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Original Article

Analysis of serum vitamin C and nitric oxide in children with septic shock

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Abstract

Background Imbalance of oxidants and antioxidants contributes to the sepsis process. Nitric oxide (NO) is an oxidant produced abundantly during sepsis and plays key role in the pathogenesis of hypotension in septic shock. Vitamin C functions as a potent antioxidant to scavenge free radicals, reduce endothelial permeability, cellular apoptosis, and endogenous vasopressor synthesis cofactor. Vitamin C prevents excessive production of NO by suppressing inducible nitric oxide synthase (iNOS) expression.

Objective To analyze for a possible correlation between serum vitamin C and NO levels in children with septic shock.

Methods This cross-sectional study was conducted in Dr. Kariadi Hospital, Semarang, Central Java, Indonesia. A total of 40 children with septic shock aged 1 month - 18 years were consecutively recruited. Serum vitamin C and serum NO levels were measured using colorimetric assay kits.

Results Of 40 children with septic shock, 21 (52.5%) were male, 21 (52.5%) were undernourished, 25 (62.5%) were on mechanical ventilators, and 10 (25%) required more than one vasoactive agent. Blood gas analysis revealed acidosis in 31 (77.5%) children. Subjects' median age was 20 (2-214) months. Mean serum vitamin C level was 7.35 (SD 2.723) μg/mL and mean serum NO was 47.25 (SD 19.278) μmol/L. There was no significant correlation between serum vitamin C levels and serum NO in children with septic shock (r=0.056; P=0.732).

Conclusion Serum vitamin C levels and serum NO has no correlation in children with septic shock. **[Paediatr Indones. 2023;63:425-32; DOI: https://doi.org/10.14238/pi63.4.2023.425-32].**

Keywords: *septic shock; vitamin C; nitric oxide*

Severe sepsis and septic shock are among the leading causes of mortality in children worldwide, with an estimated incidence of 1.2 million sepsis cases and reported mortality of up to 20% for severe sepsis.[1] There is growing evidence that an imbalance between oxidants and antioxidants contributes to sepsis pathogenesis, with consequences of impaired vascular permeability, decreased cardiac function, and altered mitochondrial respiration.[2]

Nitric oxide (NO) is the most studied oxidant. It is essential in maintaining normal cardiovascular and immune responses to infection. There are three main nitric oxide synthases (NOS) that regulate the synthesis of NO in various tissues: neuronal NOS (nNOS) found in the nervous and enteric systems, as well as vascular smooth muscle; endothelial NOS (eNOS) found in endothelium and cardiac myocytes; and inducible NOS (iNOS) produced in response to inflammation during infections and sepsis. Excessive NO expression in the endothelium by iNOS has been reported as being key to hypotension in septic shock.

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Meanwhile, non-enzymatic production of NO is from nitrite reduction occurring under acidosis conditions and following an ischemic insult in tissues.[3,4]

Vitamin C is among the well-known antioxidants studied as adjunctive therapy for its protective effects against oxidative stress-mediated cell damage and organ dysfunction in adult sepsis and septic shock. In critically ill patients, plasma vitamin C concentrations can be low due to inadequate intake and its use in chronic or acute conditions in which there is increased oxidative stress. In response to infection, vitamin C accumulates in phagocytic cells such as neutrophils to kill microbes. Moreover, the adrenal glands secrete vitamin C, which triggers the production of cortisol as part of the body's stress response. Vitamin C is lost in the cell degradation process. Vitamin C is also used in the synthesis of norepinephrine, peptide hormone, and cortisol in critically ill conditions.[5,6]

During sepsis, inflammation and endothelial damage occur due to infection that is initiated by exposure to endotoxins which bind to leukocyte and endothelial cell surface receptors. Infection during sepsis triggers the release of various inflammatory cytokines, particularly TNF-α and IL-1 that activate iNOS expression in endothelial cells and macrophages resulting in excessive NO production. Expression of these two inflammatory cytokines increases with age, with peak levels occurring at age of 13 years and lower levels found during infancy.[7,8] Exaggerated NO production causes vasodilation and vascular hyporeactivity, resulting in severe hypotension in septic shock. Vitamin C acts as an antioxidant in sepsis and septic shock that suppresses NO synthesis directly by inhibiting iNOS mRNA expression, thereby, reducing excessive NO production. Vitamin C also inhibits the adhesion of phagocyte cells to the endothelium. Phagocytic cells trigger the release of various inflammatory cytokines resulting in myocardial depression and endothelial injury. The antioxidant effect of vitamin C results in decreased endothelial permeability, increased microvascular and macrovascular function, and alleviates cellular apoptosis that produces various free radical products including NO.[4-6,9] Various trials of vitamin C administration to adults with sepsis and septic shock have yielded favorable outcomes, such as reduced organ failure, vasopressor requirement, need for mechanical ventilation, as well as improved survival. However, most of these trials involved a combination of vitamin C and other antioxidants that confounded the effect of vitamin C.[6,10] Hence, we aimed to analyze for a correlation between serum vitamin C as an inhibitor of iNOS and serum NO in children with septic shock.

Methods

This cross-sectional study was conducted from November 2020 - January 2021 at Dr. Kariadi Hospital, Semarang, Central Java, with approval by the Ethics Committee. A total of 40 children diagnosed with septic shock aged 1 month - 18 years were consecutively recruited. Subjects were divided into three age groups, consisting of Group 1 (1-12 months), Group 2 (>1 year-12 years), and Group 3 (>12 years-18 years).

Sepsis was defined according to the *Surviving Sepsis Campaign: International Guidelines for Management of Severe Sepsis and Septic Shock 2012*, as the presence of probable or documented infection with systemic manifestations of infection and septic shock as sepsis-induced hypotension persisting despite adequate fluid resuscitation.[11] We excluded children with malignant disease, immunocompromised conditions (HIV-AIDS, receiving long-term steroid treatment or immunosuppressants, including history of splenectomy), chronic kidney disease, diabetes, hypertension, severe malnourishment or obesity, and those who had undergone surgical procedures.

Demographic and clinical characteristics were collected from subjects' medical records, including gender, age, anthropometric data, underlying disease, source of infection, as well as blood and other specimen cultures. We documented the systemic manifestations of infections, nutritional status, as well as use of invasive mechanical ventilation and vasoactive agents. Blood specimens for blood gas analysis, serum vitamin C, and serum NO levels examinations were collected at the same time. Serum vitamin C and serum NO levels were measured using a colorimetric assay kit (*Elabscience®*).

Descriptive and comparative analyses were performed. Data normality was analyzed by Shapiro-Wilk test. Descriptive analyses were used to analyze characteristics, clinical and laboratory findings,

underlying disease and source of infections, use of invasive mechanical ventilation, and vasoactive agents. Parametric data were expressed as mean and standard deviation (SD), while non-parametric data were expressed as median and minimum-maximum values. We used T-test analysis and one-way ANOVA to compare normally distributed data and Mann-Whitney test to compare non-normally distributed data. Pearson's test was employed to analyze serum vitamin C and serum NO levels. Results with $P<0.05$ were considered to be statistically significant. Analyses were performed using *SPSS version 25.0* (*IBM®*) software.

Results

A total of 40 children were included (21 male, 19 female), with a median age of 20 (range 2-214) months. There were 21 (52.5%) undernourished children. The most common systemic sepsis manifestations were tachycardia, hyperthermia, and tachypnea in 62.5% of subjects. Most subjects used a mechanical ventilator (MV) (25 subjects; 62.5%). Ten subjects (25%) required > 1 vasoactive drug, and most subjects (31; 77.5%) were in acidosis conditions revealed from the blood gas analysis. Source of infections mostly originated from the respiratory tract (21; 52.5%), with sputum culture results mostly of Gram-negative bacteria (11/21), consisting of 8/11 *Pseudomonas aeruginosa* and 3/11 ESBL-producing *Klebsiella pneumonia*. Subjects' baseline characteristics are shown in **Table 1**.

Mean serum vitamin C level was 7.35 (SD 2.723) μg/mL and mean serum NO level was 47.25 (SD 19.278) μmol/L. We found no significant differences in serum vitamin C ($P=0.486$) and serum NO levels ($P=0.282$) between male and female subjects. Nor were there significant differences of serum vitamin C ($P=0.851$) and serum NO levels ($P=0.726$) between undernourished and well-nourished subjects. There was no significant difference in serum vitamin C levels among age groups ($P=0.200$) (**Table 2**). However, we found a significant difference of mean serum NO levels among age groups ($P=0.016$) (**Table 2**). Therefore, we performed a post-hoc Tukey analysis, which revealed a significant difference of serum NO levels between children in age group 1 (1-12 months) and group 3 (>12 years-18 years) that exhibited highest serum NO levels ($P=0.013$) (**Table 3**).

There is no difference in vitamin C nor NO levels between subjects using MV compared to subjects without MV (**Table 4**). Moreover, there was also no difference in vitamin C nor NO levels between subjects using 1 or 2 vasoactive drugs (**Table 5**). Subjects who were in acidotic conditions had significant higher serum NO levels (**Tabel 6**).

There was no correlation between serum vitamin C levels and serum NO levels in our subjects ($r=0.056$; $P=0.732$). Furthermore, Pearson's analysis of each age group revealed no correlations of serum vitamin C levels and serum NO levels (**Table 7**).

Discussion

In our study, there were slightly more male subjects with septic shock (52.5%) than females. Other studies also reported more male children with septic shock.[12,13] Several theories explain differences in

Table 1. Characteristics of subjects

Characteristics	(N=40)
Gender, n (%)	
Male	21 (52.5)
Female	19 (47.5)
Age, n (%)	
1-12 months	15 (37.5)
>1 year - 12 years	17 (42.5)
>12 years - 18 years	8 (20)
Median (range) months	20 (2-214)
Nutritional status, n (%)	
Undernourished	21 (52.5)
Well nourished	19 (47.5)
Mechanical ventilation, n (%)	
Yes	25 (62.5)
No	15 (37.5)
Use of vasoactive agents, n (%)	
1 drug	30 (75)
>1 drugs	10 (25)
Blood gas analysis, n (%)	
Acidosis	31 (77.5)
Non-acidosis	9 (22.5)
Source of infection, n (%)	
Respiratory	21 (52.5)
Cardiovascular	2 (5)
Gastrointestinal	7 (17.5)
Central nervous system	3 (7.5)
Genitourinary	6 (15)
Musculoskeletal	1 (2.5)

Table 2. Mean serum vitamin C and NO levels according to age group (N=40)

Age groups	Mean (SD), µg/mL	P value
Vitamin C level		
1-12 months	6.80 (2.242)	
>1 year - 12 years	7.12 (3.257)	0.200
>12 years - 18 years	8.88 (1.885)	
NO level		
1-12 months	40.53 (13.637)	
>1 year - 12 years	45.41 (16.043)	0.016
>12 years - 18 years	63.75 (19.278)	

Table 3. Post-hoc Tukey analysis of mean serum NO levels among age groups

Age groups	Mean difference, µmol/L	P value
1-12 months and >1 year - 12 years	4.878	0.718
1-12 months and >12 years - 18 years	18.338	0.013
>1 year - 12 years and >12 years - 18 years	23.217	0.053

Table 4. Mean serum vitamin C and serum NO levels according to mechanical ventilator

Variables	n	Mean serum vitamin C (SD), µg/mL	Mean serum NO(SD), µmol/L	P value
On MV	25	7.04 (2.685)	49.28 (17.048)	0.359
No MV	15	7.87 (2.800)	43.87 (22.747)	0.397

MV=mechanical ventilator

Table 5. Mean serum vitamin C and serum NO levels according to vasoactive drugs requirement

Variables	n	Mean serum vitamin C (SD), µg/mL	Mean serum NO(SD), µmol/L	P value
1 vasoactive drug	30	7.43 (2.596)	45.13 (20.542)	0.742
2 vasoactive drugs	10	7.10 (3.213)	53.60 (13.818)	0.234

MV=mechanical ventilator

Table 6. Mean serum NO levels according to blood gas analysis

Variables	n	Mean serum NO (SD), µmol/L	P value
Acidosis	31	49.74 (19.777)	0.035
No acidosis	9	38.67 (15.427)	

Table 7. Analysis of serum vitamin C and serum NO levels among age groups

Age groups	r	P
1-12 months	-0.225	0.420
>1 year - 12 years	0.190	0.464
>12 years - 18 years	-0.610	0.108

immune response, including higher natural killer (NK) cell count and a pro-inflammatory response in boys compared to girls.[14] The major source of infections in our study were respiratory (52.5%), particularly bronchopneumonia. This finding was similar to a study that found the most common site of infection in children with septic shock was the respiratory tract, whereas another study reported the site to be the central nervous system and gastrointestinal infection.[15-17]

The main pathogenic etiologies identified from subjects' sputum culture were Gram-negative bacteria, with Pseudomonas aeruginosa in 8/11 subjects and ESBL-producing Klebsiella pneumonia in 3/11 subjects. Similarly, previous studies reported that these two infectious agents were the most common causes of sepsis and septic shock in children.[18,19] Various risk factors associated with ESBL bacterial infection in septic children include a history of previous hospitalization, prolonged hospital stay,

repeated use of antibiotics, and use of indwelling devices.[20]

In our study, mean serum vitamin C levels in children with septic shock was 7.35 (SD 2.723) μg/mL, whereas the normal vitamin C level in healthy children is ≥ 50 μmol/L, which is equivalent to 8.8 μg/mL.[21] A study revealed that the mean serum vitamin C levels in septic children was 11.40 μmol/L and lower than the control group (13.80 μmol/L), which was probably due to active phagocytosis in sepsis,[22] while another study reported that mean serum vitamin C levels in septic children was 23.1 μmol/L (equivalent to 5.3 μg/mL), with more than 50% of patients having a very low vitamin C levels of <10.7 μmol/L (equivalent to 2.48 μg/mL).[23] Decreased vitamin C level in circulation during sepsis is due to its accumulation in phagocytic cells, such as neutrophils that kill bacteria, its loss in the cell degradation process, and increased use in the synthesis of endogenous vasopressors, peptide hormone, and cortisol as a body's response to physical stress during severe infections.[5,6]

During sepsis, NO is produced by activated macrophages, neutrophils, and lymphocytes. Exaggerated NO production in sepsis leads to severe hypotension and shock.[3] The mean serum NO level of children with septic shock in our study was 47.25 (SD 19.278) μmol/L. A study noted a mean serum NO level in children with septic shock who died of 33.2 (SD 18.6) μmol/L, while those who survived had lower mean NO level of 13.8 (SD 4.6) μmol/L. The cut-off serum NO level used to predict mortality in children with septic shock was 16.15 μmol/L.[24] A study in Turkey measured normal serum NO levels in healthy children aged 1-12 months [28.72 (SD 1.51) μmol/L], 1-4 years [26.03 (SD 1.42) μmol/L], 4-7 years [19.78 (SD 1.56) μmol/L], 7-11 years [20.05 (SD 1.65) μmol/L], and 11-16 years [22.29 (SD 1.60) μmol/L]. Our results were consistent with those of other studies which reported that mean serum NO levels were increased in children with sepsis and septic shock who died. They also noted that serum NO levels could be used as a predictor of outcome in children with septic shock.[15,24,25]

We found that mean serum vitamin C levels in subjects aged >12 years - 18 years was higher compared to subjects aged <12 years, but this difference was not statistically significant (P=0.200). This result was possibly due to dietary intake or vitamin C supplementation, which is likely to be adequate in older children, but was a limitation of our study, as we did not assess previous vitamin C intake of our subjects. In healthy children, vitamin C levels in the circulation tend to decrease with age due to increasing body mass and volume distribution.[26]

We observed a significant difference in mean serum NO levels among age groups, with the highest levels found in subjects aged > 12 - 18 years and the lowest levels in subjects aged 1- 12 months. Plasma NO levels in healthy children decreased with age, with the highest levels found in neonates.[27] The increase of serum NO levels in older children can be caused by the maturity of the child's immune system. Various inflammatory cytokines are released in septic shock, which is triggered by infection, particularly TNF-α and IL-1 which will subsequently activate iNOS in endothelial cells and macrophages, resulting in excessive NO synthesis. Expression of these two inflammatory cytokines increases with age, with peak levels found in children aged 13 years and lower levels detected in infancy.[8,28]

Our study showed no difference of vitamin C and NO levels between subjects with MV compared subjects without MV, neither between subjects given 1 compared 2 vasoactive drugs. Other studies found that respiratory dysfunction in children with septic shock is associated with cardiovascular dysfunction due to increased tissue metabolic demand and tissue hypoxia, as indicated by lactic acidemia and progressive multi-organ dysfunction that requires early invasive mechanical ventilation to reduce the work of breathing, provide adequate oxygenation, and improve tissue perfusion.[29,30] Moreover, a study reported that children with sepsis and three or more organ system failures had higher NO levels; the elevated NO level was associated with multiple organ failure in septic children. NO triggers vasodilation and myocardial dysfunction, and induces damage at the cellular level, and is involved in the pathogenesis of organ failure in sepsis.[31] In addition, another studies reported that elevated serum NO levels in children with sepsis and septic shock are predictors of mortality and poor outcome associated with the severity of organ dysfunction.[15,24] Adult septic patients who had high sequential organ failure assessment (SOFA) scores, had subnormal levels of plasma vitamin C.

They reported decreased SOFA scores after receiving parenteral vitamin C intervention.[32]

Blood gas analysis was performed in all subjects to assess acid-base conditions that occurred in septic shock. Most subjects (77.5%) were in an acidosis condition. Serum NO level was higher in subjects with acidosis compared to subjects who were not in acidotic condition. Metabolic acidosis is frequently observed in sepsis and associated with poor outcomes. The expression of iNOS can also be increased in acidosis, which exacerbates vasodilation and shock in sepsis. Acidosis might trigger the synthesis and release of various inflammatory cytokines as well, particularly TNF and IL-6.[33] TNF is the cytokine that induces iNOS expression and leads to exaggerated NO synthesis by endothelium in septic shock. However, the acidosis condition in most subjects might also have been due to ongoing shock that leads to cellular hypoxia and lactate accumulation.[28]

Several studies conducted on adult septic shock reported decreased NO levels after receiving vitamin C supplementation.[34] A study documented lower mortality rates in adult patients with severe sepsis receiving high-dose parenteral vitamin C compared with the placebo group. However, 66% of subjects in the study who received vitamin C also received hydrocortisone,[32] whereas the combination of vitamin C and hydrocortisone is synergistic in catecholamine production and increases sensitivity to vasopressors.[35] Therefore, the effect of vitamin C as a single agent to improve the outcome of adult patients with severe sepsis still needs further investigation.

We found no correlation between serum vitamin C and serum NO levels in children with septic shock. This finding may have been due to the production of NO through other pathways that did not involve or was not inhibited by vitamin C, which acts as an antioxidant in children with septic shock. The NO production involves enzymatic and non-enzymatic pathways. The synthesis of enzymatic NO is catalyzed by NO synthase (NOS). As an antioxidant, vitamin C inhibits the expression of inducible nitric oxide (iNOS) mRNA, thereby preventing excessive NO synthesis.[6] On the other hand, NO is also produced via non-enzymatic pathways reduced from nitrite, particularly in acidosis conditions where NO formation is not altered by NOS inhibitors. In ischemic conditions with acidosis, nitrite-mediated NO production is an alternative route in which NOS-catalyzed NO production is impaired.[4,36]

This study had limitations, as there was no control group to compare serum vitamin C and NO levels in septic children to those without septic shock children. Also, we did not measure serum lactate levels that might have been useful for assessing acidic conditions in tissue during septic shock.

In conclusion, there is no correlation between serum vitamin C and serum NO levels in children with septic shock. Other factors that might modulate NO production during septic shock need further investigation.

Conflict of interest

None declared.

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