 2,3 DPG concentration have been claimed to be the underlying causes of rheological changes (12,13).

NO, an important regulator of vascular tone, is also found to be a contributor to hemorheological changes in sepsis. Human erythrocytes are capable of synthesizing their own NO, and inhibition of eNOS activity in human erythrocytes alters hemorheological parameters. Many findings on hemorheological effects of NO suggest that it improves erythrocyte deformability while decreasing erythrocyte aggregation, thereby favoring blood flow by

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decreasing viscosity (14–16). However, contradictory reports are also available (17), and Mesquita et al. (18) reported a dose-dependent effect on erythrocyte properties. It was previously reported that inhibition of eNOS by selective inhibitors in human blood diminished erythrocyte deformability; however, that effect was minute and partially observed only under lower shear stress conditions.

Endothelin is another important actor for vascular tonus, and has been found to affect erythrocyte physical behavior. Sakashita et al. (19) have reported that endothelin-1 and a specific type-B endothelin receptor agonist (IRL 1620) both improved the impaired filterability of mechanically stressed red blood cells. Schut et al. (20) have reported that cyclosporine-treated renal transplant patients had low erythrocyte deformability, accompanied by elevated endothelin levels. Rossenbach et al. (21) have reported improvement of erythrocyte aggregation (by capillary microscopy), accompanied by a decrease in endothelin-1 level after lipid apheresis treatment. Walter et al. (22) have reported that endothelin-1 has no effect on blood viscosity.

Although there is a considerable number of studies focused on the relationship between sepsis and erythrocyte deformability, research considering its impact on erythrocyte aggregation is limited (4).

This study was conducted to test the hypothesis that nonselective NO synthesis blocker (L-NAME), inducible NO synthesis blocker (L-canavanine), or endothelin receptor antagonist (bosentan) will reverse the effects of sepsis on hemorheological parameters.

2.1. Materials and methods

2.1. Animals

Forty-eight male Sprague-Dawley rats (250–300 g) were used in 8 groups: saline (control), endotoxin, bosentan, L-NAME, L-canavanine, endotoxin + bosentan, endotoxin + L-NAME, and endotoxin + L-canavanine. The rats were administered intraperitoneally with bosentan (30 mg kg^{-1}), L-NAME (3 mg kg^{-1}), or L-canavanine (100 mg kg^{-1}) 2 h after they received saline or *E.coli* endotoxin (4 mg kg^{-1}). All chemicals were obtained from Sigma and dissolved in saline before administration. Blood was withdrawn into heparinized tubes, and four main parameters contributing to blood viscosity were measured at the 4th hour of endotoxemic state.

The animals were kept in a room where the ambient temperature and relative humidity were stable. Water and food were available ad libitum.

Ethical permission was obtained from the Ethical Committee for Animal Experiments, Hacettepe University, and all procedures were performed in accordance with the Helsinki Declaration.

2.2. Erythrocyte deformability

Erythrocyte deformability was determined by laser-assisted optical rotational cell analyzer (LORCA; Mechatronics, Holland) at 30 Pa shear stress and 37 °C (23). To measure erythrocyte deformability, blood was withdrawn from the heart under light anesthesia into a heparin-coated injector (15 U/mL). Approximately 10 mL of blood was drawn from each animal, of which 25 µL was mixed with 5 mL of PVP medium (polyvinylpyrrolidone, 360,000 MW, 92,300 mOsm/kg). From this suspension, 1 mL was placed into the LORCA, into the chamber formed by the gap between two concentric glass cylinders. In this system, the rotating outer cylinder creates a shear stress on the suspension, causing the erythrocytes in it to change shape (deform) from natural biconcave form to ellipsoid. A continuous laser beam traverses through the suspension to the receiver panel. The cell suspension causes diffraction of the beam and its pattern changes from circular into ellipsoid, with increasing deformation in erythrocytes. This deformation is expressed by the elongation index (EI). A higher EI indicates better deformability, which is calculated with a computerized ellipse-fitting program (24,25). A lower EI means attenuated deformability.

2.3. Erythrocyte aggregation

Erythrocyte aggregation was determined within 30 min of the withdrawal of blood from the animals, since erythrocyte aggregation deteriorates with time. Aggregation parameters were measured by LORCA at 37 °C. Prior to each measurement, blood samples were oxygenated for 10–15 min to mimic arterial blood composition. Following oxygenation, 1 mL of whole blood was placed into the chamber formed between the glass cylinders. Since whole blood does not allow the laser beam to traverse through the chambers as in erythrocyte deformability, aggregation measurement relies on back-scattered light. With the aggregation of erythrocytes, the intensity of this laser light decreases, and this decrease is recorded as a syllectogram. LORCA software calculates the amplitude (AMP, representing the total extent of aggregation, measured in au unit), aggregation half-time (t½, time that elapses until the peak intensity is reduced by half, reflects the kinetics of aggregation, represented in seconds) and aggregation index, which is an index incorporating both AMP and t½ parameters in its formula (AI, a larger index represents greater and/or quicker aggregation, shown in % as unit) and from this syllectogram (23–25). An increase in AMP and a decrease in t½ increase AI and vice versa.

2.4. Plasma viscosity

The plasma of the withdrawn blood is obtained by centrifugation, and 0.5 mL of plasma was used for measurement. Plasma viscosity (mPa.s) was measured by a cone-plate viscometer (Brookfield LVDV-II + PRO, USA) at 120 rpm (900 s^{-1} shear rate) and 37 °C.

2.5. Statistical analysis

All statistical analyses were performed by using SPSS 12.0. ANOVA and Tukey tests were used to compare the groups. The results were expressed as mean ± SEM (standard error of means). $P < 0.05$ was accepted to be statistically significant.

3. Results

Endotoxin application lowered AMP significantly compared to the control ($P < 0.05$). AMP is an indicator of the magnitude of erythrocyte aggregation and its value was found to be 14.97 ± 0.87 au in the control group, whereas it was 5.14 ± 1.26 au in the Etx group. Bosentan (9.97 ± 2.4 au), Lname (13.08 ± 1.89 au), and Lcan (12.65 ± 2.06 au) groups demonstrated similar results with a slight attenuation from the control. Concomitant applications of endotoxin with these blockers yield intermediate AMP values between lone endotoxin and lone blocker application groups. Here, Etx + Bos yield 7.13 ± 0.64 au, Etx + Lname 8.84 ± 2.49 au, and Etx + Lcan 7.44 ± 1.52 au (Figure 1).

Following endotoxin application, aggregation half time (t½) (inversely related with aggregation speed) was significantly increased in the Etx group (43.28 ± 6.98 s) compared to the control (7.04 ± 0.76 s) ($P < 0.05$). This shows a prolongation of the aggregation time. Blockers show similar results to the control group (Bos: 2.8 ± 0.82 s, Lname: 6.62 ± 1.67 s, Lcan: 8.65 ± 1.46 s). In these blocker groups, Bos had the lowest value. Administration of blockers with endotoxin presents a reversal of the endotoxin's effect. Here, they demonstrate similar results to the control group (Etx + Lname: 9.23 ± 1.58 s, Etx + Lcan: 9.5 ± 1.38 s), except for the Etx + Bos group (23.05 ± 3.97 s), which has double the t½ value of the control group (Figure 2).

AI, which is a combination of AMP and t½, is lowered in the endotoxin group (9.08 ± 2%) compared to the control (37.31 ± 2.02%) ($P < 0.05$). Blocker groups show similar results (Lname: 40.83 ± 4%, Lcan: 33.45 ± 4.27%) as the control, except for the Bos (56.19 ± 5.1%) group, which is higher than other blockers, as well as the control. AI values of concomitant application of endotoxin with blockers show a reversal effect (Etx + Lname: 33.85 ± 4.71%, Etx + Lcan: 31.67 ± 4.13%) to control values. The Etx + Bos group (16.13 ± 3.45%) is an exception that demonstrates a much lower value than those of the concomitant application groups (Figure 3).

Plasma viscosity did not change significantly during endotoxemia. The control group (control: 1.35 ± 0.05 mPa.s) and endotoxin group (1.32 ± 0.05 mPa.s) show similar results. Blocker groups had higher plasma viscosity values without statistical significance (Bos: 1.54 ± 0.14 mPa.s, Lname: 1.43 ± 0.11 mPa.s, Lcan: 1.39 ± 0.09 mPa.s). Concomitant application of endotoxin with blockers gave results similar to the saline and endotoxin groups (Etx + Bos: 1.27 ± 0.04 mPa.s, Etx + Lname: 1.28 ± 0.04 mPa.s, Etx + Lcan: 1.28 ± 0.12 mPa.s, $P > 0.05$) (Figure 4).

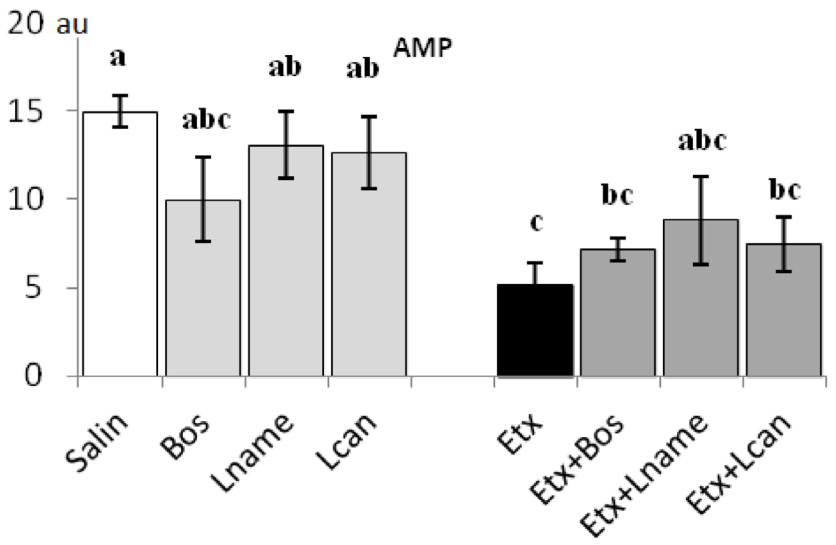

Figure 1. Effect of endotoxin and blockers on erythrocyte aggregation amplitude (AMP). N = 6 for each treatment group. $P < 0.05$ was accepted as statistically significant. Salin = saline, Bos = bosentan, Lcan = L-canavanine, Etx = endotoxin, Etx + Bos = endotoxin & bosentan, Etx + L-NAME = endotoxin & L-NAME, Etx + L-Can = endotoxin & L-canavanine. AMP is presented in au unit. Statistically homogeneous groups are marked as a, b, c.

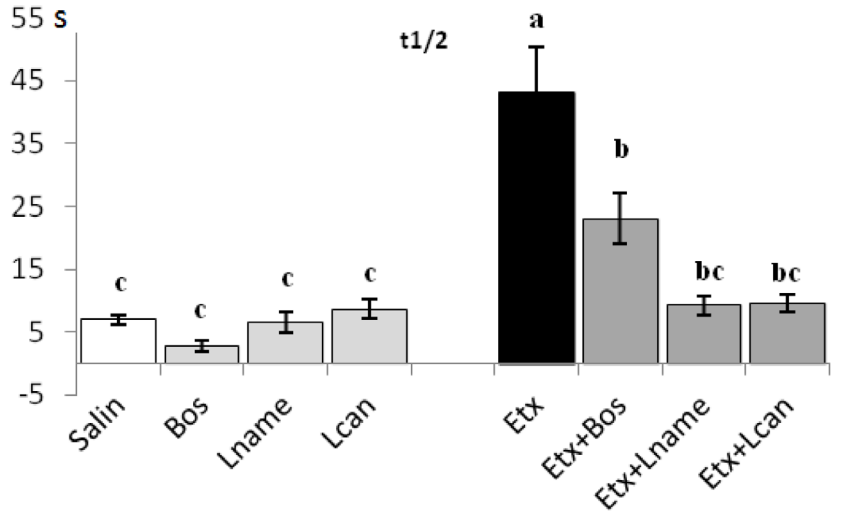

Figure 2. Effect of endotoxin and blockers on erythrocyte aggregation half time (t½). N = 6 for each treatment group. P < 0.05 was accepted as statistically significant. Salin = saline, Bos = bosentan, Lcan = L-canavanine, Etx = endotoxin, Etx + Bos = endotoxin & bosentan, Etx + L-NAME = endotoxin & L-NAME, Etx + L-Can = endotoxin & L-canavanine. t½ is presented in seconds. Statistically homogeneous groups are marked as a, b, c.

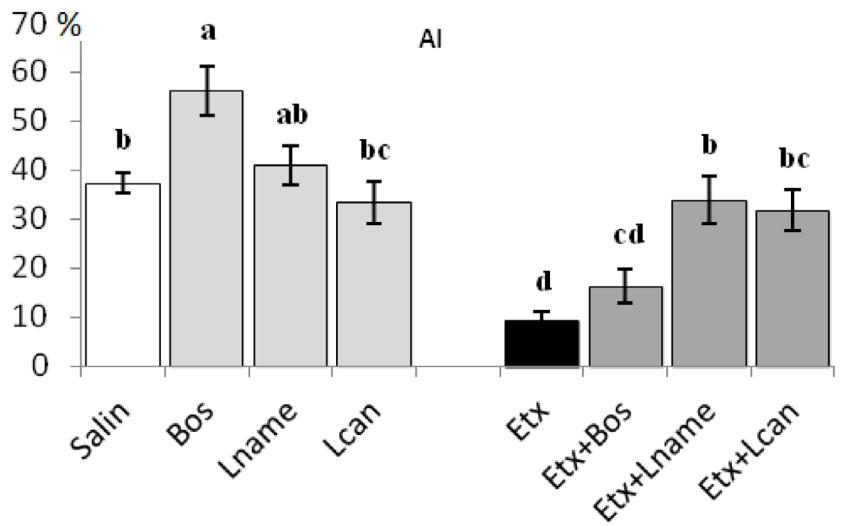

Figure 3. Effect of endotoxin and blockers on erythrocyte aggregation index (AI) in % unit.
N = 6 for each treatment group. P < 0.05 was accepted as statistically significant. Salin = saline, Bos = bosentan, Lcan = L-canavanine, Etx = endotoxin, Etx + Bos = endotoxin & bosentan, Etx + L-NAME = endotoxin & L-NAME, Etx + L-Can = endotoxin & L-canavanine. AI is presented in % unit. Statistically homogeneous groups are marked as a, b, c.

Erythrocyte elongation index, which is a marker of erythrocyte deformability, did not change significantly during endotoxemia (0.584 ± 0.006) compared to the control (0.578 ± 0.007). Erythrocyte elongation was similar in blocker groups, with a slight increase (Bos: 0.592 ± 0.002, Lname: 0.588 ± 0.005, Lcan: 0.594 ± 0.003). Erythrocyte deformability values at different shear rates reveal similar results without statistical significance (Figure 5).

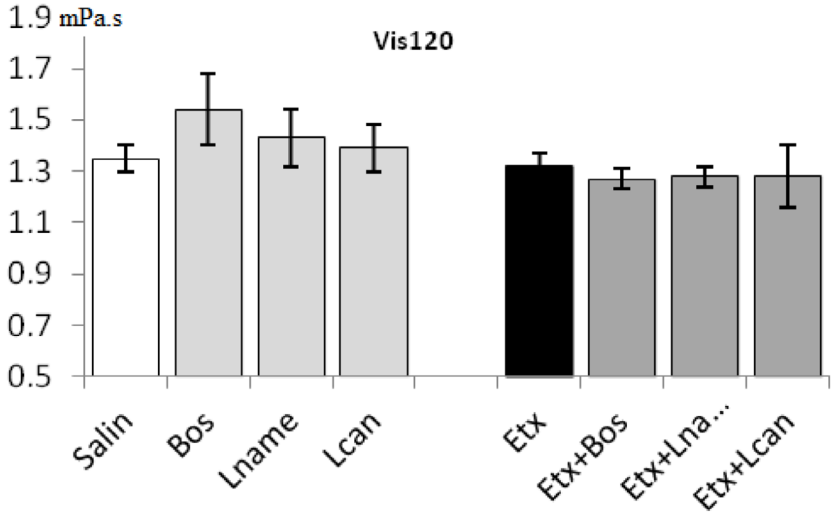

Figure 4. Effect of endotoxin and blockers on plasma viscosity (Vis).
N = 6 for each treatment group. No statistically significant difference was found among groups. Salin = saline, Bos = bosentan, Lcan = L-canavanine, Etx = endotoxin, Etx + Bos = endotoxin & bosentan, Etx + L-NAME = endotoxin & L-NAME, Etx + L-Can = endotoxin & L-canavanine. Vis is presented in mPa.s unit.

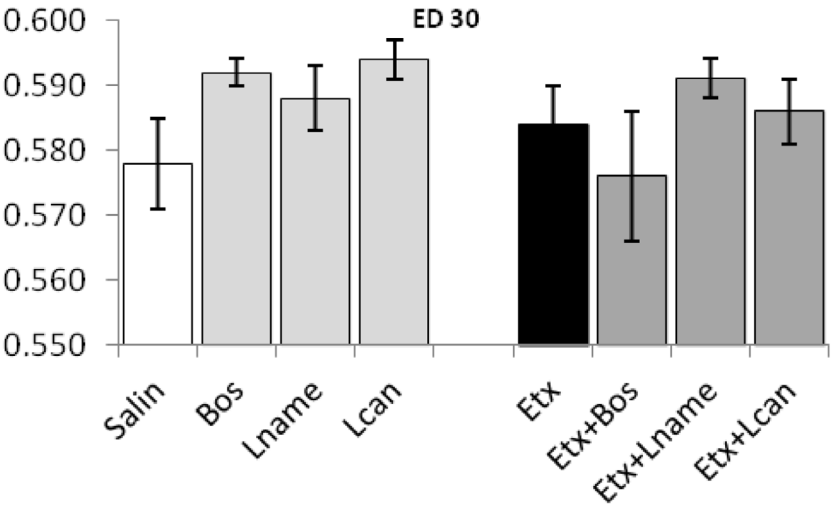

Figure 5. Effect of endotoxin and blockers on erythrocyte deformability.
N = 6 for each treatment group. No statistically significant difference was found among groups. Salin = saline, Bos = bosentan, Lcan = L-canavanine, Etx = endotoxin, Etx + Bos = endotoxin & bosentan, Etx + L-NAME = endotoxin & L-NAME, Etx + L-Can = endotoxin & L-canavanine. ED is presented as erythrocyte deformability index numerically.

4. Discussion

This study was performed to test the hypothesis that blockers of NO synthesis and endothelin receptor may alleviate the effects of sepsis on hemorheological parameters. For this reason, nonselective NO synthesis blocker L-NAME, inducible NO synthesis blocker L-canavanine, and endothelin receptor antagonist bosentan were used.

Endotoxin administration significantly increased aggregation half-time (t½), and lowered erythrocyte aggregation amplitude (AMP) and aggregation index (AI) compared to the control group, indicating a slower and weaker aggregation pattern. Plasma viscosity and erythrocyte deformability were not changed significantly during endotoxemia.

L-NAME and L-canavanine alone had no significant effect on hemorheological parameters. However, when applied during sepsis, they both prevented the changes and kept AI and t½ values at normal levels, keeping AMP half way between normal and sepsis levels. Therefore, it is possible to say that the application of the blockers of NO synthesis acts as an augmenting factor for AMP, AI, and t½ values for the etx-administered animals.

Bosentan administration during sepsis could cause only a partial recovery in aggregation parameters. When considered together, blockers of NO synthesis show a common trend for recovery against the alteration of endotoxin for all the erythrocyte aggregation parameters.

There are limited previous reports on the effects of sepsis on erythrocyte aggregation parameters. Our finding that AMP and AI fall, whereas t½ rises, contradicts these reports. Sordia et al. (4) found a marked increase in erythrocyte aggregability in septic shock in rats. They explained this increase with enhanced infiltration of globulins from the interstitial environment into the bloodstream because of increased permeability of the capillary wall. Thus, the accumulation of high molecular weight, fibrinogen, and globulins in microvessels was characterized by increased erythrocyte aggregability (4). Similarly, Baskurt et al. (3) reported higher erythrocyte aggregation in septic rats, and Reggiori et al. (2) reported higher AI and shorter t½ in septic patients.

The reason behind the dissimilarity between the results obtained in our study and those of previous studies may lie in the blood sampling time. In Sordia et al. (4), blood was withdrawn when the blood pressure fell to 60 mmHg; in Baskurt et al. (3), 18 h after operation (cecal ligation and puncture); in Reggiori et al. (2), within 24 h of admission to the intensive care unit. This period is within the progress of sepsis, when the animal enters the hypodynamic phase of sepsis. In contrast, our blood sampling was performed at the fourth hour, which is within the hyperdynamic phase. In addition, their LPS dose (10 mg/kg) was higher than our application (4 mg/kg). Moreover, difference in the techniques applied (i.e. filterability versus LORCA) may yield such dissimilar results. LORCA is an ectacytometric method that uses a laser beam passing through an erythrocyte suspension rotating dynamically in a glass chamber (24), whereas filterability methods rely on measurement of filtration fraction or filtration time of erythrocytes from porous media (19). Therefore, filterability results may be hampered by interactions of white blood cells and thrombocytes in the filtration media under different conditions. Consequently, it is not convenient to compare the result of those two tests mutually.

Erythrocyte deformability did not change significantly in this study. Reduction of deformability was observed in other studies focusing on sepsis and hemorheology (4), (9). In Sordia et al. (4), erythrocyte deformability was reduced, and they correlated this reduction observed with filterability to the presence of oxygen radicals, change in ionic compositions and toxins, and production of inflammatory mediators. However, in both studies, LPS dosage was higher (10 mg/kg) and was observed at the progressed stages of septic shock, as with erythrocyte aggregation. Thus, the dissimilar results to our study can be explained by this condition.

Production and control of NO is vital for maintaining cardiovascular homeostasis, which is also valid for the proper functioning of the erythrocytes. Excessive production of NO is unfavorable for red blood cells because of the resultant oxidative stress (26). However, there are contradictory results in the studies considering NO donors and NOS inhibitors in their functioning on erythrocyte deformability (15,27,28). In addition, NO donors and its inhibitors exhibit different results on deformability when applied in vivo or ex vivo (29).

Most of the findings in the literature on sepsis, NO, and hemorheology focus on erythrocyte deformability. Our findings present another aspect of this issue, erythrocyte aggregation. Nonselective NO synthase inhibition by L-NAME and inducible NO synthase inhibition by L-canavanine alleviated the effects of endotoxin on erythrocyte aggregation without altering the values in the control animals. However, endothelin receptor antagonist bosentan did not perform such a restoration. This finding suggests that the restoration effects of these blockers occur via their modulation of NO synthesis, rather than through the endothelin pathway.

Since no statistically significant results were observed in plasma viscosity, it can be suggested that plasma chemistry in septic state or its treatment with certain selected blockers did not interfere with plasma proteins to any detectable limit.

An increase in blood viscosity due to deterioration in any of the parameters that contribute to it, such as erythrocyte aggregation, hinders blood flow. On the other hand, a certain level of blood viscosity is thought to be essential for normal circulation, since there are accumulating data on the correlation between moderate increase in blood viscosity and cardiovascular benefits (30). It should be clarified whether a decrease in erythrocyte aggregation in septic shock is a deleterious outcome or not. Furthermore, it should be assessed in further studies with a larger sample size whether its reversal by NO synthase blockers is beneficial or potentially harmful.

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