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ORIGINAL ARTICLE

Ibuprofen reduces plasma nitrite/nitrate levels in a rabbit model of endotoxin-induced shock

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Abstract

OBJECTIVES: The purpose of this study was to investigate the effects of ibuprofen on plasma nitrite/nitrate levels, as indirect indicators of nitric oxide, in correlation with blood pressure in a rabbit model of endotoxin-induced shock.
METHODS: A total of 28 rabbits were randomly divided into four groups. Control group received physiological saline, while endotoxin (ETX, E. Coli, 055:B5, 2 mg/kg, i.v.) was administered to the rabbits in the other groups: group II receiving only ETX, in addition to ETX group III received ibuprofen (30 mg/kg) 30 minutes after ETX administration, whilst the group IV received ibuprofen (30 mg/kg) 30 minutes before ETX. Arterial blood pressure and plasma levels of nitrite/nitrate were determined immediately before (time 0) and 30, 60, 90, 120, 180, 240, and 300 minutes after ETX administration.
RESULTS: ETX administered groups had significantly higher plasma levels of nitrite/nitrate than the control group, at all consecutive measurements except at time 0. Treatment with ibuprofen, either before or after ETX, partly restored the elevated levels of nitrite/nitrate. ETX also caused a significant decrease in blood pressure which was prevented in ibuprofen treated groups.
CONCLUSION: Results from this study indicate that administration of ibuprofen prevents sudden reductions in blood pressure by inhibiting excessive production of nitric oxide in rabbit model of endotoxin-induced shock and this may be of importance for providing crucial time for therapeutic intervention and survival in septic shock.

Abbreviations:

BAL	bronco-alveolar lavage
COX	cyclooxygenase
EDTA	Ethylenediaminetetraacetic acid
eNOS	endothelial NOS
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
LPS	lipopolysaccharide
MAP	mean arterial pressure
NADH	Nicotinamide adenine dinucleotide
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal NOS
iNOS	inducible NOS

Introduction

Endotoxic shock occurs following infection by gram-negative bacteria and it is a serious abnormal condition characterized by hypotension, poor tissue perfusion and multi-organ dysfunction. Despite medical advances including modern antibiotic therapy and optimum intensive care management, gram-negative septic shock continues to be a major cause of morbidity and mortality [13, 17]. Recent findings have revealed that endogenous mediators that result in an inappropriate inflammatory response in the host has more prominent role(s) than traditionally believed harmful influence of an invading microorganism in the pathophysiological process of septic shock [14, 18]. Sepsis due to bacterial infections and experimentally induced endotoxin shock initiate immune responses by activation of intracellular signal transduction cascades resulting in production and release of numerous biochemical mediators including nitric oxide (NO), cytokines, prostaglandins, leukotrienes, toxic eicosanoids, platelet activating factor, and toxic oxygen radicals [6, 14, 18]. It is well established by many preclinical and clinical studies that NO is the predominant mediator of sepsis and septic shock [27, 29]

NO, a pervasive gas, is a unique intra– and extracellular messenger involved in regulation of a wide variety of important biological functions including in the cardiovascular, the central and peripheral nervous system and the immune system [5, 26]. The potent vasodilator NO is synthesized from L–arginine by three distinct isoenzymes [5, 22] termed nitric oxide synthase (NOS): constitutive neuronal NOS (nNOS) and constitutive endothelial NOS (eNOS), and inducible NOS (iNOS). The eNOS mediated NO production play a role in the modulation of basal vascular tone and it displays an antithrombotic effect by inhibiting platelet aggregation and leukocyte adhesion to the endothelial surface. iNOS is stimulated by bacterial lipopolysaccharide (LPS) and causes excess production of NO. This outcome along with several cytokines may mediate cardiovascular dysfunction observed in endotoxic shock [4, 7, 10, 23, 27]. In a later stage of sepsis, inflammatory mediators induce a calcium-independent isoform of NO synthase (iNOS) that has been closely associated with the hypotension and the catecholamine hyporesponsiveness observed in experimental septic shock [10].

In early stages of sepsis, NO regulates vascular tone. In a later stage of sepsis NO synthase is closely associated with the hypotension and these excessive productions of NO leads to vasodilatation, vascular collapse, reduced oxygenation of tissues, and the multiple organ failure. The hypothesis linking NO and the pathogenesis of septic shock, and fact that NO overproduction in sepsis can last for several days makes NO–targeted therapeutic attempts as promising strategy for the therapy of septic shock [8, 27].

Ibuprofen is one of the most commonly used cyclooxygenase (COX) inhibitors and it may be useful for controlling the NO pathway in endotoxic shock [27]. COX inhibition has been shown *in vitro* to down regulate iNOS expression and NO production. Ibuprofen was reported to fail to increase blood pressure in septic patients [4] or in volunteers challenged with endotoxin [28]. These divergent results suggest two possibilities; either ibuprofen does not decrease NO production *in vivo*, or it decreases NO production but other vasoregulatory pathways compensate for this loss and blood pressure remain unaffected [27]. So, it is still difficult to incorporate all data on NO production and overall beneficial effects of NO synthase inhibitors into the field of septic shock.

The purpose of this study was to investigate the effects of the ibuprofen, nonsteroidal anti-inflammatory drug acts by inhibiting isoforms of cyclo-oxygenase 1 and 2, on plasma nitrite/nitrate levels in a rabbit model of endotoxin-induced shock.

Materials and Methods

The protocol of this study was approved by the Local Ethics Committee.

A total of 28 New Zealand rabbits (weighing 3–4 kg) were used in this study. They were anesthetized by intramuscular injection of ketamine hydrochloride (35 mg/kg; Ketalar, 50 mg/ml, Eczacibasi, Istanbul, Turkey) and xylasine hydrochloride (5 mg/kg; Rompun ampul, 23.32 mg/ml, Bayer, Istanbul, Turkey). Anesthesia was maintained by additional doses of ketamine and xylasine. 24G canules were inserted into the femoral artery and vein and monitored by Siemens SC 6000 monitor (Danvers, MA, USA).

The rabbits were randomly divided into four groups of seven animals. Control group received only physiological saline. Endotoxin (*Escherichia coli* O55: B5, Sigma Chemical Company, USA, 2 mg/kg, i.v.) was administered to the animals in the other three groups, in sterile physiological saline (0.5 ml/kg) and time of administration was registered as minute 0. Rabbits in the group II received only ETX, while rabbits in groups III and IV received ibuprofen (30 mg/kg/5 ml) in addition to the ETX, 30 minutes after (group III) and before ETX (group IV). Sodium salt of ibuprofen (Sigma) was dissolved in sterile saline and injected in groups III and IV by slow infusion. Arterial blood pressure and plasma levels of nitrite/nitrate were determined immediately before (time 0) and 30, 60, 90, 120, 180, 240, and 300 minutes after ETX administration.

In all groups, 1 ml blood samples (to obtain plasma nitrite/nitrate levels) were taken simultaneously at 0, 30, 60, 90, 120, 180, 240 and 300th minutes and replacement was done with 1 ml isotonic saline by intravenous infusion for every 1 ml blood sample taken.

Blood samples were collected in EDTA containing tubes for nitrite/nitrate determination. Tubes were centrifuged immediately at 1000 g (10 min) to remove the plasma. Plasma samples were placed in 0.5–1 ml portions into Eppendorf tubes and kept at –80 ^{o}C until analyzed.

Plasma nitrite/nitrate levels were measured using the method described by Grisham et al [11]. Briefly, plasma samples were thawed on ice and a 100 µl of plasma sample was added to 50 µl (0.5 M) HEPES, 10 µl (5 mM) NADH and 316 µl distilled water. Then into the reaction, mixture was pipetted 15 µl (10 U/ml) nitrate reductase and 60 minutes incubation at 37 ^{o}C followed. After that 10 µl (750 U/ml) LDH, 50 µl (10 mM) pyruvic acid, and 1000 µl Greiss reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) were added. After 10 minutes incubation at room temperature, the absorbance was read at 543 nm against the blank. The results were expressed as µmol/l. The intra-assay coefficient of variation was 5.2% and interassay coefficient of variation was 10.2%.

All data are presented as mean ± SEM. Parameters were compared over the study period using analysis of variance for repeated measurements. Where the F values were found to be significant, the data were compared with Tukey's B test. Comparisons between the four groups at the same time point were analyzed by analysis of variance followed by a Tukey's B post hoc test when appropriate. $p < 0.05$ was considered statistically significant.

Results

There were no significant differences between the groups with respect to the mean arterial pressure (MAP) at baseline. ETX injection caused a progressive decrease, evident at respective measurement times, in MAP in all groups (*Table 1*). At 30th minute, MAP values in group II and group III was significantly lower than control group and from their respective initial MAP values at time 0 (*Table 1*, $p < 0.05$).

There was a significant recovery in group III MAP values when measured at 60th minute, which remained restored until 120th minute, but a significant fall was progressed afterwards. There also was a parallel recovery in MAP values of group IV, but it was only modest compared to the group III (*Table 1, Fig. 1*).

Plasma Nitrite/Nitrate Levels

There was no statistically significant difference between the groups' plasma levels of nitrite/nitrate at min 0th. The levels of plasma nitrite/nitrate levels of the control group remained relatively stable throughout the study period.

ETX administration caused significant increases in plasma nitrite/nitrate levels in groups II, III and IV, which were injected with ETX. As shown in *Table 2*, the nitrite/nitrate levels were significantly higher in only ETX administered group. The increase in plasma levels of nitrite/nitrate was significant at 30th minutes in all these groups; and it progressively increased in only ETX injected group while ibuprofen was partly able to prevent the increase, being more effective in group III (*Table 2, Fig 2*).

In group III, plasma nitrite/nitrate levels showed increase at min 30th, and these higher levels remained statistically significant when compared with the group I values until 90th min. After this time, the levels in group III did not show statistically difference than levels of group I until end of the study period. Values and progressive changes of plasma nitrate/nitrite levels are presented in *Table 2*.

Discussion

In this study, we determined the levels of nitrite/nitrate as the final metabolites of NO during septic shock in a rabbit model, and also examined the vasorelaxant effect of NO by measuring respective blood pressure values.

Table 1: Mean arterial pressure (mmHg) values in each group after intravenous administration of bacterial endotoxin

Study Period (Minutes)	GROUP I	GROUP II	GROUP III	GROUP IV
0	69.3 ± 4.8	70.1 ± 3.1	66.4 ± 3.1	73.3 ± 3.5
30	66.9 ± 4.5	29.0 ± 2.6	40.1 ± 6.9	62.4 ± 5.0
60	65.8 ± 4.0	32.9 ± 1.1	59.7 ± 7.1	45.9 ± 16.8
90	71.9 ± 6.0	31.3 ± 5.2	63.8 ± 6.5	41.8 ± 4.0
120	75.9 ± 6.0	36.5 ± 7.2	59.8 ± 3.5	44.3 ± 4.1
180	79.9 ± 4.7	28.9 ± 9.9	49.2 ± 5.4	32.5 ± 2.8
240	73.2 ± 4.9	29.2 ± 14.4	46.3 ± 3.7	28.5 ± 1.4
300	74.7 ± 4.9	11.0	39.7 ± 5.4	16.9 ± 1.5

Table 2: Plasma nitrite/nitrate (μmol/l) levels in each group after intravenous administration of bacterial endotoxin.

Study Period (Minutes)	GROUP I	GROUP II	GROUP III	GROUP IV
0	75.2 ± 2.4	84.2 ± 11.1	83.0 ± 11.0	87.4 ± 7.7
30	74.0 ± 1.7	186.0 ± 4.8	112.8 ± 5.7	139.0 ± 6.8
60	75.4 ± 5.7	185.2 ± 9.9	112.2 ± 8.7	161.0 ± 3.9
90	91.4 ± 4.9	180.6 ± 16.6	121.8 ± 12.1	181.0 ± 13.8
120	86.8 ± 7.0	189.4 ± 13.5	111.8 ± 14.3	135.0 ± 10.8
180	94.4 ± 5.9	203.0 ± 5.7	109.4 ± 5.9	133.6 ± 12.6
240	107.8 ± 3.6	206.0 ± 5.8	105.2 ± 0.6	110.4 ± 5.3
300	73.8 ± 2.3	215.6 ± 4.8	107.0 ± 1.6	98.8 ± 6.7

Nitrite/nitrate levels were significantly higher and mean blood pressure values were significantly lower in all ETX–administered groups, and treatment with ibuprofen, either before or after ETX, restored the elevated nitrite/nitrate levels and this effect was paralleled by reversal of hypotension.

It is well known that excessive amounts of inflammatory cytokines are produced during septic shock and these in turn induce overproduction of NO [7, 16, 29]. NO is a labile radical gas with extremely short half-life and this poses a considerable obstacle for its analytical assessment. However, there is good evidence that determination of its final metabolites, namely, nitrite/nitrate reliable provides information on NO production [15] and this approach has proven valid in evaluation of the amount of NO production in ETX administered rats [25].

Peripheral vascular changes and cardiac dysfunction are among clinical findings of septic shock [2, 6, 19]. Increased production of NO by iNOS has been held responsible for the cardiovascular derangements during sepsis [12]. In patients with serious sepsis, NO both acts together with the other COX metabolites in preventing the hyperactivity of endogen vasoconstrictor agents and helps to increase reproduction of these COX metabolites [20].

In an endotoxin shock dog model, immediate after application of endotoxin, a sudden fall in mean arterial pressure, sustaining more than 2 hours has been demonstrated [1, 17]. When ibuprofen was applied at 120th minute, arterial pressure and ventricular function were reported to be corrected without changes in venous compliance [1]. In their experimental canine septic shock model Coran et al. [9] reported that mean arterial pressure was significantly higher in animals that received ibuprofen before the shock than in those of the control group. They indicated that mean arterial pressure continued to be around 90% of the initial arterial pressure level. They also found that, in animals receiving ibuprofen after the endotoxin; arterial pressure fell about 50% initially, then increased gradually and continued to be around 70% of initial values.

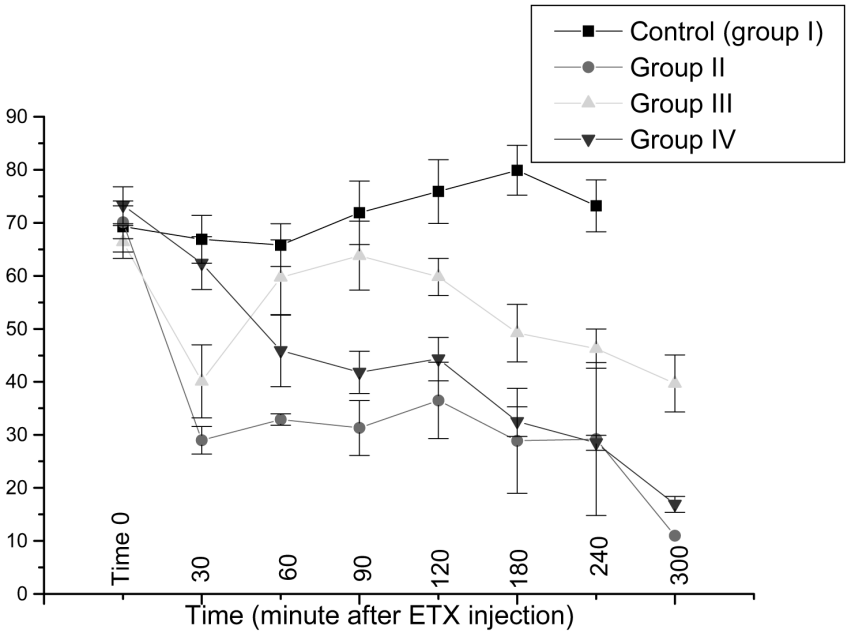

Figure 1: Time dependent changes in mean arterial pressure (mmHg) values of rabbits in each experimental group after administration of endotoxin (ETX: 2 mg/kg, i.v.)

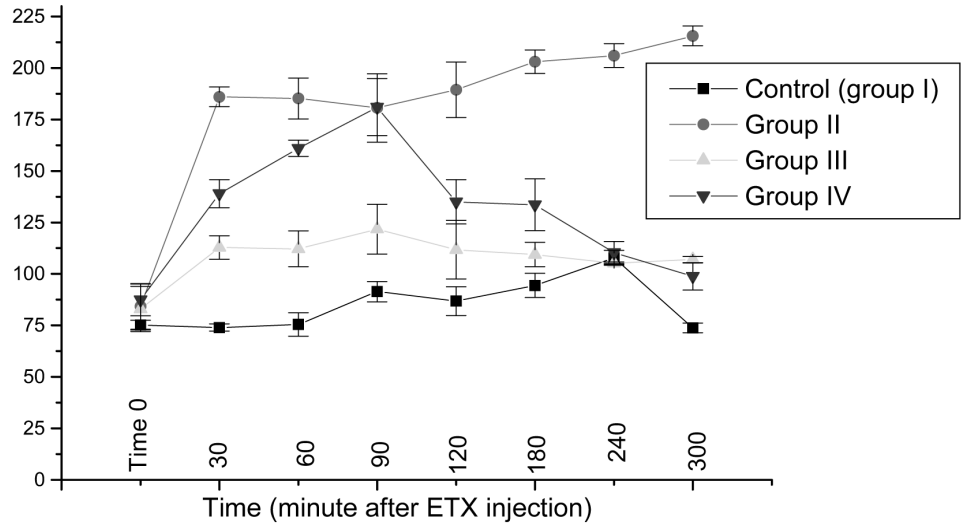

Figure 2: Time dependent changes in plasma nitrite/nitrate (μmol/l) levels of rabbits in each experimental group after administration of endotoxin (ETX: 2 mg/kg, i.v.).

In this study, plasma nitrite/nitrate levels of the control group slightly increased starting from the first minutes but did not reach to statistically significant level throughout the study period. It was thought that this increase might be due to the invasive practice. In group III, nitrite/nitrate levels showed increase at 60th minutes, but after this point, it was close to the group I values. The levels of nitrite/nitrate in group III were lower than in group IV until 180th min ($p < 0.05$). However, in group IV, the levels were remained higher until min 240th compared to the respective values of group I. Therefore, ibuprofen administration after exposure to endotoxin worked better than administration beforehand. Thus, there was evident reduction in mean arterial pressures of groups that received endotoxin and administration of ibuprofen partly reversed the ETX–induced hypotension. However, this ibuprofen induced restoration of blood pressure was for only limited time period, from 30th minute to 120th minutes, and this was followed by a rapid fall in blood pressure, and sudden death. Interestingly, hypotension restoring effect of ibuprofen was more prominent when it was given 30 minutes after ETX challenge than its administration 30 minutes before ETX. We have no explanations to this, and its effect on blood pressure values were accompanied by parallel changes in nitrite/nitrate levels. It may be due to the clearance of early administered ibuprofen and providing lower plasma levels than its later application.

Our findings that ibuprofen restored the ETX–induced hypotension and maintained blood pressure for a limited duration are in agreement with the findings of previous studies indicating use of a selective inhibitor of inducible NO synthase restores mean arterial pressure, and offers a therapeutic approach to managing hypotension in endotoxic shock [24, 30]. A study conducted *in vitro* on the human umbilical vein epithelial cells revealed that ibuprofen inhibits cNOS but activates iNOS [2]. However, another study performed *in vitro* on rat macrophages showed that just like ibuprofen, NSAIDs inhibit the iNOS gene expression [3]. In another *in vitro* study; when brain glial cells are incubated with LPS, IFN–gamma and ibuprofen, it was observed that both iNOS activity and the cyclooxygenase enzymes (COX–1 and COX–2) are not directly catalyzed by ibuprofen. However, *in vivo*, it may reduce the effect of both enzymes [21]. A research conducted on rats; ibuprofen was administered 1 hour before endotoxin and TxB_2 in plasma and NO in plasma and bronco-alveolar lavage (BAL) were investigated. The NO levels in the plasma were similar in both groups, the local NO levels in BAL gradually decreased in the group taking only endotoxin, but no decrease was observed in the group taking ibuprofen [12].

In conclusion, although there were no direct data showing that ibuprofen affected the nitrite/nitrate generation, results from this rabbit model of endotoxin-induced shock model revealed that the non-selective COX inhibitor ibuprofen prevents sudden reductions in blood pressure by inhibiting excessive production of nitric oxide and able to restore endotoxin-induced hypotension for about an hour and this may be of importance for providing critical time for therapeutic intervention and survival in septic shock.

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