Implications of Nitric Oxide and Carbon Monoxide in Neonatal Sepsis

1Shadia El-Sallab, 1Hesham Abdel-Hady, 2Ekbal Abu-Hashim and 1Mohamed Matter

Nitric oxide (NO) and carbon monoxide (CO) are proposed to regulate physiologic vascular tone, blood pressure, and tissue perfusion. To investigate their role in the pathogenesis of neonatal sepsis, plasma levels of NO and CO in 45 neonates with clinical evidence of sepsis plus positive blood culture as well as in 20 healthy neonates were assessed. Significantly higher plasma levels of NO and CO in sepsis group compared with control group were found (56.4 ± 21.1 vs. 33.4 ± 12.1 μmol L⁻¹, p < 0.0001; 44.0 ± 12.0 vs 23.7 ± 8.5 nmol L⁻¹, p < 0.0001, respectively). This increase in NO and CO levels occurred independent of gestational age, onset of sepsis, and gram stain of the isolated organism. Moreover, there were significantly higher plasma levels of NO and CO in septic infants who required inotropic support compared with those who did not require this support (p<0.0001). Plasma CO was higher in non-survivors compared with survivors (p=0.003). Significantly positive correlation between plasma NO and CO was observed. In conclusion, plasma NO and CO was increased in neonatal sepsis. This increase is independent of gestational age, onset of sepsis and gram stain of the isolated organism. Assay of plasma CO and NO may help to assess the severity of sepsis and progression to septic shock. The role of nitric oxide synthase (NOS) and heme oxygenase (HO) inhibitors in the management of neonatal sepsis should be investigated in further trials.

Key words: Nitric oxide, carbon monoxide, plasma, sepsis, newborn

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Introduction
Neonatal sepsis is a life-threatening condition and it is one of the main causes of morbidity and mortality in newborns (Klein and Remington, 1990). Several lines of evidence indicate that endogenously produced mediators are involved in the pathogenesis of sepsis (Bane, 1981). Both NO and CO are proposed to regulate physiologic vascular tone, blood pressure, and tissue perfusion (Moncada et al., 1991; Morita et al., 1995). NO is an omnipresent physiologic agent formed from the conversion of l-arginine and oxygen to NO and citrulline by a family of 3 NOS enzymes (Cannon et al., 1990). The constitutive forms of NOS present in endothelial and neuronal cells produce basal levels of NO that mediate homeostatic functions, such as resting vascular tone, neurotransmission, and inhibition of neutrophil and platelet adhesion. Infectious and inflammatory insults lead to NO production by the inducible form of the enzyme (iNOS) found in macrophages, monocytes, neutrophils, and Kupffer cells. Nitric oxide (NO) has a role in host defense by killing pathogens. Excessive levels, however, lead to pathologic loss of vascular tone, a central feature of sepsis-induced lethality (Lovenstein et al., 1994; Wheeler and Bernard, 1999; Terregino et al., 2000).

CO has been implicated to be a new endogenously produced mediator similar to NO. It is produced almost exclusively from heme catalysis by microsomal HO enzyme (Manes, 1997). Similar to NOS system, three isoforms of NO have been characterized, which are encoded by distinct genes; whereas the isoforms HO-2 and HO-3 are constitutively expressed, the isoform HO-1 is highly inducible (Choi and Alam, 1996; Bauer et al., 1998).

Materials and Methods

Materials: Study included 45 newborn infants with sepsis. They were consecutively admitted in the Neonatal Intensive Care Unit, Mansoura University Children's Hospital in the period from February 2001 to July 2001. Infants were eligible for enrolment if they had clinical evidence of sepsis plus positive blood culture. Infants with obvious major congenital abnormalities, metabolic disease, hyperbilirubinemia, birth asphyxia or receiving corticosteroids were excluded.

Twenty normal healthy newborns of matched gestational age, postnatal age and sex served as a control group, none of them had evidence of disease that might alter plasma NO and CO concentrations. Informed parental consent was obtained before enrollment. Complete obstetric histories were obtained and examinations were performed at the time of admission. The neonatal clinical course was followed prospectively and data were recorded on predetermined proform sheets. The following laboratory investigations were done: complete blood count, C-reactive protein, blood culture, plasma NO and CO estimation.

Sample collection: Plasma samples were obtained using ethylene diamine tetra acetate (EDTA) as an anticoagulant. Centrifuged at 1000 x g within 30 minutes of collection. Samples were stored at -20 °C until analysis was done.

Plasma NO assay: Nitrite/nitrate (NO⁻ / NO₃⁻) has been confirmed to be a good indicator for NO production (Evans et al., 1993). The plasma concentrations of NO were assayed by enzyme immunoassay kit (R&D System Inc., USA). This technique determines the total NO based on the enzymatic conversion of nitrates to nitrite by nitrate reductase. The reaction is followed by colorimetric detection of nitrite as an azo dye product of the Griess Reaction.

In brief, samples were two-fold diluted into reaction buffer. Twenty-five μl of NADH were added to 60 μl of nitrate standard or samples and incubated for 30 minutes at 37 °C. Fifty μl of Griess Reagent I (sulfanilamide) were added and then 50 μl of Griess Reagent II (N-1-naphthyl ethylenediamine in 2 N hydrochloric acid) were added and then incubated for 10 minutes at room temperature. The optical density was determined using a microplate reader set at 540 nm. Plasma NO concentrations were determined from a linear standard curve obtained from standards of known concentration. The detection limit for assay for NO was 1.0 μmol L⁻¹, the concentration range of the standard curve was 1.0 to 500.0 μmol L⁻¹ (Megash and Shiloah, 1982).

Plasma CO assay: Carbon monoxide concentration was measured using simple, sensitive spectrophotometric method (Chalmers, 1991). CO is trapped with hemoglobin (Hb) to form carboxyhemoglobin (COHb) and subsequently estimated by dilution reduction. One ml of Hb solution (0.25 ml of fresh-packed erythrocytes in 50 ml of 0.24 mol L⁻¹ ammonia solution) was mixed with 0.25 ~ 1ml of a sample or an equivalent amount of water, which was used as a blank to measure the endogenous CO present in the Hb solution. Then 0.1 ml of 20 % sodium dithionite solution was added to both the test sample and water-containing blank solution, vortex-mixed, and let stand for 10 minutes. The absorbance at 541 and 555 nm against a reference corvette containing water was read and the ratio of 541 to 555 reading was measured. Then % COHb was calculated from a standard curve derived by mixing 100 % HbO₂ and 100 % COHb in different proportions. Carbon dioxide concentration in m of the sample was determined as follows (Shi et al., 2000a; Shi et al., 2002):

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

Where

\[
\text{COHb} = \text{Carboxyhemoglobin, Hb} = \text{Hemoglobin}
\]

The detection limit of the assay was 3.5 nmol L⁻¹. The concentration range of the standard curve was 3.6 to 500 nmol L⁻¹.

Statistical analysis: The data were described as mean ± SD. The independent samples t-test was used for group comparisons of continuous variables and Chi square test for categorical variables. To test for associations Pearson's correlation coefficient were used. Statistical significance was determined at p < 0.05 and all p-values reported were of the two-sided type. The statistical analyses were completed on SPSS for windows (SPSS Version 10.0.1, Inc. Chicago, IL).

Results
Demographic data and perinatal characteristics of the studied groups showed that no differences were found between septic and control groups regarding gestational age, birth weight, postnatal age, sex, type of delivery and place of delivery, but results of premature rupture of membranes were statistically significant at p < 0.0001 (Table 1).

Eighteen infants (40 %) had early onset sepsis and 27 (60 %) had late onset sepsis, 15 (33 %) had gram-positive sepsis and 30 (67 %) had gram-negative sepsis. Klebsiella pneumonia was the commonest organism (64%) isolated from blood cultures followed by Staphylococcus epidermidis (20 %), Staphylococcus aureus (19 %), Escherichia coli (9 %), group B Streptococcus (4 %) and
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Table 1: Demographic data and clinical characteristics of the studied population

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Septic group (n = 45)</th>
<th>Control group (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (weeks)</td>
<td>36.3 ± 4.0</td>
<td>37.6 ± 2.2</td>
</tr>
<tr>
<td>Birth-weight (Kg)</td>
<td>0.32 ± 0.80</td>
<td>0.56 ± 0.94</td>
</tr>
<tr>
<td>Postnatal age (days)</td>
<td>11.4 ± 7.7</td>
<td>12.2 ± 4.3</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>29.0 (62.2%)</td>
<td>08.0 (40.0%)</td>
</tr>
<tr>
<td>Cesarean section (%)</td>
<td>24.0 (53.3%)</td>
<td>11.0 (55.0%)</td>
</tr>
<tr>
<td>Inborn (%)</td>
<td>28.0 (57.8%)</td>
<td>15.0 (75.0%)</td>
</tr>
<tr>
<td>Premature rupture of membranes*</td>
<td>18.0 (40.0%)</td>
<td>00.0 (0%)</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SD and as number (%) for categorical variables # *: p < 0.0001

Table 2: Plasma nitric oxide (NO) and carbon monoxide (CO) levels in septic and control groups

| Groups                      | Nitric oxide (μmol L⁻¹) | Carbon monoxide (μmol L⁻¹)
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Septic</td>
<td>55.4 ± 21.1</td>
<td>44.0 ± 12.0</td>
</tr>
<tr>
<td>Control</td>
<td>33.4 ± 12.1 *</td>
<td>23.7 ± 8.5 *</td>
</tr>
<tr>
<td>Septic full-term infants</td>
<td>57.4 ± 19.8</td>
<td>41.3 ± 11.9</td>
</tr>
<tr>
<td>Septic preterm infants</td>
<td>56.4 ± 23.4</td>
<td>48.0 ± 11.4</td>
</tr>
</tbody>
</table>

*: p < 0.0001

Table 3: Plasma nitric oxide (NO) and carbon monoxide (CO) levels in relation to the onset of sepsis and gram stain of the organism

| Groups                      | Nitric oxide (μmol L⁻¹) | Carbon monoxide (μmol L⁻¹)
<table>
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<tbody>
<tr>
<td>Early onset sepsis</td>
<td>51.6 ± 18.1</td>
<td>40.5 ± 10.2</td>
</tr>
<tr>
<td>Late onset sepsis</td>
<td>59.2 ± 22.7</td>
<td>46.3 ± 12.7</td>
</tr>
<tr>
<td>Gram-positive sepsis</td>
<td>58.7 ± 22.7</td>
<td>46.2 ± 11.7</td>
</tr>
<tr>
<td>Gram-negative sepsis</td>
<td>58.2 ± 20.3</td>
<td>42.9 ± 12.5</td>
</tr>
</tbody>
</table>

Table 4: Plasma nitric oxide (NO) and carbon monoxide (CO) levels in relation to inotropic support requirements and outcome

| Groups                      | Nitric oxide (μmol L⁻¹) | Carbon monoxide (μmol L⁻¹)
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Inotropic support required</td>
<td>24.1 ± 23.2</td>
<td>46.6 ± 12.9</td>
</tr>
<tr>
<td>No inotropic support required</td>
<td>31.3 ± 12.2 *</td>
<td>35.1 ± 13.2 *</td>
</tr>
<tr>
<td>Survivors</td>
<td>60.5 ± 20.3</td>
<td>33.1 ± 14.4</td>
</tr>
<tr>
<td>Non-Survivors</td>
<td>25.4 ± 21.5</td>
<td>43.6 ± 12.5 *</td>
</tr>
</tbody>
</table>

*: p < 0.05, **: p < 0.001

Fig. 1: Correlation between plasma nitric oxide and carbon monoxide levels in the studied population (n = 65)

Pseudomonas aeruginosa (4%). Twenty-four patients (53%) required inotropic support and twenty-three patients (51%) succumbed.

Plasma NO and CO levels were significantly higher (p < 0.0001) in septic infants compared to controls (Table 2). Elevation of plasma NO > 41.7 μmol L⁻¹ and CO > 30.1 mmol L⁻¹ were significantly associated with sepsis. There were no statistically significant differences between plasma NO and CO levels in septic full-term and septic preterm infants (Table 2). There was a significant positive correlation between plasma NO and CO levels (r = 0.65, p < 0.0001) (Fig. 1).

Discussion

Some important findings have been emerged from this work, first, plasma NO and CO were significantly elevated in newborn infants with sepsis, with a significant positive correlation between plasma NO and CO and with no significant difference between full-term and preterm infants (Table 2). Second, no significant differences were found in plasma levels of NO and CO between early or late onset sepsis or gram-positive or gram-negative sepsis (Table 3). Third, plasma NO and CO were significantly higher in septic newborns requiring inotropic support compared with those not requiring inotropic support and plasma CO levels were significantly higher in those who died compared to survivors (Table 4).

Plasma NO was reported to be elevated in adult patients with severe sepsis or shock (Ochoa et al., 1991; Battafarano and Dunn, 1992), in septic children hospitalized in intensive care settings (Wong et al., 1995; Spack et al., 1997), and in septic full-term infants (Shi et al., 2006b). On the other hand, some researchers did not find elevation of NO metabolites in patients with sepsis (Terregino et al., 2000). However, this study was performed on patients with presumed sepsis on arrival to the emergency department and before the development of septic complications such as altered perfusion or shock. Some sources suggest that constitutive NOS may be down regulated in the early stages of inflammation, and that the time for increased production by the iNOS may take from 4 to 12 hours (Curran et al., 1991; Shieh et al., 2000).

In the present work plasma CO levels were significantly related to NO production. Similar findings were reported by Shi et al. (2006b). It was reported that NO is an important stimulator of NO and so it increases CO production (Foresti and Montielli, 1999). Moreover, this study shows the first evidence that septic preterm infants have similar changes in plasma NO levels as septic full-term infants. Similar to these findings, Shi et al. (1993), found no differences in plasma NO levels between gram-positive sepsis and gram-negative sepsis.

NO plays an important role in the mediation of the main cardiovascular features of septic shock (Kibboum and Griffith, 1992). Excessive formation of NO is associated with profound vasodilatation, hypotension, hyporeactivity to catecholamines and inadequate tissue perfusion (Teale and Atkinson, 1992; Bhagat et al., 1999). Moreover, a broad range of evidence has suggested that much of the depressant effect of proinflammatory cytokines and septic serum on myocardial tissue is mediated through NO-dependent mechanisms (Kumar et al., 1999). Current results confirms the findings of previous studies that highly elevated NO concentration were related to the occurrence of septic shock and organ failure (Shi et al., 1993; Wong et al., 1995; Spack et al., 1997, Duke et al., 1997).

These findings demonstrate that high level of plasma NO is a dangerous sign of septic shock, and would support the concept that overproduction of NO should be controlled. Inhibition of excessively produced NO may eventually prevent the detrimental hemodynamic effects associated with septic shock (Wolkow, 1998). There has been much recent enthusiasm regarding the possibility of using inhibitors of NOS for the treatment of septic shock (Grover et al., 1999). On the other hand, NO is a physiologically important molecule at low concentrations that is
produced by the constitutive enzyme and that maintains blood vessel vasodilator tone. Complete inhibition of NO production during sepsis is detrimental (Gradel et al., 2001). A more selective iNOS inhibitor, ONO-1714, was found to improve blood pressure without impeding tissue perfusion in animal studies (Mita et al., 2001). Evaluation of specific inhibitors of iNOS represent one potentially fruitful area for further investigation (Chang, 2001). In this study, plasma NO levels were not significantly different between survivors and non-survivors (Table 4). This finding is similar to that of Duke et al. (1997) who reported that the serum concentrations of NO were not associated but positively associated with illness severity but there was no association with mortality. These results are in contradistinction to Shi et al. (1993) who reported that higher plasma NO levels were related to severity of illness, pediatric risk of mortality (PRISM) scores, and occurrence of septic shock. There are very few reports regarding the levels of CO during sepsis in humans. Very recently, Shi et al. (2000b) found that plasma CO levels in newborns with sepsis were higher than in healthy neonates. However, this study included only 7 full-term newborn infants with sepsis. Earlier, an animal study, Shi et al. (1997) demonstrated that in newborn rat endotoxicosis, CO levels in the circulation as well as in the liver, kidney and lung were found to be significantly increased and Mucumure et al. (1998) reported significantly elevated CO-Hb levels during septic shock. Here reported the first evidence that septis preterm infants have similar changes in plasma CO levels as septic full-term infants. An interesting finding is that no differences between plasma CO levels in neonates with early onset and those with late onset sepsis and that the elevation of CO levels were independent of the gram stain of the organism isolated (Table 3). This may imply that both gram-negative and positive organisms are capable of activating HO system and increasing CO production in a fashion similar to activation of iNOS and production of NO. Recent studies have suggested that CO generated by HO-2 might contribute to the regulation of vascular tone under basal condition and that the relatively large amount of CO generated by markedly increased HO-1 activity could contribute to the reduction of vascular tone during sepsis. This action is mediated via activation of guanylate cyclase and the production of cyclic guanosine monophosphate[cGMP] (Morita et al., 1998). It has been hypothesized that both NO and CO release could contribute to the abnormal vasodilatation of the septic state. Thus, both stress-inducible vasodilator systems, iNOS and HO, can contribute to the critical balance of vasoconstrictor and dilator forces regulating microvascular perfusion in septic shock (Shi et al., 2000b). Administration of HO inhibitors, zinc protoporphyrin (ZnP), resulted in an increase in arterial blood pressure in animals (Johnson et al., 1995). ZnP was shown to abrogate the endotoxin-induced hypotension and metabolic derangement (Shi et al., 2000a). On the other hand, Downdar et al. (1997) suggested that HO-dependent CO production might be a hepatic adaptive response to sepsis. Pannen et al. (1998) found that endogenous CO has a protective role in hepatic microcirculatory dysfunction after hemorrhagic shock in rats. Moreover, Otterbein et al. (1995) have indicated that induction of HO-1 might protect against oxidative damage of endotoxemia, and administration of HO inhibitors such as ZnP to decrease HO activity below basal levels could make rats more susceptible to endotoxin-induced death. Thus we believe that the clinical meaning of CO and the use of HO inhibitors in neonatal sepsis worth further investigation. The higher plasma CO levels in nonsurvivors compared to survivors indicate the prognostic value of plasma CO levels (Table 4). This is a unique finding reported for the first time. If such findings is validated, measurement of plasma CO may add to standard clinical evaluation and management: This test may help in the triage of septic newborns to a certain level of care (e.g. SCBU or NICU) and may help targeting certain patients for new interventions such as HO inhibitors. In conclusion, plasma NO and CO levels are elevated in newborn infants with sepsis. This elevation is independent of gestational age, gram stain of the isolated organism, and whether it is early or late onset sepsis. The elevation in CO production is related to increased NO production. Assay of plasma CO and NO may have some clinical value to assess the severity of sepsis and progression to septic shock. The role of NOS and HO inhibitors in the management of neonatal sepsis should be investigated in further trials. Plasma levels of NO and CO can be used to decide which patient should be a candidate for these trials.

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