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The nitric oxide pathway antagonists in septic shock: Meta-analysis of controlled clinical trials

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1. Introduction

Nitric oxide [1] is the gaseous agent previously denominated "endothelial-derived relaxing factor" noted to be responsible for the vasodilation and hypotension in distributive shock and, particularly, in septic shock. Nitric oxide (NO) is a free radical (NO^-) synthesized by a family of three isoforms of the nitric oxide synthase (NOS). The isoform located in the leukocytes (iNOS or NOS-2) is inducible by interferon-gamma and tumor necrosis factor-alfa, and is calcium-independent. The other two are the constitutively calcium-dependent neuronal (nNOS or NOS-1) and endothelial (eNOS or NOS-3) enzymes. Activated macrophages during sepsis will convert L-arginine to L-citrulline resulting in production of the NO radical, detectable in plasma as nitrates (NO_3^-), nitrites (NO_2^-) and nitrotyrosine [2]. NO can buffer reactive oxygen species (ROS) and also react with superoxide to create highly toxic peroxynitrite. NO freely permeates cell membranes including vascular smooth muscle, where activates the guanylate cyclase (sGC), generating the second messenger cGMP which will modulate calcium channels, protein kinases and phosphodiesterases [3]. In this way, increased intracellular levels of cGMP will cause relaxation of vascular smooth muscle and will lower blood pressure.

Unwanted hemodynamic NO effects in septic shock as hypotension can be opposed in more than one way. Non-selective NOS inhibitors (NOSi) as N^G-monomethyl-L-arginine (L-NMMA) will block NO production. Guanylate cyclase inhibitors as methylthioninium chloride (methylene blue, MB) will interfere NO action. A limited number of small pilot clinical trials were used to restore blood pressure during septic shock. Confirmation trials were subsequently developed and shown to be effective in restoring hemodynamics, but such effect did not improve the overall outcome both with L-NMMA and MB. In fact, it showed a deleterious effect for this population in terms of mortality [4]. Other NO effects [5] as amplification of macrophage activity, reduced platelet aggregation and free radical scavenging could be beneficial and must account for unwanted consequences when NO production or its effects are blocked. For this reason, other approaches as the use of NO scavengers have been tried. While the NO molecule is removed from the blood stream [6], the intracellular NO activity may continue exerting its beneficial effect [7]. NO scavengers have been tried in clinical trials as treatment for septic shock, but the outcomes have been equally disappointing. Our intention was to obtain a quantitative summary of these diverse antagonistic agent effects along the NO pathway using the available clinical trials. The number of available trials were limited and sometimes were small-sized. When this is the case, the use of a tool as meta-analysis may be particularly useful in obtaining meaningful conclusions.

2. Methods

Electronic searches in Medline, EMBASE and Google Scholar were carried out using the medical subject headings, text words and Boolean operators "nitric oxide synthase" and "clinical trial" and "distributive shock" or "septic shock" up to December 2016. The inclusion criteria were: controlled randomized clinical trials (RCTs) whose intervention included either a NOS inhibitor or a sGC/cGMP inhibitor or a NO scavenger in distributive shock or specifically septic shock. The outcomes analyzed in these RCTs were: hemodynamic (blood pressure, heart rate, cardiac output, systemic vascular resistance, pulmonary vascular resistance, etc) survival, adverse events (acute respiratory or renal deterioration, significant liver damage, severe hematologic abnormalities, cardiac failure, ischemia or dysrhythmia) and disposition issues (length of stay in ICU and/or hospital). Preclinical studies, dose-finding studies, case reports, case series, RCTs related exclusively to different outcomes, opinion articles and reviews were excluded. A hand-search of the references was added. Two independent researchers examined the citations abstracts for inclusion and exclusion criteria. The quality of the included citations was reviewed by the same researchers using the Jadad score [8] and the Cochrane Collaboration tool for risk of bias [9] (Table 1). Any discrepancies were discussed by the two researchers until an agreement was reached.

The size of the effect for quantitative outcomes was extracted from mean and standard deviation plus population size for each study. It was calculated as Difference in means (DM) with a confidence interval (CI) and p value. A neutral DM was 0, a 95% CI crossing 0 was considered not significant, p values were significant if <0.05. The size of the effect

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Table 1
Quality of report assessment.

Study	Randomized	Blinded	Withdrawals	Total Jadad	Sequence generation	Allocation concealment	Blinding	Incomplete outcomes addressed	Free of selective reporting	Free of other bias
Petros [11]	1	1	1	3	+?	+?	+?	+	+	−
Bakker/Watson [12,13]	2	2	1	5	+	+	+	+	+	+
López [14]	1	2	1	4	+?	+	+	+	+	+
Kirov [15]	2	0	1	3	+	+	−	+	+	+
Memis [16]	2	1	1	3	+	?	−	+	+	+
Kinasewitz [17]	1	0	1	2	?	?	−	+	+	+
Vincent [18]	2	0	1	3	?	?	−	+	+	+

+? Author mention it was done, but no method was specified; ? unknown.

for dichotomous outcomes was calculated from incidence rate and size of the groups and expressed as odds ratio (OR), 95% CI and *p* value. An OR of 1 represented a neutral effect for the outcome. A CI crossing 1 was considered not significant, as well as *p* values above 0.05. The choice of fixed vs. random effects analysis was decided in favor of the later because the heterogeneity of the actions. When the number of studies with usable data for a given outcome was three, a fixed effect analysis was performed because of the uncertainty of the between-studies variance. For outcomes addressed only by one or two studies or presented in graphics with unclear quantification, the data was presented in tables or in a narrative way. A heterogeneity test was performed including the comparison of Cochrane's Q with the degrees of freedom (df), the variance of true effects (T^2) and real difference in effect size (I^2) but this information was not used to decide the type of analysis (fixed vs. random) to be used. The software utilized was the Comprehensive Meta Analysis V2 (Biostat™, Englewood, USA). This report was written following the PRISMA checklist [10].

3. Results

The literature search rendered 41 citations. After the abstracts exam, 4 reviews were ruled out, as well as 3 animal studies, one opinion article, 9 related to other type of outcomes and 15 uncontrolled studies (case reports and case series). Out of the 9 retrieved, one was a dose-finding study [11] and two papers were unified because related to the same study [12,13]. Seven was the final number of studies considered. The search flowchart is represented in Fig. 1. The quality of the studies is depicted in Table 1 using a numerical scale and a descriptive analysis. The summary of characteristics of the RCTs included is shown in Table 2. The initial physiologic score is similar among the studies except Memis et at., which includes lesser sick patients in both groups.

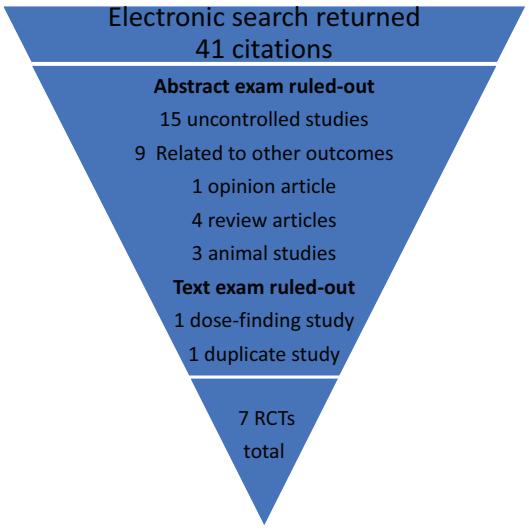

Fig. 1. Literature search algorithm.

All the included RCTs contributed to the analysis of survivors by day 28 (Fig. 2), which is worse for the study (NO pathway inhibition) group (OR 0.79; 95% CI 0.64 to 0.97; $p = .02$), and the result is based in the three strongest for this outcome (López et al., Vincent et al. and Bakker/Watson et al.).

All of the RCTs included addressed directly or indirectly the changes in blood pressure and other hemodynamic parameters but often times not in a fashion amenable for quantitative comparison. Graphic chart-time representation was the usual means. Less frequent was numerical data provided at certain points in time (Kirov et al. and Memis et al.). Petros et al. quantified (*p* value) a significant increase in MAP, SVR, CVP, PVR and decrease of CO, heart rate and DO_2 before and after the NOS inhibitor L-NMMA was used but they did not compare with the control group besides showing the graphics. A summary of the hemodynamic parameters trend differential comparing study vs. control group is offered in Table 3. The most consistent effects in the intervention group were the elevation of blood pressure, deceleration of heart rate and elevation of systemic vascular resistance. Cardiac output was most frequently decreased. Pulmonary pressure and vascular resistance, frequently increased.

Other way of assessing effectiveness was by looking at the number of patients that achieved resolution of shock (ROS, meaning normalization of blood pressure and weaning of vasopressors) by 72 h after the intervention was initiated. It was used by three studies and a favorable meta-analysis result for the intervention group is shown in Fig. 3 (OR 2.02; 95% CI 1.52 to 2.68; $p < .01$).

The number of adverse effects results (Fig. 4) is detrimental for the intervention group, based on the three RCTs with usable data for this outcome (OR 2.22; 95% CI 1.35 to 3.6; $p<0.01$).

The length of stay (LOS) as days in the ICU among the survivors (Fig. 5) is shorter in the control group (DM −2.46; 95% CI -4.24 to −0.69; $p = .01$) based on three studies with usable data.

Our meta-analysis concludes that the interventions along the NO pathway interfering NO synthesis, interfering NO action in target tissues or removing circulating NO are effective to restore blood pressure and for the resolution of shock in short term. But on the long run perspective, such agents are deleterious for the survival rate of these patients, they have more frequent adverse effects and have longer ICU stay among the survivors.

4. Discussion

New drugs RCTs have systematically failed to prove a benefit in septic shock patient survival. Small trials often times suggest possible advantages that are denied by confirmatory larger trials. The absence of a prior power analysis by the small studies could be the explanation for these contradictory results. Nitric oxide production has been associated to hypotension resistant to fluid resuscitation and ultimately multiple organ dysfunction. Non-selective opposition to NO production or to its effects has not resulted in improved long-term outcome and survival despite the fact blood pressure is improved in the short-term. Several explanations can be offered for such adverse outcome.

Table 2
Summary of the included studies.

Study	Action – Agent	Target population	$N = 841$ score study	$N = 791$ score control	Centers	Description	Outcomes
Petros [11]	NOS inhibitor N^G-monomethyl-L-arginine	Septic shock	5 APACHE II 31	6 APACHE II 27	1	0.3 mg·kg^{-1} plus 1 mg·kg^{-1} plus 1 mg·kg^{-1}·h^{-1} × 6 h	Main: hemodynamics Others: survival
Bakker/Watson [12,13]	NOS inhibitor N^G-methyl-L-arginine hydrochloride	Septic shock	156 SAPS II 51	156 SAPS II 55	48	5 mg·kg^{-1}·h^{-1} titrated to a maximum 20 mg·kg^{-1}·h^{-1} × 72 h	Main: efficacy as ROS Others: organ dysfunction, survival
López [14]	NOS inhibitor N^G-methyl-L-arginine hydrochloride	Septic shock	439 SAPS II 52	381 SAPS II 52	126	2.5 mg·kg^{-1}·h^{-1} titrated to a maximum 20 mg·kg^{-1}·h^{-1} × 7–14 days	Main: survival Others: ROS, adverse events
Kirov [15]	Guanylate cyclase inhibitor Methylene blue	Septic shock	10 SAPS II 57.8	10 SAPS II 57.7	1	2 mg·kg^{-1} plus 0.25 mg·kg^{-1}·h^{-1} titrated to a maximum 2 mg·kg^{-1}·h^{-1}	Main: hemodynamics Others: ROS, survival, LOS ICU
Memis [16]	Guanylate cyclase inhibitor Methylene blue	Severe sepsis	15 APACHE II 13 SOFA 6.2	15 APACHE II 14 SOFA 7.06	1	0.5 mg·kg^{-1}·h^{-1} × 6 h	Main: cytokine levels Others: Blood pressure, survival
Kinasewitz [17]	NO scavenger Pyridoxilated hemoglobin polyoxyethylene	Distributive shock	33 APACHE II 33	29 APACHE II 30	15	0.25 ml·kg^{-1}·h^{-1} for a maximum 100 h	Main: survival Others: hemodynamics, adverse events, LOS ICU
Vincent [18]	NO scavenger Pyridoxilated hemoglobin polyoxyethylene	Distributive shock	183 SOFA 13.8	194 SOFA 12.8	61	0.25 ml·kg^{-1}·h^{-1} for a maximum 150 h	Main: survival Others: adverse events, ROS

NO, nitric oxide; NOS, nitric oxide synthase; ROS, resolution of shock; LOS, length of stay; APACHE, Acute Physiology and Chronic Health Evaluation; SAPS, Simplified Acute Physiology Score; SOFA, Sequential Organ Failure Assessment.

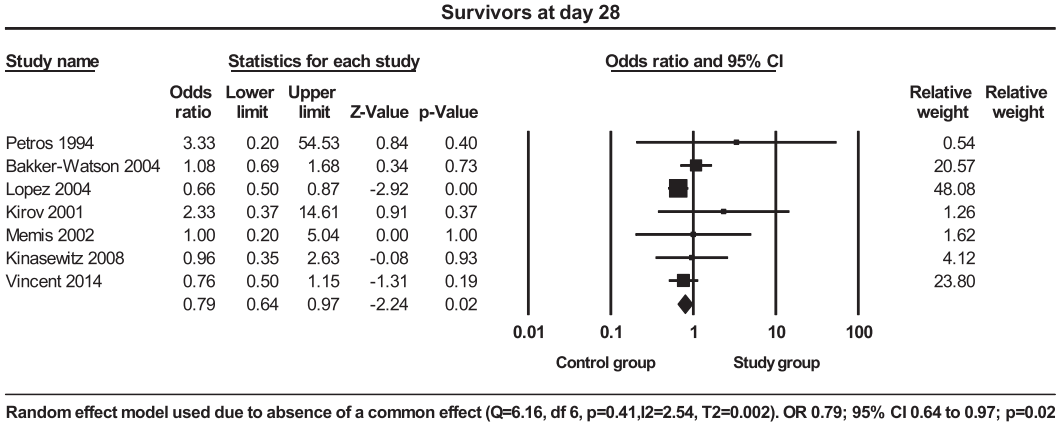

Fig. 2. Survival al 28 days.

A physiological reason for this is that NO produced by iNOS enhances microbial killing by macrophages [20] and neutrophil recruitment [21]. In addition, iNOS must nowadays be considered as an integral component of the development, differentiation, and function of B and T lymphocytes, and also of non-hematopoietic cells [22]. NO interfering of platelet aggregation (in fact there, is no expression whatsoever of NOS in human platelets) and free radical scavenging [23] could be beneficial in septic shock and must account for unwanted consequences when NO production or its effects are blocked.

It has been argued that methylene blue is not a vasoconstrictor by itself but re-balances the vascular system by blocking the cGMP system to NO effects and enhancing cAMP response to norepinephrine (NE) [24]. In an experimental model in rats, the combination of MB and NE preserved microvascular integrity and did not so when NE was used alone [25]. Methylene blue RCTs have been remarkably small. Larger trials with the previous consideration included in its design could be considered in the future.

Certain limitations of these RCTs should be pointed-out and some alternative strategies may be suggested. If NO immunoregulatory effects are beneficial for septic patients, selective iNOS activity preservation with selective iNOS inhibitors could be beneficial [26]. Lange et al. [27] reported an early eNOS expression vs. late iNOS expression during sepsis, in correspondence with the worse outcome that patients in hypodynamic phase had in López et al. RCT, meaning that relatively more iNOS was inhibited during such phase. May be NO donors will be salutatory for these patients, providing tailored cardiovascular support by usual agents (vasopressin, norepinephrine) in the interim [26].

In López et al. trial there is low-grade evidence (post hoc analysis) that the low dose treatment initial group could have better outcome

Table 3
Hemodynamic parameters.

	MAP	SVR	CO	HR	CVP	PAOP	PAP	PVR	DO_2
Petros [12]	+	+	−	−	+	+		+	−
Watson [13,14]	+	+	−	−			+	+	−
López [15]	+	+	−	−			+	+	
Kirov [16]	+	+	+	−	=	−	=	=	=
Memis [17]	+			=	=				
Kinasewitz [18]	+	=	−	−		+	+		
Vincent [19]	+	+	−	−	+	+	=		

MAP, mean arterial pressure; SVR, systemic vascular resistance; CO, cardiac output; HR, heart rate; CVP, central venous pressure; PAOP, pulmonary artery occlusion pressure; PVR, pulmonary vascular resistance; DO_2 oxygen delivery.

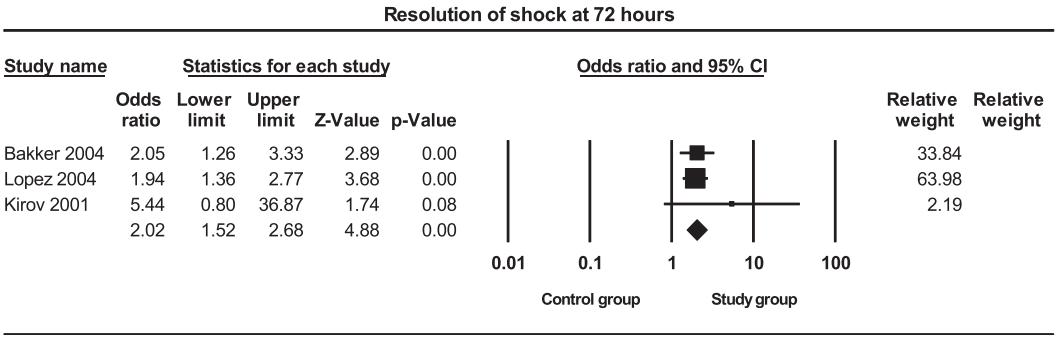

Fig. 3. Resolution of shock at 72 h.

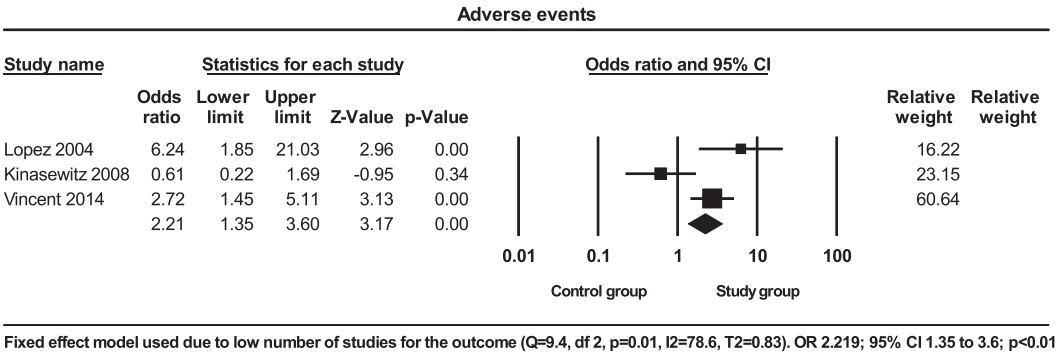

Fig. 4. Number of adverse effects.

compared to the high dose group, which was the standard not only in this one, but in other included trials. This suggests that the doses used in these trials could have been excessive.

A phase I trial in patients with severe sepsis using intravenous ascorbic acid reduced the proinflammatory biomarkers C-reactive protein and procalcitonin and improved pathophysiologic scores [28]. The reason for this could be that ascorbate increases overall nitric oxide concentration by releasing nitric oxide from adducts and by acting through tetrahydrobiopterin (BH4) to stimulate eNOS, although must be emphasized that at the same time, it also undesirably diminishes endothelial expression of iNOS [29]. A single action may have desirable and undesirable effects that will need to be gauged.

When BH4 availability is a limiting factor, eNOS becomes enzymatically uncoupled and generates superoxide. NO reaction with superoxide creates peroxynitrite [30]. Peroxynitrite interacts with lipids, DNA, and proteins via direct oxidative reactions or via indirect, radical-mediated mechanisms, triggering significant oxidative injury, cellular necrosis and apoptosis [31]. The novel drug R-100 has a mixed action as superoxide scavenger and peroxynitrite degradation catalyst as well as nitric oxide donor. It improved pulmonary function in a sheep model of septic shock caused by *Pseudomonas aeruginosa* and smoke inhalation [32].

The use of biomarkers as surrogates of outcome is an interesting possibility that was scarcely used in these trials. Bakker/Watson trial provided arginine and nitrate levels, Memis provided Tumor Necrosis Factor, interleukines (1beta, 2R, 6 and 8) and NO oxidative species.

NO role in sepsis is a complex matter with favorable and deleterious actions. We also now know that NO is generated by bacterial NOS or nitrite reductases and helps bacterial pathogens to resist antimicrobial activities of myeloid cells or even modulates host cell immune responses. Any future trial will be technically difficult to perform, and should focus in adequate dose, selective receptor action, timely NO inhibition/

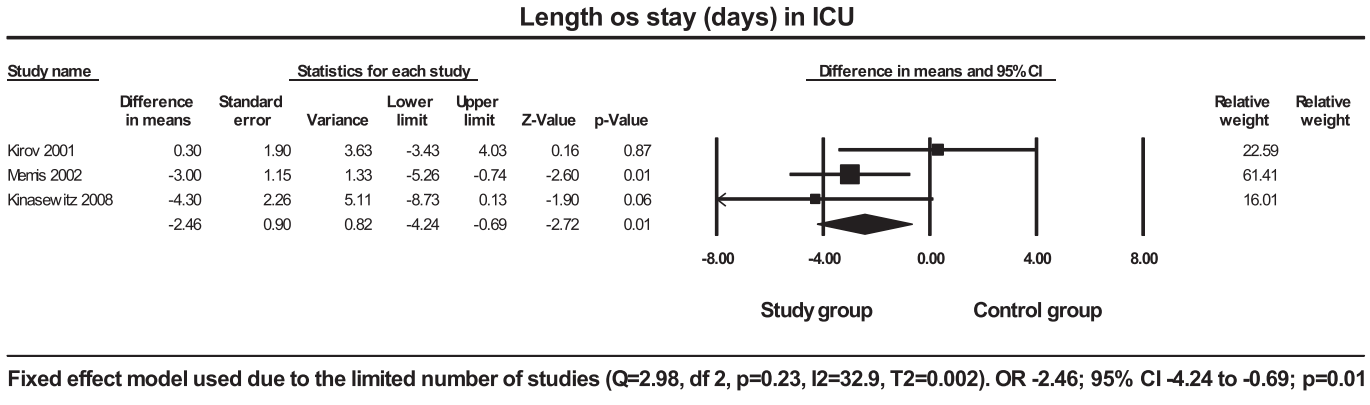

Fig. 5. Length of stay in ICU.

donation, appropriate tissue- or cell-specific populations as targets and could include the use of biomarkers as surrogates of outcome.

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Conflict of interest

The authors have no Conflict of Interest to declare.

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