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The Effects of Inducible Nitric Oxide Synthase Blockers on Survival and Organ Injury in a Murine Caecal Ligation and Puncture Model of Septic Shock

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ABSTRACT

Introduction: Septic shock is a serious circulatory disorder with an unacceptable mortality. The detrimental effects of endotoxin such as hypotension and unresponsiveness to both vasodilators and vasoconstrictors were explained by the production of nitric oxide (NO) in copious amounts. Thus it appears feasible to inhibit excessive NO production by using inducible nitric oxide synthase (iNOS) inhibitors in the treatment of septic shock. Therefore, we assessed our hypothesis in a caecal ligation and puncture (CLP) mice model of septic shock in which the overall survival was monitored.

Methods: Male Swiss albino mice (27-41 g, n=15 per group) were randomly allocated into five groups. The first group consisted of sham-operated animals treated with solvent (saline) while the second group (control) underwent CLP procedure. All animals in the third, fourth and fifth groups underwent CLP, but were also given one of the following iNOS inhibitors: AET (S-(2-aminoethyl)-isothiourea bromide hydrobromide; 240 mg/kg/day), SMT (S-methylisothiourea hemisulfate, 10 mg/kg/day) or aminoguanidine (15 mg/kg/day) twice daily for the following seven days commencing just after the surgery. The survival rates were then recorded and analyzed accordingly by using the log-rank test. Histopathological examinations of liver and spleen were also performed at the end of the experimental protocols.

Results: At 168 h (7 days) after the surgery the survival rate was 93.3% in sham-operated animals but decreased to 33.3% ($P<0.01$ vs. sham-operated) in controls which underwent CLP. However, the survival rates were 66.6% for the third and 60.0% for the fourth and the fifth groups (i. e. iNOS inhibitor treated) which were not significantly different than that of controls.

Conclusion: In our study, although an apparent improvement has been observed with new iNOS inhibitor usage, the magnitude of the beneficial effects has failed to reach statistical significance. Although SMT, AET and aminoguanidine had beneficial effects in many studies; we showed that selective iNOS inhibitors have no beneficial effects in a murine CLP model of septic shock.

Key words: septic shock, caecal ligation and puncture, iNOS inhibitors, SMT, AET, aminoguanidine

Introduction

Sepsis is a severe systemic inflammatory response to an infection. The causative agents are microorganisms or their toxins, which spread from a local infection site and enter the blood stream. In majority of the cases, the clinical outcome is worsened by the development of multiple organ dysfunctions that leads to an unrelenting state of acute circulatory failure, namely the septic shock. Despite many attempts to combat its high mortality, the septic shock is still among the major causes of death in intensive care units (1).

The controversial role NO in the pathophysiology of sepsis and septic shock is currently under scrutiny (2). The pro– and antiinflammatory effects of NO, as well as its oxidant and antioxidant properties together with its role as a "vital poison" for the immune and inflammatory network all have been the source of fascination and confusion in sepsis research. Currently it is becoming clearer that NO may act both as friend and foe in septic states (3).

NO, produced at copious amounts by iNOS (4) contributes significantly to the deleterious effects of endotoxin such as hypotension, vascular hyporesponsiveness to vasoconstrictors) and vasodilators during sepsis (5). Non-selective nitric oxide synthase inhibition by using NG-substituted L-arginine analogues (e.g. NG-nitro-L-arginine methyl ester: L-NAME) is shown to exacerbate endotoxin-induced organ ischaemia and mortality due to concomitant inhibition

of the constitutive nitric oxide synthase (6). Furthermore, a large randomized prospective trial of NG-methyl-L-arginine hydrochloride was discontinued early because of increased mortality in patients receiving the study drug (7). On the other hand, when the inducible enzyme was blocked selectively by using L-canavanine, the mice challenged with a lethal dose of endotoxin were reported to express significant improvements in haemodynamic and metabolic parameters as well as increased survival (8).

Although nitric oxide plays a crucial part in the hypotension and vascular hyporesponsiveness that characterize septic shock, its critical role in the subsequent multiple organ dysfunction is yet unknown. The liver and spleen are essential for managing immune defense during sepsis. Even the prognosis of sepsis is markedly worsened by hepatic dysfunction, which also serves as a reliable independent predictor of mortality in the intensive care unit (9). Inhibiting iNOS is expected to have some possible impact on diseases linked to septic shock because endotoxin is the main inducer of iNOS, which produces large amounts of nitric oxide to cause extensive tissue damage and organ injury.

Isothioureas are relatively new and the potent inhibitors of iNOS with variable isoform selectivity (10). In our quest to better understand the effects of selective iNOS inhibition in experimental septic shock, we attempted to investigate the effects of two different relatively new isothioureas and aminoguanidine on the survival and organ injury in CLP-induced sepsis model in mice. The rational behind the selection of experimental CLP model was to mimic the clinical situation of bowel perforation since CLP is becoming regarded as superior in reflecting various facets of the systemic response to local (11).

Thus, we propose to test the effects of relatively novel, selective iNOS inhibitors in CLP-induced experimental models of septic shock in mice by evaluating the overall survival and histopathological alterations in spleen and liver.

Methods

Animals

Swiss albino mice (25–45 g) were obtained from Laboratory Animals Husbandry Facility of The Department of Pharmacology, Hacettepe University Faculty of Medicine and were housed under environmentally controlled conditions at 21±2°C and 30–70% relative humidity with a 12-h dark/12-h light illumination sequence (the lights were on between 07:00 and 19:00 h) with *ad libitum* access to tap water (drinking bottle) and standard pellet rodent chow (Korkutelim Yem Sanayi, Antalya, Turkey) and used with strict adherence to all of the Guiding Principles in the Care and Use of Animals together with the Recommendations from the Declaration of Helsinki and Tokyo throughout the studies. This project was approved by the Institutional Experimental Animal Care and Use Ethics Committee of Hacettepe University before the commencement of any intervention (Approval Number: 2005/13-9).

Mice Model of Septic Shock Induced by Caecal Ligation and Puncture

Polymicrobial sepsis was induced by adhering to the same protocol of our previous report (12) which was based on the detailed guiding of other publications (11). In brief, mice were fasted overnight, but were allowed *ad libitum* access to drinking water prior to the experiment. The animals were then anaesthetized with chloralhydrate (400 mg/kg, i. p.) and placed on a heat-insulated-cork sheet-covered operating table. The animals were allowed to breathe room air spontaneously. After the obtundation of all responses to painful stimuli (pinching of the plantar skin by a tweezer), a 1-cm midline abdominal incision was made. The caecum was then exposed, ligated by using atraumatic 3-0 silk sutures just distal to the ileocaecal valve to avoid any intestinal obstruction, and punctured twice with a 22-Gauge needle. The punctured caecum was gently squeezed to expel a small amount of fecal material and the abdominal incision was then closed in two layers by using atraumatic 4-0 silk sutures. Sham-operated animals underwent the same surgical procedure except that the caecum was neither ligated nor punctured. Immediately after the surgery all animals have received a subcutaneous injection of 1 ml of sterile saline (NaCl 0.9%, w/v, dissolved in pyrogen-free distilled water).

Experimental Protocols

When the animals have recovered from the surgery, they were randomly allocated into different treatment groups (n=15 per group) to receive twice daily intraperitoneal injections of either (i) aminoguanidine (AG) (15 mg/kg per day), (ii) S-(2-Aminoethyl) isothiourea dihydrobromide (AET) (50 mg/kg per day), or (iii) S-Methylisothiourea hemisulfate (SMT) (10 mg/kg per day) until the tissue harvesting (a maximum of 7 days depending on the survival time of an individual animal). Likewise, the animals in sham and CLP operated control groups (n=14 per group) have received equal volume of sterile saline.

Survival rates were recorded at 12 h intervals for the following 168 hours (7 days) after the surgery and all living animals at the end of our observation period were put down humanely. Since our previous studies have revealed a direct correlation between the enlargement of spleen with the presence and severity of septic challenge, the spleens of all animals were isolated and weighed as an indication of success of the surgical intervention.

At the end of the protocol and in some randomly selected animals' livers were also isolated with spleens and the tissues were fixed in 10% formaldehyde solution immediately after removal and embeded into paraffin blocks. The histopathological sections were then prepared and stained with hemotoxylin-eosin. The slides were examined under conventional light microscopy (Zeiss Axioskop, Germany) by an appropriately blinded expert pathologist.

Drugs

Chloralhydrate, sodium chloride, hematoxylin (Merck, USA), eosin, aminoguanidin, S-(2-Aminoethyl) isothiourea dihydrobromide, S-Methylisothiourea hemisulfate (Sigma, USA), formaldehyde (Carlo Erba, Italy), and paraffin (Shandon, UK) were commercially purchased. All drug solutions were daily prepared, kept in dark containers until the injections in order to

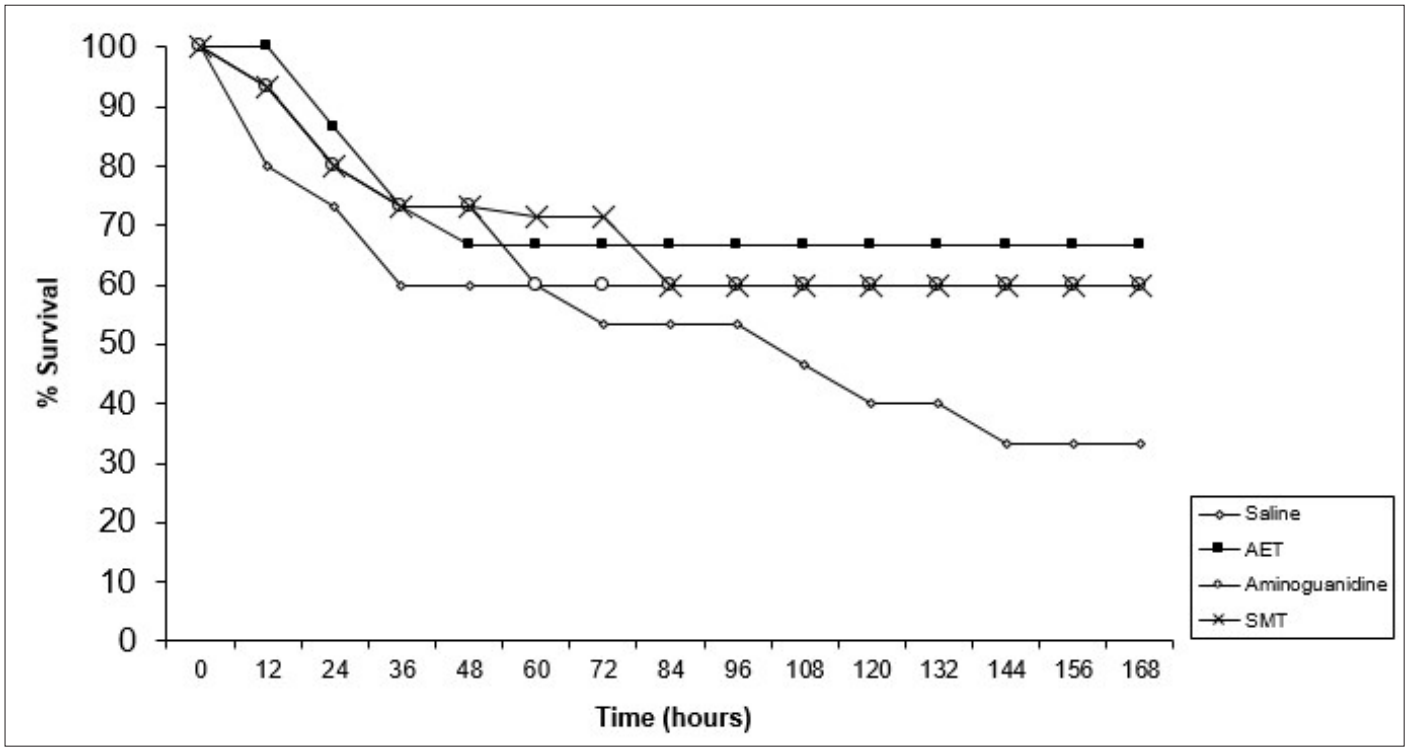

Figure 1. Survival rates obtained from mice that underwent CLP surgery and treated either with saline or NOS inhibitors AET, SMT or aminoguanidine. n=14-15 in all groups.

protect them from light-induced decomposition and warmed to the body temperature (approximately 37°C) before injections.

Statistical Analysis

Fisher's exact test for 2×2 tables with Yates' correction was used to compare the percent of survivals at selected (i. e. 168 h after CLP) time points. Cox regression analysis (SPSS 12.0 for Windows) was also used to compare the survival rates in each group. The data related to the corrected weights of the spleen were analysed by using one-way Analysis of Variance procedure while the rest of pair-wise comparisons were performed by using either the Mann Whitney *U*-test or Student's *t*-test where appropriate, depending on the similarity of the standard deviations of the populations. The differences were considered to be statistically significant when the two-tailed *p* value was less than 0.05.

Results

The effects of iNOS inhibition on survival and spleen weight in CLP Model

The survival at 168 h after the surgical procedure in saline-treated sham-operated animals was 92.9% (13 out of 14) in comparison to 33.3% (5 out of 15) which was significantly ($P<0.05$) different than the survival observed in animals that underwent CLP and treated with saline. As indicated by the survival curves, the deaths have occurred consequently on a linear basis beginning by the 12 h with no apparent aggregation at one time period (Figure 1).

As mentioned earlier, CLP resulted in 33.3% (5 out of 15) survival at 168 h in saline-treated animals. Although treatment with one of the iNOS inhibitors (i.e. AG, AET or SMT) resulted in an apparently better overall survival at 168 h (the overall survivals were 60% for AG, 66.6% for AET and 60% for SMT), the differences between these groups and saline-treated CLP-operated animals have failed to reach a statistical significance. Similarly, Cox regression analysis applied to these survival curves has also indicated no significant difference between them (Figure 1).

Table 1. Spleen weight (g per kg body weight) after CLP for 168 h (7 days) in saline and iNOS inhibitors treated animals. One-way ANOVA applied to all CLP groups indicated the effect of iNOS inhibitors are not significant on spleen weight.

Group	Treatment	Calculated Spleen Weight (g/kg) (Standard Deviation)
CLP	Saline	7.48 (4.48)
	AMG	7.32 (4.45)
	AET	8.92 (5.04)
	SMT	6.82 (4.36)

In saline-treated animals, CLP has significantly increased the weight of spleen (g/kg body weight; sham-operated: 5.5±0.6, n=14 versus CLP: 8.13±0.8, n=14; $P<0.05$) while this increase was not reversed by iNOS inhibition (Table 1).

The Effects of iNOS inhibition on Spleen and Liver Histopathology in CLP

CLP resulted in a marked congestion in the spleen of all randomly selected animals (n=6) accompanied by fibrosis (2 out of 6) and capsulitis (1 out of 6). When the animals were treated with

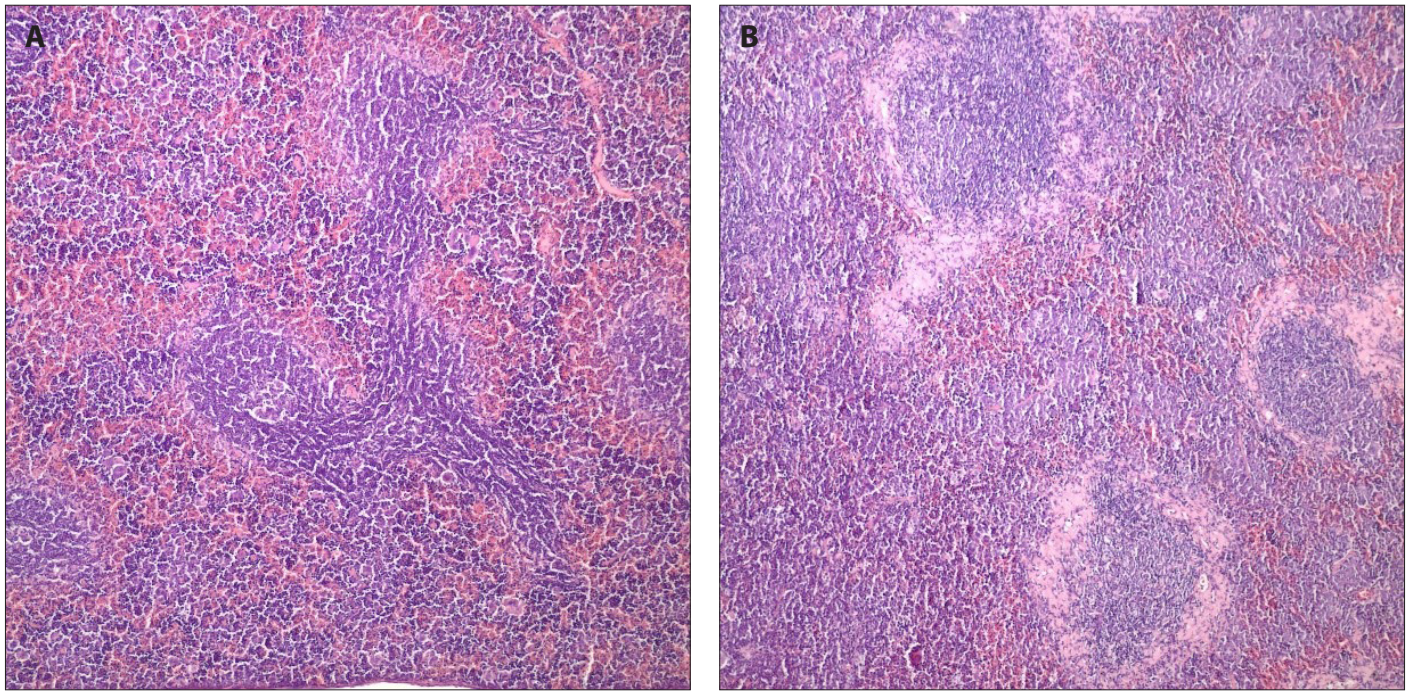

Figure 2. Hematoxylin and eosin staining showing the significant congestion of the red pulp in the spleen of an animal underwent CLP (200x, on the right).

Table 2. Summary of histopathological findings after CLP for 168 h (7 days) in saline and iNOS inhibitors treated animals

		Mice number	Liver							Spleen		
	Treatment		Congestion	Capsulitis	Necrosis	Regeneration	Fibrosis	Degeneration	Other	Fibrosis	Congestion	Capsulitis
CLP	Saline	1	0	0	0	0	0	0	0	0	1	0
		2	1	0	1	0	0	0	0	0	1	0
		3	1	1	1	0	0	0	0	1	1	0
		4	0	0	1	1	0	0	0	0	1	0
		5	0	0	1	1	0	0	0	1	0	0
		6	0	1	1	1	0	0	0	0	1	1
	AMG	1	0	1	2	1	0	1	0	0	1	1
		2	0	0	1	1	0	0	0	0	1	0
		3	0	1	1	1	0	0	0	0	2	1
	AET	1	0	1	2	2	0	1	mitosis	1	1	1
		2	0	1	1	1	0	0	mitosis	1	1	1
		3	0	1	1	1	0	1	oval cells	0	0	1
	SMT	1	0	1	2	1	0	1	0	0	1	1
		2	0	0	1	1	0	0	0	0	1	0
		3	0	1	1	1	0	0	0	0	1	1

one of the iNOS inhibitors, the capsulitis and congestion were moderately enhanced in spleen. However, AG or SMT treatments have prevented fibrosis in spleen while AET did not reverse the fibrosis (n=3) (Table 2).

Similarly, CLP has resulted in mild focal "spotty" necrosis (5 out of 6 randomly selected animals) accompanied by a minimum degree of congestion and capsulitis in liver (2 out of 6 randomly selected animals) with no peripheral fibrosis or any other degeneration. However, AG, AET or SMT consistently resulted in moderate capsulitis and regeneration in the liver accompanied by severe focal necrosis (Table 2).

Histopathological evaluations of spleen and liver did not reveal significant alterations except marked increased mitosis in AET group (Figure 3, Table 2).

Discussion

In this study none of the harmful effects, observed in CLP-induced sepsis model, were not abolished by selective inhibitors of inducible nitric oxide synthase.

NO is believed to play a key role in the pathogenesis of septic shock. In several experimental models, endotoxin has been shown to increase the constitutive release of nitric oxide by the

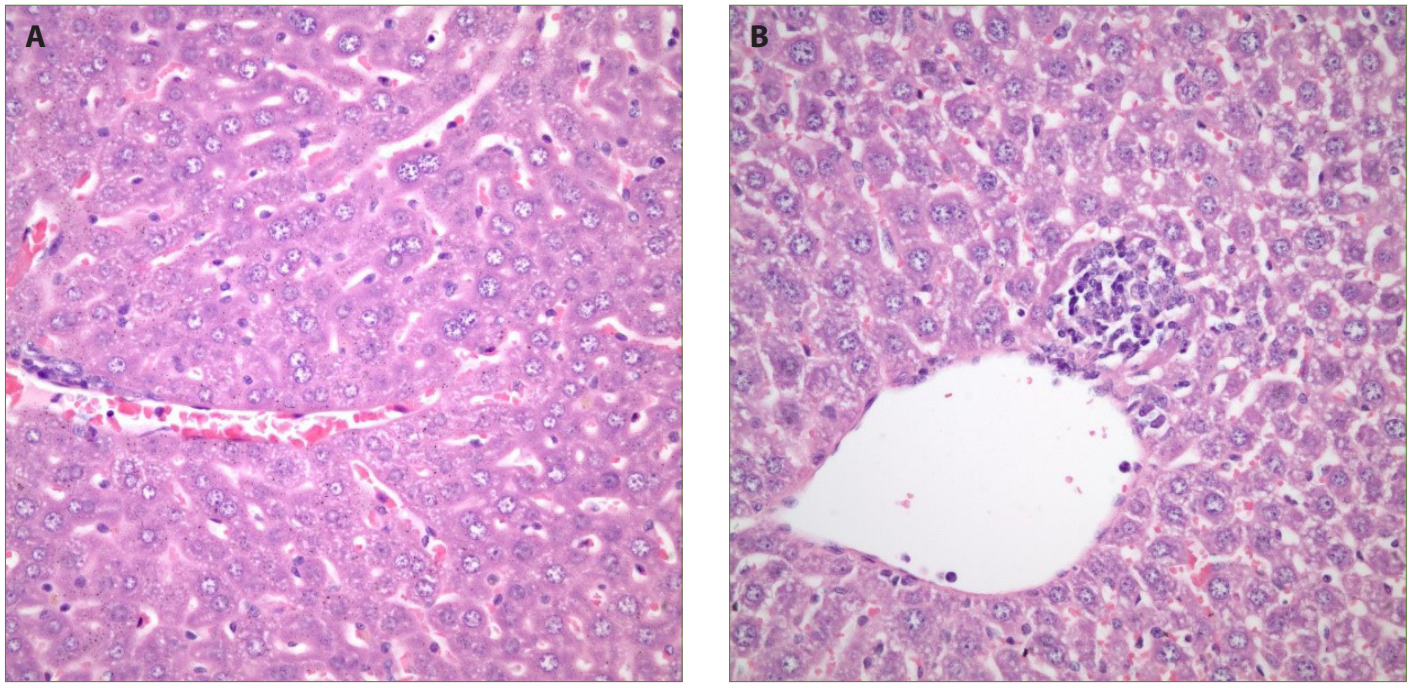

Figure 3. Hematoxylin and eosin staining showing the presence of pericentral fibrosis in the liver of an animal underwent CLP (200x, on the right).

endothelium and the activity of iNOS (4). NO, produced at copious amounts by iNOS may contribute significantly to the deleterious effects of endotoxin such as hypotension, vascular unresponsiveness (vasoplegia), cardiodepression, and organ injury–dysfunction in septic shock (3). The pharmacologic inhibition of NO production has been recently proposed as a potentially interesting adjunct to septic shock therapy. The inhibition of both endothelial and inducible nitric oxide synthases by non-selective inhibitor *L*-NAME (*N*G-substituted *L*-arginine analogue) has been shown to exacerbate endotoxin-induced adverse effects, organ ischemia and accelerate death. It has been proposed that these deleterious effects might reflect the loss of the regulatory functions of constitutive, endothelial nitric oxide synthase (eNOS) on the microcirculation, platelet aggregation and endothelium-leukocytes interactions, thereby favoring tissue hypoperfusion, microthrombi formation and leukocyte infiltration (6). Taken together, basal release of nitric oxide by eNOS has an important role in the regulation of regional blood flow (beneficial effects), while the excessive generation of nitric oxide by inducible nitric oxide synthase inhibited regional blood flow (harmful effects).

More recently, attention has been focused on the inhibition of iNOS as this isoform is selectively increased during sepsis and has a greater significance. When this enzyme was blocked selectively by *L*-canavanine, the mice challenged with a lethal dose of endotoxin were reported to exhibit significant improvements in hemodynamic and metabolic parameters as well as increased survival in low doses (13). However, the high dose of L-canavanine appeared somewhat less protective which indicate possible nonspecific toxicity of L-canavanine at high doses. In contrast to this beneficial effect in low doses of the iNOS inhibitor, MacMicking et al. reported that although the hypotension and early mortality were reduced, there was no significant difference between the knockout mice deficient from inducible nitric oxide synthase and normal wild type with regard to lipopolysaccharide/*Clostridium parvum*-induced hepatic injury and they concluded that nitric oxide was not the key mediator in septic shock (14). In our study we investigated the effects of relatively novel selective iNOS inhibitors, chemically unrelated to L-canavanine, on survival and organ injury in a murine caecal ligation and puncture model of septic shock in order to solve this paradoxical situation.

It is widely accepted that endotoxin produces multiple organ failure which is a frequent cause of death among patients who succumb to endotoxic shock (5,15). This study demonstrated that CLP-induced sepsis experimental model in mice produced significant blood congestion in liver and spleen which was reflected as weight increase in these organs and inflicted some degree of inflammatory injury. Based on our own data in this manuscript as well as results reported in the literature, CLP produces severe hemocongestion in the liver parenchyma, profound hydropic degeneration, inflammatory lymphocytic infiltration around the bile canaliculi together with the formation of minimal parenchymas injury in the form of spotty necrosis in liver (12). CLP also resulted in severe congestion, minimal fibrosis, and capsulitis in the spleens of animal. Although aminoguanidine and one analogue, l-amino-2-hydroxy-guanidine, reduced organ injury and metabolic acidosis in endotoxemic rats (16), SMT, AET and aminoguanidine did not reverse these histopathologic effects and organ injury in our experimental CLP model.

Thiourea derivatives, such as SMT and AMT, also produced some beneficial hemodynamic and metabolic effects in endotoxic shock rats and aminoguanidine was also shown to improve survival of endotoxemic mice (17). In our study, although an apparent improvement has been observed with new iNOS inhibitor usage, the magnitude of the beneficial effects on survival have failed to reach statistical significance. None of the harmful effects observed in CLP-induced experimental sepsis model were not abolished by selective inhibitors of inducible nitric oxide synthase inhibitors.

In accordance with the current knowledge about NO, however our previous reports indicate a potential beneficial effect of NO induced local vasodilation in the mesenteric circulation (3) which may decrease bacterial translocation (18) and improve the overall survival (19) since splanchnic ischaemia increases mortality in septic shock (20), many deleterious effects of endotoxin such as hypotension, vascular hyporesponsiveness to vasoconstrictors and vasodilators are explained by the excess production of nitric oxide in endotoxin-induced experimental septic shock models (4,5).

We believe that future studies should be designed to assess the effects of selective iNOS inhibition in experimental models of septic (like as CLP) rather than endotoxic shock. In conclusion, the data presented herein confirm that selective iNOS inhibition does not give any survival advantage or organ protection in this CLP-induced septic shock model.

AUTHOR CONTRIBUTIONS:

Concept: NU, ABI; **Design:** NU, ABI; **Supervision:** ABI; **Fundings:** ABI; **Materials:** NU, CS, ABI; **Data Collection and/or Processing:** NU, CS; **Analysis and/or Interpretation**: NU, CS; **Literature Search:** NU; **Writing Manuscript**: NU, CS, ABI; **Critical Review:** ABI.

Ethics Committee Approval: This project was approved by the Institutional Experimental Animal Care and Use Ethics Committee of Hacettepe University before the commencement of any intervention (Approval Number: 2005/13-9).

Informed Consent: Basic animal experimental research

Peer-review: Externally peer-reviewed.

Conflict of Interest: Authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

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