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## Plasma Levels of Nitric Oxide and Carbon Monoxide in Critically Ill Children with Septic Syndrome

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Vascular tone alteration in sepsis is attributed to nitric oxide (NO) overproduction. Endogenously released carbon monoxide (CO) has been recently proposed to induce excessive vascular relaxation. We aimed 1) to study plasma levels of NO, CO and blood methemoglobin (methHb) in sepsis and sepsis induced organ failure in critically ill children. 2) to determine their relationship to disease severity and outcome. Plasma levels of NO, CO and blood methHb were assessed in 30 critically ill and 14 healthy children. Patients were classified into septic and sepsis induced multi-organ failure (MOF) groups. All patients showed significantly higher plasma levels of NO, CO and blood methHb when compared to controls ( $p < 0.001$ ). Sepsis induced MOF group attained higher plasma levels of NO and CO and blood methHb compared to septic group ( $p < 0.001$ ). Children requiring vasopressors showed increased plasma levels of NO, CO and blood methHb compared to those not requiring it ( $p < 0.001$ ,  $< 0.05$ ,  $< 0.001$ , respectively). Significant positive correlations existed between PRISM score with plasma NO, CO and blood methHb. Non-survivors exhibited higher plasma levels of NO, blood methHb ( $p < 0.001$ ) and CO ( $p < 0.05$ ) compared to survivors. In conclusion, plasma NO, CO levels and blood methHb were elevated in sepsis and sepsis induced MOF. They can be used as prognostic markers for disease severity and outcome. We recommend adding plasma CO and blood methHb to standard laboratory tests in Pediatric Intensive Care Units (PICU).

**Key words:** Nitric oxide, carbon monoxide, blood methemoglobin, sepsis, multi-organ failure, pediatric intensive care unit

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## INTRODUCTION

Sepsis remains a serious problem with high rate of mortality, despite of recent progress in critical care (Shi *et al.*, 2003).

Septic syndrome is characterized by alterations in vascular tone and in severe sepsis the peripheral vasculature is markedly refractory to the vasoconstrictive effects of alpha-1 adrenergic receptor agonist agents (Tsuneyoshi *et al.*, 1996). The desensitization of blood vessels in inflammatory conditions is mediated by endogenous substances that lead to excessive vasodilatation and hypotension. This is the hallmark of patients with septic shock (Bakker *et al.*, 2004).

Both Nitric Oxide (NO) and Carbon monoxide (CO) are proposed to regulate the physiologic vascular tone, blood pressure and tissue perfusion (El-Sallab *et al.*, 2002).

Nitric oxide is a free radical that is synthesized from L-arginine by a family of enzymes termed nitric oxide synthetase (NOS) (Krafte-Jacobs *et al.*, 1997). Three distinct forms of NOS had been identified: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Wheeler and Bernard, 1999). During critical illness, proinflammatory cytokines and bacterial lipopolysaccharides stimulate iNOS which results into sustained production of NO for a prolonged period of time. Excessive NO levels lead to pathologic loss of vascular tone which is a central feature of sepsis induced lethality (Mitaka *et al.*, 2003).

Nitric oxide in the blood stream reacts with hemoglobin (Hb) to form methemoglobin (metHb) which is continuously reduced to hemoglobin by methemoglobin reductase. Normally this process is very efficient, resulting in little detectable circulating methemoglobin (<1% of total hemoglobin). However, circulating oxidants, exogenous nitrites, or a deficiency of methemoglobin reductase may result in methemoglobinaemia. In septic shock, it was assumed that circulating methemoglobin concentrations could serve as an indicator of endogenous NO overproduction (Krafte-Jacobs *et al.*, 1997).

Recently, biomedical interest in CO gas has grown rapidly as a new endogenously produced mediator participating in the pathogenesis of sepsis syndrome (Wunder *et al.*, 2005). Overproduction of CO has been proposed to induce vascular relaxation and hence a fall in blood pressure by activating guanylyl cyclase and increase cellular concentration of cyclic guanosine monophosphate, an action which is similar to NO (Reade *et al.*, 2005). CO is produced almost exclusively from heme catabolism by microsomal heme-oxygenase enzyme (HO). Three isoforms of HO have been identified;

inducible HO-1 and two constitutively expressed HO-2 and HO-3 (Perella and Yet, 2003). HO-1 plays a protective role in hyper perfusion and ischemia/reperfusion injuries. (Chen *et al.*, 2003).

In the present study, we aimed to 1) study plasma levels of NO, CO gases and blood metHb in sepsis and sepsis induced multi-organ failure (MOF) in critically ill children. 2) Determine the potential relationship between the levels of these mediators with disease severity and outcome.

## MATERIALS AND METHODS

The study was conducted on 30 infants and children with a clinical diagnosis of sepsis, recruited from the Pediatric Intensive Care Unit, Pediatric Hospital, Ain Shams University. They were collected in the period between March to June, 2006. Their mean age was (2.6±3.32 year). They were 19 males and 11 females. They were further classified into:

**Group 1 (Septic):** Sixteen children (10 males and 6 females). Their mean age was (2.1±1.7 year). Septic criteria were: rectal temperature < 36°C or > 38.5°C, heart rate > 90 beats min<sup>-1</sup>; respiratory rate > 20 breaths min<sup>-1</sup>; WBCs > 12,000 cells mm<sup>-3</sup> or < 4000 cells mm<sup>-3</sup> or > 10% band forms. The diagnosis of septic shock was made when the patients had hypotension (systolic blood pressure < age × 2 + 60 mm Hg) or poor capillary refill > 3 sec in addition to sepsis syndrome (Marik, 2003).

**Group 2 (Sepsis induced MOF):** Fourteen infants and children (8 males and 6 females). Their mean age was (1.4±2.1 year). They were suffering from sepsis as well as one or more organ failure, diagnosed according to the multiple organ failure index (Proulx *et al.*, 1994):

**Cardiovascular:** Mean Arterial Blood Pressure (MAP) < 5th percentile for age  
Or requirement for vasopressor/inotropic agents after volume resuscitation.

**Pulmonary:** PaO<sub>2</sub>/FIO<sub>2</sub> < 300 and ventilator requirement.

**Renal:** Oliguria < 1 mL kg<sup>-1</sup> h<sup>-1</sup>. For 8 h if < 30 kg or < 0.5 mL kg<sup>-1</sup> h<sup>-1</sup> for 8 h if > 30 kg or serum creatinine > 1 mg dL<sup>-1</sup>.

**Hematologic:** Prothrombin Time (PT), Partial thromboplastin time > 1.5× normal and platelet count of < 100,000 × 10<sup>3</sup> thrombocytes mm<sup>-3</sup> (10<sup>9</sup> L<sup>-1</sup>).

**Hepatic:** Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) > 100 units L<sup>-1</sup> and total bilirubin > 1.0 mg dL<sup>-1</sup> (excluding neonates).

**Central nervous system:** Glasgow Coma Scale < 12 (in the absence of sedation).

Pediatric Risk of Mortality (PRISM) score was calculated for all children included in the work on study entry in its third version. It was based on 17 physiological variables. The measured variables included: systolic blood pressure, heart rate, temperature, papillary reflexes, mental status, acid-base and blood gases (pH, PaO<sub>2</sub>, PaCO<sub>2</sub>), chemistry tests (random blood glucose, serum potassium, creatinine and blood urea nitrogen BUN) and hematology tests (white blood cell count TLC, platelet count, prothrombin time PT and partial thromboplastin time PTT) (Pearson, 2002).

Fourteen healthy sex and age matched children were enrolled in the study and served as control group. They were recruited from Pediatric Outpatient Clinic, National Research Center.

**Blood collection:** Two milliliter of venous blood were collected, one part was withdrawn into tubes containing EDTA for complete blood count and another part was left without addition of anticoagulant, centrifuged at 500 x g for 15 min. for renal and hepatic profiles. Two milliliters of arterial blood on heparin were collected for blood gas analysis.

All patients and controls had completed the following:

- Full history taking and thorough clinical examination.
- Chest X-rays (posteroanterior and lateral views).
- Complete blood picture using coulter counter (coulter Micro-Diff 18, Fullerton, CA, USA).
- C-reactive protein applying Nephelometric technique (Dade Behring Instruments)
- Capillary blood gases using Blood Gases Analyzer-Ion Selective Electrode Method (ABOT, Germany): pH, PO<sub>2</sub>, PCO<sub>2</sub> and HCO<sub>3</sub>.
- Liver function tests: Serum bilirubin, albumin, SGOT and SGPT (Beckman instruments, Brea, California).
- Kidney function tests: Serum urea, creatinine and uric acid (Beckman instruments, Brea, California).
- Serum sodium and potassium (Beckman instruments, Brea, California).
- Repeated blood culture for gram positive and negative organisms (Becton Dickinson Microbiology System, Sparks, Maryland).

**Plasma NO assay:** (R and D System inc., USA, catalog No. DE 1600): The plasma concentrations of NO were assayed by enzyme immunoassay kit. This technique determines the total NO based on enzymatic conversion of nitrates to nitrites by nitrate reductase. The reaction is followed by colorimetric detection of nitrite as an azodye product of the Griess reaction. Samples were diluted by adding 100 µL sample+ 100 µL reaction buffer (1x). The diluted sample was ultra-filtered through a 10,000 molecular weight cut off filter to eliminate proteins. Two hundred microliter reaction buffer (1x) were added to the blank well, while 50 µL of nitrate standard were added. To both the standard and the sample wells, 25 µL of NADH and 25 µL of nitrate reductase were added, mixed well by tapping the sides of plate wells and incubated for 30 min at 37°C. Then 50 µL of Greiss reagent 1 added to all wells except the blank one. Fifty microliter of Greiss reagent I I was added. Wells were incubated for 10 min. at room temperature. Optical density of each well was determined at 540 nm. A standard curve was created. The concentration read from the standard curve was multiplied by the dilution factor (Mitaka *et al.*, 2003).

**Plasma CO assay:** CO concentrations were measured using simple, sensitive automated spectrophotometric method, Ciba Corning 800 system (Chalmers, 1991). COHb% was calculated from a standard curve. Carbon monoxide concentration was calculated as follows:

$$\text{CO (nmol L}^{-1}\text{)} = \frac{\text{COHb\%} \times \text{Hb (mg L}^{-1}\text{)} \times 4000}{100 \times 64456}$$

(El-Sallab *et al.*, 2002)

**Methemoglobin determination:** Using automated spectrophotometric method, Ciba Corning 800 system (Chalmers, 1991).

**Statistical analysis:** A standard computer programme (SPSS for Windows, Release 10.0; SPSS; Chicago, IL) was used for data entry and analysis. All data were expressed as mean±SD. Comparison of different variables was done using Student t test or Mann Whitney test for non-parametric data. One way ANOVA test was used to compare more than two groups as regarding quantitative variable. Correlation study (r) was performed to find the association between different parameters. For all tests p<0.05 = significant, p<0.01 = highly significant and p>0.05 = non significant.

## RESULTS AND DISCUSSION

Sepsis is a life threatening condition and it is one of the main causes of death in Pediatric Intensive Care Unit

(Bakker *et al.*, 2004). Recent studies have pointed to NO and CO as endogenously produced mediators that play important roles in the pathogenesis of sepsis and septic shock in children (Chen *et al.*, 2003).

This study demonstrated that plasma NO levels were significantly increased in all children with sepsis and sepsis induced MOF when compared to healthy controls (Table 1). This data was compatible with Spack *et al.* (1997), El-Sallab *et al.* (2002) and Mitaka *et al.* (2003). During critical illness, proinflammatory cytokines and bacterial lipopolysacchrides stimulate the production and activity of an inducible isoform of NO synthetase from a variety of cell types resulting in concomitant increase in generation of NO (Sarady *et al.*, 2004).

Mitaka *et al.* (2003), noted that although NO is a potent vasodilator that leads to visceral blood flow improvement, yet its overproduction plays an important role in the pathogenesis of impaired cellular function and tissue damage in sepsis. In addition, it could be toxic for macrophages, endothelial cells and renal proximal tubular cells through its interaction with intracellular iron or sulphur containing enzymes that results in loss of intracellular iron and inhibition of mitochondrial respiration, citric acid cycle and DNA synthesis (Groeneveld *et al.*, 1999).

In the current study, MOF group of children showed significantly higher plasma NO levels when compared to the septic group (Fig. 1). End organ damage during sepsis may be partially dependant on NO molecule which reacts with free oxygen and hydroxyl radicals to form peroxynitrite radicals. These in turn induce epithelial cell damage with derangement of cellular respiration leading to cytopathic hypoxia, severe tissue ischemia and multiple organ dysfunction syndromes (Khan *et al.*, 2002). Also, NO overproduction causes DNA deamination and strand breaking leading to energy depletion and cell death

Table 1: PRISM score and Laboratory parameters of all children included in the study

Variables Mean±SD	Patients N = 30	Controls N = 14	p-value
PRISM score	10.97±3.76	4.21±1.05	<0.001
NO (µmol L <sup>-1</sup> )	29.44±16.11	8.40±2.5	<0.001
Blood MetHb (g dL <sup>-1</sup> )	1.33±0.53	0.31±0.1	<0.001
COHb (g dL <sup>-1</sup> )	0.76±0.297	0.11±3.6	<0.001
CO (mmol L <sup>-1</sup> )	26.98±12.16	9.80±1.8	<0.001

Table 2: Correlations between the different laboratory parameters and the PRISM score in the critically ill children included in the study.

Variables	PRISM score		NO µmol L <sup>-1</sup>		CO mmol L <sup>-1</sup>	
	r	p	r	p	r	p
Blood (metHb g dL <sup>-1</sup> )	0.702	<0.01	0.931	<0.01	0.698	<0.01
NO (µmol L <sup>-1</sup> )	0.812	<0.01	-	-	0.682	<0.01
CO (mmol L <sup>-1</sup> )	0.468	<0.01	0.682	<0.01	-	-

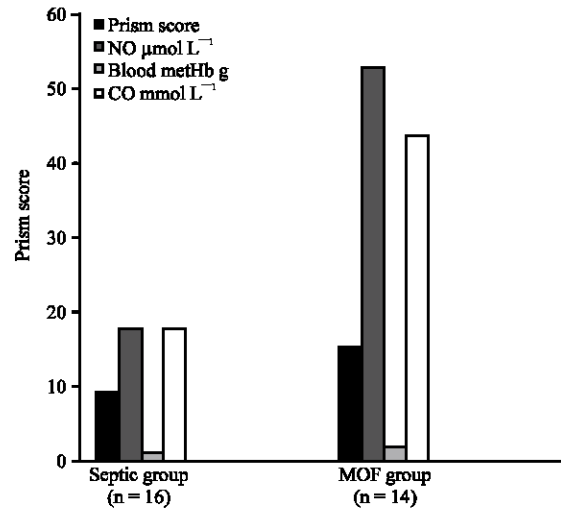


Fig. 1: Comparison of PRISM score and different laboratory parameters among the studied groups

Table 3: Comparison of the different studied clinical and laboratory parameters between children requiring and those not requiring vasopressors

Variables Mean±SD	Patients on vasopressors N = 20	Patients not on vasopressors N = 10	p-value
PRISM score	11.9±4.1	9.1±1.9	<0.05
NO (µmol L)	35.3±16.9	17.7±1.5	<0.001
Blood (metHb g dL <sup>-1</sup> )	1.6±0.4	0.8±0.1	<0.001
CO (mmol L)	31.6±12.4	17.7±2.9	<0.05

(Doughty *et al.*, 1998). Yang *et al.* (2002), demonstrated that the change of nitrate concentrations was correlated with the severity of sepsis and development of MOF. This was in accordance to our results which showed a highly significant positive correlation between high plasma NO levels and the PRISM Score (Table 2).

The present study revealed that plasma NO levels were significantly higher in patients requiring vasopressors compared to those not requiring it (Table 3). This result was consistent with Kumar *et al.* (1999), El-Sallab *et al.* (2002) and Mitaka *et al.* (2003). They noted that excessive NO production was considered to be largely responsible for vasoconstriction-resistant vascular relaxation, catecholamine hypo-reactivity together with impaired oxygen delivery to the microvasculature observed in septic shock. Moreover, a broad range of evidences have suggested that much of the depressant effect of pro-inflammatory cytokines and septic serum on myocardial tissue is mediated via NO dependant mechanism (Kumar *et al.*, 1999).

Present results showed greater plasma NO levels in non-survivors (NS) than survivors (Fig. 2) and hence it could be used as a prognostic marker for sepsis outcome. This came in agreement with Spack *et al.* (1997),

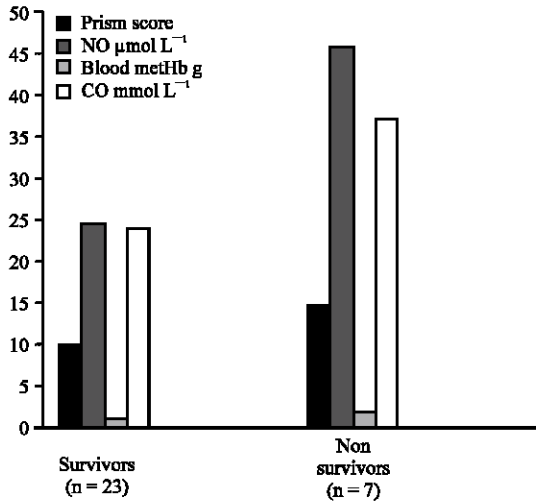


Fig. 2: Comparison of the different studied parameters between survivors and non-survivors

Shi *et al.* (2000), Mitaka *et al.* (2003) and Bakker *et al.* (2004). However, Krafte-Jacobs *et al.* (1997), showed no significance in NO levels when compared in NS versus survivors.

The validity of measurement of circulating NO as a prognostic marker for sepsis outcome has been questioned. NO has an extremely short half life in solution and its direct assessment is difficult because NO is degraded to nitrite and nitrate which in turn may be influenced by other factors such as nitrogen balance and renal function. Therefore, isolated NO measurement may not reliably reflect changes in NO synthesis (Jia *et al.*, 1996). For this reason we investigated another marker of NO metabolism, which is metHb. Blood metHb concentration was significantly higher in all children recruited from PICU when compared to controls (Table 1). In comparison to the septic group of children, the MOF group showed higher levels of metHb (Fig. 1). It gave significant positive correlations with the PRISM scoring system and plasma NO levels (Table 2). In addition, its concentration was significantly higher in patients requiring vasopressors compared to those not requiring it (Table 3). NS showed significant elevation in metHb levels when compared to survivors (Fig. 2). These results were in accordance with Ohashi *et al.* (1998). They suggested that blood metHb concentration might be used as a useful marker in sepsis or septic shock. Contrary to our results, Krafte-Jacobs *et al.* (1997) research did not validate metHb as a marker for sepsis to replace NO measurement.

Few studies have examined the pathophysiology of CO in sepsis. However, there is growing evidence that this diatomic gaseous molecule serves an important role

as a second messenger in the cascade of mediators participating in the pathogenesis of sepsis (Morse *et al.*, 2001; Reade *et al.*, 2005). The present study showed that plasma CO levels were significantly higher in all children with critical illness as compared to controls (Table 1). Patients with organ failure had highly significant increase in the levels of CO compared to the septic group (Fig. 1). These findings indicate that there is a progressive increase in the plasma CO levels with increase in the severity of the condition. In confirmation, our critically ill children showed a significant positive correlation between plasma CO levels and the PRISM scoring system. Moreover, plasma CO levels gave significant positive correlations with plasma NO levels and blood metHb (Table 2). Also, plasma CO levels were significantly higher in septic children requiring vasopressors than those not requiring it (Table 3). Higher levels of CO were attained in non survivors as compared to survivors (Fig. 2). This was in agreement with El-Sallab *et al.* (2002).

Shi *et al.* (2003), implied that both gram negative and positive organisms were capable of activating HO system leading to increase in CO production in a fashion similar to activation of iNOS and production of NO. CO shares many chemical and biological properties with NO. El-Sallab *et al.* (2002), noted that in sepsis, under basal conditions, generated CO may modulate blood vessel tone by activating guanyl cyclase resulting in significant increase in cyclic GMP. Sarady *et al.* (2004), reported the emergence of cytoprotective properties for CO in sepsis. CO has antioxidant and anti-inflammatory functions. It may suppress lipopolysaccharide induced lung alveolitis (Kim *et al.*, 2005) and reduce the expression of alanine aminotransferase a marker of liver injury (Mayer *et al.*, 2003). However, large amounts of CO generated by increased HO-1 activity, could contribute to the reduction in the vascular tone during sepsis (Shi *et al.*, 2003). NO proved in many studies to be the main stimulant for HO-1 activation. Moreover, many investigators believe that the harmful effect of excessive NO production in sepsis is mediated via increase in CO production (Reade *et al.*, 2005).

In conclusion, the present study provided evidences that plasma NO, CO and blood metHb levels are elevated in sepsis and sepsis induced MOF. Positive correlations that existed between these markers and illness severity highlight their prognostic role in assessment of illness severity and prediction of outcome.

Since both plasma CO and blood metHb concentrations are measured by the automated spectrophotometer, which is easily feasible and gives rapid results, therefore, we recommend adding both markers to the standard laboratory tests for evaluation of

pediatric sepsis in PICU. Larger studies are needed to confirm our observation that blood metHb concentration could serve as a sensitive indicator of NO overproduction.

The role of NOS and HO inhibitors in the management of pediatric sepsis should be evaluated on wide scale.

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