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Original Article

Serum nitric oxide and pediatric sepsis outcomes

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Abstract

Background Sepsis is the complex pathophysiologic responses of the host against systemic infection. Sepsis can cause severe conditions such as septic shock and multiple organ failure. Although we have a better understanding of the molecular basis of sepsis as well as aggressive therapy, the mortality rate remains high, between 20-80%. Nitric oxide (NO) is one of the mediators associated with cardiovascular failure, apoptosis and organ dysfunction in sepsis.

Objective To evaluate for a possible correlation between NO levels and outcomes in pediatric sepsis.

Methods A prospective cohort study was conducted at the pediatric intensive care unit (PICU) of Prof. Dr. R.D. Kandou General Hospital in Manado, from June to November 2012. Forty children aged one month to five year old, fulfilled the *International Pediatrics Sepsis Consensus Conference 2005* criteria were recruited. Nitrite oxide metabolites (nitrite and nitrate) levels were measured using a calorimetric assay kit (Cayman®, Catalog No.780001) from venous blood specimens collected at admission. All patients received antibiotics empirically within an hour of the diagnosis. Outcomes of patients recorded were survivor or died, and length of stay in PICU.

Results Mann-Whitney U test revealed a significant difference between median serum NO levels in survivors and those who died (18.60 vs. 36.50 μM/L, respectively; P=0.016).

Conclusion Serum NO concentration is higher in those who died than in survivors of pediatric sepsis. Specific NO inhibition may be beneficial in decreasing morbidity and mortality in this condition. **[Paediatr Indones. 2014;54:213-8.]**.

Keywords: *nitric oxide, sepsis, mortality, pediatric*

Sepsis is one of leading causes of admission to pediatric intensive care units (PICUs) in the world with significant morbidity and mortality in patients and an elevated cost for society.[1,2] In United States, sepsis and septic shock are major causes of morbidity and mortality, with an estimated 42,000 cases per year and an associated mortality rate of 10,3% with more than 4,300 cases.[2-4] However, in developing countries, the mortality rate of sepsis in children from PICU is higher than 50%.[5] The incidence of sepsis was reported to be highest in children less than 1 year of age (5.16/1,000), and decreased to 0.2/1,000 in children 10-14 years of ages.[2]

The sepsis syndrome is characterized by alterations in vascular tone and, in the most severe forms of septic shock, the peripheral vasculature is markedly refractory to the vasoconstrictive effects of alpha1-adrenergic receptor agonist agents.[6] The prevailing theory is that sepsis represents an uncontrolled-inflammatory response with loss of the normal homeostatic balance between systemic inflammation and the anti-inflammatory re-

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sponse.[7,8] Cytokines and mediators that are released may cause collapse of circulation, pan-endothelial vascular trauma, and increased of vascular permeability,[9] leading to septic shock and multiple organ failure.[7] Recently, it has been proposed that impaired cellular oxygen utilization, termed cytopathic hypoxia, rather than inadequate oxygen delivery, may play an important role in the development of multiple organ dysfunction syndrome (MODS).[7,10] It may lead to suggest that, in early sepsis is associated with microcirculatory disturbances, in late course of disease the problem seems to be related to mitochondrial dysfunction. Nitric oxide (NO) is known to be mediator in microcirculation and cytopathic hipoxia.[7,11,12]

Nitric oxide, a potent endogenous vasodilator produced by various mammalian cells, has been postulated to play a role in the hemodynamic derangements of sepsis syndrome.[6,13,14] The inducible isoform of NO is responsible for increased NO output after induction by lipopolysaccharide and various pro-inflammatory cytokines [i.e., tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interferon gamma (IFN-γ)],[14-18] which are of pathogenic importance during the sepsis syndrome.[6,19] Although NO is known to have a role in sepsis, its action has not been elucidated by recent studies. Some studies reported a positive correlation between NO metabolites and sepsis,[20] septic shock,[6,21] organ failure,[21] severity of inflammation,[6] and mortality.[22] However, others reported no correlation between NO metabolites and sepsis,[23] severity of sepsis,[20] or mortality.[20,24] We hypothesized NO concentration to be higher in sepsis those who died than survivors. This study was undertaken to evaluate a possible correlation between NO levels and outcomes in pediatric sepsis.

Methods

A prospective cohort study was conducted at the PICU, Prof. Dr. R.D. Kandou General Hospital in Manado, from June to November 2012. The study protocol was approved by the Ethics Committee of the hospital. All patients' parents provided written informed consent following a full explanation of the study and reading of the information sheet. Infants and children, aged one month to five years, who fulfilled the *International Pediatrics Sepsis Conference Consensus criteria (2005)*[3] were consecutively recruited. We excluded infants and children with diagnoses of severe dehydration, severe malnutrition, obesity, immunodeficiency disorders, trauma, scald burns, acute pancreatitis, and patients with a history of liver and kidney diseases in the 6 months prior to the study.

At time of admission, subjects' blood specimens were drawn in order to evaluate complete blood counts, urea and creatinine levels, blood cultures, and NO metabolites (nitrite and nitrate) levels. Clinical information was also recorded (age, gender, nutritional status and diagnosis) at that time. All subjects were treated with early goal-directed therapy. Combination antibiotics comprising of the 3rd generation cephalosphorines, such as ceftriaxone or cefotaxime, and gentamicin, were given empirically within an hour of the diagnosis. The antibiotic regimen was appropriately changed after blood culture and sensitivity test results became available. During treatment, all subjects were followed-up daily until patients either recovered enough to be moved to the pediatric ward or died. Patients were considered to have recovered from sepsis if they were free from a systemic inflammation response for two days. Outcomes of patients were recorded (survivor or died and length of stay in PICU). None of the children were treated with inhaled NO or nitrovasodilators.

Venous blood specimens were collected at admission and immediately centrifuged at 1,500 rpm for 15 minutes. Serum samples were then removed and stored at –30 degrees Celsius until they were used for NO metabolite assays. Nitrate and nitrite measurements were made using the nitrate/nitrite colorimetric assay kit (*Cayman*®, catalog No. 780001). Two hundred μL of assay buffer and up to 80 μL of sample dilutions (diluted sample 1:2 with distilled water) were added to the wells. Ten μL of enzyme cofactor mixture, followed by 10 μL nitrate reductase mixture were added to each well. Plates were then covered and incubated for 1 hour at room temperature. We then added 50 μL of *Griess Reagent R1* followed immediately by 50 μL of *Griess Reagent R2* to the wells. Plates were incubated for 10 minutes at room temperatures to develop the color. Absorbance was read at 550 nm using a plate reader. Serum NO metabolites were plotted on the nitrate standard curve. The detection limit for this assay was 2.5 μM. Nitric oxide metabolites are stable and most eliminated by

the kidney. As such, indexing of NO metabolites to serum creatinine concentrations is considered to be representative of endogenous NO.

Statistical analysis consisted of descriptive and comparative analyses. Normality of data was analyzed by *Kolmogrov-Smirnov* test. Descriptive analyses were used in order to analyze the characteristics, clinical and laboratory findings, diagnoses as well as antibiotic use, and were reported in a distribution table. Parametric data was expressed as mean, standard deviation (SD), while non-parametric data was expressed as median, and minimum-maximum values.

We used the *Mann-Whitney* rank sum test to compared the ratio of serum NO metabolites to serum creatinine concentrations in pediatric sepsis between survivors and those who died. Data were considered to be statistically significant for $P < 0.05$. All analyses were performed using *SPSS version 19.0*.

Results

The final PICU dataset was comprised of 40 children with a median age of 8.5 (range 2 - 70) months, of whom 22 were boys. Thirty-three children were categorized in the under 24 months' old grouping. Subjects' nutritional status were as follows: 28 well-nourished, 11 undernourished, and 1 overweight. Two children had culture-positive sepsis, *Streptococcus pneumoniae* and *Enterobacter aerogenes*, respectively. The sources of sepsis included bronchopneumonia (33/40), meningoencephalitis (11/40) and acute diarrhea (11/40). Thirty-one children were treated with a combination of cefotaxime and gentamycin. During treatment, 31 children survived and were allowed to move to pediatrics ward, while 9 children died. The median length of PICU stay was 5 (range 1-15) days (**Table 1**).

Table 1. Baseline characteristics of subjects

Characteristics	Overall n=40	Survived n=31	Died n=9
Gender			
Male, n	22	15	7
Median age (range), months	8.5 (2 - 70)**	10.11 (7.2)*	8 (2 – 26)**
1 to < 24 months, n	35	25	9
24 to < 72 months, n	5	5	0
Nutritional status			
Overweight, n	1	1	0
Well-nourished, n	28	22	6
Undernourished, n	11	8	3
Sources of sepsis, n			
Bronchopneumonia, n	33	26	7
Meningoencephalitis, n	11	7	4
Acute diarrhea, n	11	7	4
Median creatinine (range), mg/dL	0.65 (0.2 – 2.5)**	0.7 (0.3 – 2.5)**	0.61 (0.28)*
Blood cultures, n			
S.pneumoniae, n	1	1	0
E. aerogenes, n	1	1	0
Antibiotics			
Cefotaxime+ gentamycin, n	31	23	8
Ceftriaxone+ gentamycin, n	9	8	1
Median length of PICU stay (range), days	5 (1 – 15)**	5 (2 – 15)**	1 (1- 12)**

Note: * parametric data: mean (SD);** non-parametric data: median (range)

Table 2. Ratio of serum NO metabolites to serum creatinine concentration in survivors and those who died with pediatric sepsis.

Variable	Survived	Died	P value
Median ratio of serum NO to serum creatinine (range), μM/L	18.60 (4.20-59.30)	36.50 (12.90-93.00)	0.016

Mann-Whitney U test

The Mann-Whitney rank sum test analysis revealed that the ratio of serum NO metabolites to serum creatinine concentrations in those who died was higher than in survivors within pediatric sepsis (**Table 2**).

Discussion

Nitric oxide metabolites concentrations are influenced by their synthesis and elimination rates. As NO metabolites are excreted predominantly via the kidneys, differences in serum NO concentrations may occur as a result of renal dysfunction.[6,25,26] Renal dysfunction in sicker children may be a consequence of increased disease severity.[6] We attempted to correct any confounding effects of renal dysfunction in our patients by indexing serum concentrations of NO metabolites to serum creatinine concentrations to be a better proxy for the level of endogenous NO.

Nitric oxide has an important role in microvascular regulation.[27] Generated in physiologic amounts, NO has clearly been shown to have a beneficial role in organ perfusion by acting as a regulator of vascular tone and blood flow distribution. In basal amounts, NO is also considered to be important in immune-mediated disease by protecting various tissue against injury by endotoxin-induced inflammation. However, inducible nitric oxide synthetase is generated in response to inflammatory stimuli, and is able to produce vastly increased amounts of NO. This NO relaxes vascular tone, causes vascular hyporeactivity against vasopressor agents, depresses myocardium, and can lead to multiple organ dysfunction and death.[25-29] Nitric oxide can also react with free oxygen and hydroxy radicals to form toxic peroxynitrite radicals which can induce damage to endothelial cells.[15]

In our study, we found that the ratio of serum NO metabolites to serum creatinine concentrations in those who died was significantly higher than in survivors. This finding was consistent with the role of endogenous NO overproduction in sepsis pathogenesis. Our findings were also in agreement with other study which reported that NO concentrations in pediatric sepsis were higher in non-survivors than in survivors and could be used as predictor of mortality.[30] In addition, a study showed that NO concentrations in non-survivors were higher than in survivors. The difference was not statistically significant on the first day of examination, but it was significant on the 2nd and 3rd days of examination.[21] A study on adults also reported that serial NO concentrations were higher in sepsis non-survivors than in survivors (P=0.005) and was related to the severity of disease.[27] However, other studies reported no significant differences in NO concentration between survivors and non-survivors.[25,29] All these other studies had different subject characteristics from our study, though most studies did not index the NO metabolites to serum creatinine concentrations in order to correct for renal dysfunction.

Interestingly, all those who died in this study had septic shock during the course of disease (data not shown). Mortality due to cardiovascular failure caused by septic shock may occur in the initial stages of sepsis. Nitric oxide is also known as a potent vasodilator and can decrease cardiac contractility.[21] Our findings are consistent with others reports, such as Galley *et al.*[31] who reported that NO concentration was elevated in non-survivors due to shock septic and decreased over the study period only in patient who survived, and other studies[6,28] which reported that the ratio of serum NO metabolites to serum creatinine concentration was higher in the hypotensive sepsis group than in the non-hypotensive group (P<0.05). Nitric oxide concentrations also increased in sepsis patients who needed vasopressor agents (dopamine, dobutamine, or nor-ephinephrine). They concluded that NO was associated with cardiovascular failure.[6,21,28,32] An animal study reported that NO and peroxynitrite were involved in cytopathic hypoxia. Microvascular dysfunction and cytopathic hypoxia are reported to be good predictors for mortality.[33]

Baines *et al.* were also in agreement with our findings in that they found a relationship between NO and mortality risk in children with meningococcemia by calculating PRISM scores (Spearman's rho=0.72; P=0.0003).[26] Mitaka *et al.* reported that NO concentrations were related to APACHE II and SOFA scores in adults,[25] while Groeneveld *et al.* showed relationships between NO concentrations and TNF-α (Spearman's rho=0.69; P< 0.0001) or IL-6 (Spearman's rho=0.44; P<0.0001) in sepsis. Nitric oxide is reportedly increased when excess of cytokine pro-inflammation is produced during the first phase of sepsis but tends to decrease when cytokine anti-inflammation is produced during the secondary phase.[34] Doughty *et al.* reported that

children with elevated IL-6 concentration had higher NO concentrations than those with undetected IL-6 concentration in sepsis, but both were still higher than controls ($P<0.005$).[35] Interleukin-6 concentration has been reported to be associated with mortality in pediatric sepsis.[36]

Exogenous NO influences the endogenous NO levels.[37] We did not calculate the effect of oral intake before admission to PICU since our subjects were categorized as critically ill and did not receive exogenous NO during treatment (dietary nitrite, inhaled NO or nitrovasodilators), Our findings were consistent with another study which reported that oral intake contributed little NO metabolites to critically ill patients admitted to the hospital.[37]

In addition to nitrite and nitrate as NO metabolites, NO also reacts with reactive oxygen species (ROS) to form reactive nitroso species (RNS), such as nitrosotyrosine (NT) and nitrosothiol.[38] A study reported that some septic shock patients had NO concentrations within normal limits, but higher NT concentrations.[38] Wong *et al.* also reported that variable NO concentrations in septic children occurred because the biological effects of NO are short lived and are likely to occur at local tissue and intracellular levels.[6] All these possibilities may explain why some of our septic patients who died had lower NO concentrations. Because of the NO concentration variability in our patients, we should not use NO concentration as a single predictor of mortality in pediatric septic patients.

Limitations of our study are the single blood examination of NO, lack of measurements of other NO bioreactives, such as NT and nitrosothiol, and no calculation of the synthesis effect of NO. Further study is needed with serial examinations of NO metabolites (nitrate, nitrite, NT, and nitrosothiol) in order to more accurately evaluate the role of NO as a predictor of mortality. In conclusion, serum NO level is higher in those who died than in survivors pediatric sepsis. Specific NO inhibition may be beneficial in decreasing morbidity and mortality in this condition.

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