

Serum Nitric Oxide in Children with Dengue Infection

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SUMMARY One hundred and ten patients (M/F=67/43) from King Chulalongkorn Memorial Hospital and the provincial hospitals of Uttaradit, Ayudhaya, and Sakonnakorn, who were clinically diagnosed with dengue infection and serologically confirmed by ELISA anti-Dengue IgM and IgG were recruited. Their serum NO level was measured using commercially available assay kits to investigate its correlation with the severity of the dengue infection: dengue fever (DF), DHF I/II, and DHF III/IV or dengue shock syndrome (DSS). Serum NO levels were also measured in 38 healthy controls (M/F=19/19). Serum NO levels in dengue patients were lower than those of the controls (control = 168.18 ± 24.10 $\mu\text{mol/l}$, DF = 124.94 ± 36.79 $\mu\text{mol/l}$, DHF I/II = 99.69 ± 33.42 $\mu\text{mol/l}$, and DHF III/IV = 120.63 ± 46.26 $\mu\text{mol/l}$; $p < 0.05$). Serum NO levels in patients with DHF I/II were significantly lower than in those with DHF III/IV. These preliminary data revealed that levels of serum NO in dengue patients were significantly lower than those of normal controls. Patients with DSS had higher NO levels than those with DHF I/II. The decreased NO in dengue patients could be due to endothelial damage rendering the endothelium incapable of producing NO. Endothelial function seems to play a role in the pathogenesis of dengue infection. Further studies are required to see whether serum NO levels could play a role in the course of the disease and could help predict the severity of dengue infection.

The World Health Organization (WHO) estimates 50-100 million illnesses from dengue infection annually, including 250,000-500,000 cases of dengue hemorrhagic fever (DHF) and 24,000 deaths. In addition, more than two-fifths of the world's population (2.5 billion people) live in dengue endemic or high-risk areas.¹ DHF has been a major cause of severe illness and death among children in Southeast Asia. The clinical picture is characterized by high continuous fever, hemorrhagic manifestations (positive tourniquet test, petechiae, epistaxis, hematemesis and melena), and hypovolemic shock in cases of dengue shock syndrome (DSS).

Increased capillary permeability and damage of endothelial cells as a response to dengue viral in-

fection can manifest as any combination of hemocoagulation, pleural effusion or ascites.² In order to maintain the homeostasis of the body and respond to the invasion by countless foreign organisms, blood vessels secrete a variety of cytokines, such as nitric oxide (NO), which regulates the contraction of both smooth muscle cells and vascular tissue. NO can lead to vasodilatation and reduction of systemic vascular resistance, which could lead to hypotension, shock, and inevitably death if not corrected.

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NO levels are elevated in many infectious diseases including dengue infection. When the dengue virus was co-cultured with human Kupffer or spleen cells, increased production of NO was detected.^{3,4} Although NO levels in DHF patients were similar to those of healthy controls, elevated levels of NO were found in dengue fever (DF) patients.⁵ However, no previous reports have as yet described the levels of NO in patients with DHF III and IV, conclusively known as DSS. This study attempted to correlate NO levels in three groups with different severity ranging from DF, DHF I/II, to DSS, and to compare the results with a control group.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. The subjects who participated in this study were informed of the objective of the study and a written consent was obtained from them before their recruitment.

Population

Patients who were clinically diagnosed with DF or DHF during the period of January 2003 to January 2004 were recruited from King Chulalongkorn Memorial Hospital and three other provincial hospitals (Ayudhaya, Sakonnakorn, and Uttaradit). Only sera, which were serologically confirmed by anti-Dengue IgM and IgG ELISA (NovaTec Immunodiagnostica GmbH[®], Technologie & Waldpark, Dietzenbach, Germany) were included in this study. Healthy age-matched controls were obtained from previous dengue patients who came in for a six months follow-up and from healthy children who were on their routine vaccination visit. The mean ages \pm SD of patients with DF ($n = 37$), DHF I ($n = 19$), DHF II ($n = 17$), DHF III ($n = 28$), DHF IV ($n = 9$), and healthy controls were 9.97 ± 2.87 , 10.89 ± 2.37 , 11.07 ± 2.89 , 9.14 ± 3.67 , 9.27 ± 4.12 , and 10.71 ± 2.15 years, respectively. Clotted blood samples were obtained on the day of admission during the febrile stage of the disease. Sera were separated and kept at -70°C until tested. The dengue patients were classified into different groups based on the clinical severity: DF, DHF grade I, II, III, and IV according to WHO criteria.¹

Nitric oxide measurement

The concentration of NO is indirectly measured by determining both nitrate and nitrite levels in the sample. Total serum NO was measured by utilizing a nitric oxide ($\text{NO}_2^-/\text{NO}_3^-$) assay kit (Saliva Sac[®], Pacific Biometrics Inc., Seattle, WA, USA), following the manufacturer's instructions. This assay determines nitric oxide based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction. The Griess reaction is based on the two-step diazotization reaction in which acidified NO_2^- produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to *N*-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 nm.

Data analysis

Statistical analysis was performed with SPSS software (version 11.5). Data were expressed as mean \pm standard deviation. Differences between groups were analyzed by one-way ANOVA and *t*-test. The statistical test used was the Spearman non-parametric 2 tailed correlation test. *P*-values of less than 0.05 were considered statistically significant.

RESULTS

Demographic and laboratory data of healthy controls and patients with dengue infection recruited in this study are shown in Table 1. Serum NO levels of the control group, patients with DF, DHF I, II, III, and IV were 168.18 ± 24.10 , 124.94 ± 36.79 , 102.01 ± 26.89 , 97.11 ± 40.20 , 122.39 ± 50.06 , and 115.15 ± 33.58 $\mu\text{mol/l}$, respectively. The analysis showed that serum NO levels of the control group were significantly higher than those of the groups with dengue infection (Fig. 1). DF and DSS patients had comparable serum NO levels which were significantly higher than those in patients with DHF I/II (Fig. 1).

DISCUSSION

Nitric oxide, a gaseous molecule, is a product accompanying L-citrulline from the conversion of L-arginine and oxygen by P450-like enzymes called NO synthases (NOS).⁶ Currently, NOS are classified into

Table 1 Demographic and laboratory data of healthy controls and patients with dengue infection

	Control (n = 38)	DF (n = 37)	DHF I (n = 19)	DHF II (n = 17)	DHF III (n = 28)	DHF IV (n = 9)
Male/Female	19/19	22/15	10/9	12/5	16/12	7/2
Hematocrit (%)	NA	38.68 ± 4.20	42.25 ± 6.48	38.67 ± 6.62	43.85 ± 5.47	45.80 ± 9.08
Platelets (cell/mm³)	NA	76,763	57, 333	58, 250	58,778	30,833
Albumin (g/dl)	NA	3.68 ± 0.43	3.42 ± 0.31	3.68 ± 0.51	3.08 ± 0.62	3.40 ± 1.07
AST (U/l)	22.50 ± 4.31	103.31 ± 71.87	152.58 ± 95.95	219.53 ± 137.96	387.61 ± 962.73	3,808.11 ± 7,322.09
ALT (U/l)	9.13 ± 1.64	53.53 ± 33.49	89.53 ± 74.05	112.47 ± 85.48	136.93 ± 313.59	1,106.56 ± 2,040.54

three isoforms, namely: endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS). In addition to constitutive expression, eNOS in arterial and venous endothelial cells can also be induced by specific extracellular stimuli such as shear stress, adenosine, and bradykinins.⁷ The main physiologic function of eNOS-derived NO is vasodilatation. iNOS can be found in several cell types, such as macrophages as well as endothelial cells, and is expressed when appropriate stimuli such as lipopolysaccharide (LPS) or interferon gamma (IFN- γ) are applied.⁸ In response to inflammatory cytokines from cellular injury and inflammation, iNOS is stimulated to produce NO independent of changes in intracellular calcium. NO released from macrophages and vascular endothelial cells plays an important role in adjusting the diameter of blood vessels, remodeling blood vessels, and inhibiting leukocyte adhesion, platelet aggregation, and contractile cell proliferation.⁹⁻¹¹

Endothelial cells and macrophages are among the targets of the dengue virus. Damage to endothelial cells in dengue infection may reduce effectiveness of the permeability of the endothelial barrier, and hence disrupt the regulation of the vascular tone. Dengue virus antigen can be found in Kupffer cells and sinusoidal lining cells in the liver.¹² Dengue virus serotype 2 has been shown to have the highest virus replication in human cultured endothelial cells. Previous reports suggested that dengue virus-infected monocytes or macrophages resulted in T-cell activation and cytokine production.^{13,14} The dengue virus is capable of inducing apoptosis, immune complex formation, complement activation, and chemokine

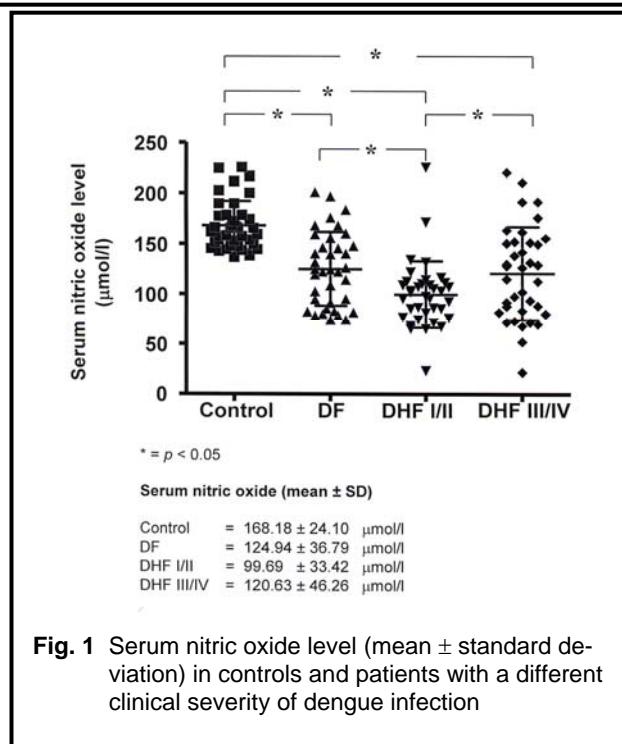


Fig. 1 Serum nitric oxide level (mean ± standard deviation) in controls and patients with a different clinical severity of dengue infection

production leading to shock, vasculopathy, thrombopathy and disseminated intravascular coagulation (DIC) in severe infection.¹⁵⁻¹⁷

From this study it has become clear that levels of serum NO in the control group were higher than those in patients with dengue infection ranging from dengue fever to all grades of DHF. The decreased NO in dengue patients could be due to endothelial damage rendering the endothelium incapable of producing NO. Endothelial cell apoptosis caused by the presence of cross-reactive anti-endothelial cell anti-

body (AECA) via a NO-mediated mechanism was demonstrated in dengue virus infections.^{18,19} Damage of endothelial cells as well as failure of eNOS and iNOS could be involved in dengue infection. In addition, levels of transcript for eNOS in vascular endothelial cells are reduced by inflammatory stimuli, such as tumor necrosis factor alpha (TNF- α),²⁰ which has been reported to be elevated in dengue infection.²¹ In agreement with Valero's finding,⁵ NO levels in DF patients are significantly higher than in DHF I and II. The underlying mechanism may be explained by NO leaking together with plasma into the interstitium as a result of increased vascular permeability in DHF patients. A previous study by Valero *et al.* showed that different dengue serotypes did not yield significantly different NO levels.⁵ NO appears to be involved in the progression of dengue infection independent of the serotype of the dengue virus.

The pattern of low serum NO levels in the DHF I/II group and high serum NO levels in the DSS group is similar to the NO pattern studied in patients with and without septic shock by Endo *et al.*²² It appears that both the increase and the decrease of NO levels might be due to eNOS and iNOS. When compared to DHF I/II, higher serum NO levels in DSS may be explained by the fact that NO generation protects the liver from ischemic-reperfusion (I/R) injury and NO may confer a protective mechanism against apoptotic stimuli in DSS. In liver transplantation, NO seems to contribute to the protective effects of ischemic preconditioning, a paradoxical phenomenon by which brief periods of vascular occlusion protect the liver from subsequent I/R injury.^{23,24} An inhibitory or enhancing effect of NO-mediated apoptosis is dependent on NO concentration, cell type, and the oxidative milieu.²⁵⁻²⁹ In addition, iNOS-synthesized NO can enhance the expression of Bcl-2 and Bcl-xL and protect endothelial cells from apoptosis.³⁰ These effects of NO might play certain roles in counteracting I/R injury and apoptosis in DSS.

Taken together, these preliminary data demonstrate that NO might not be the sole contributing factor for the severity of dengue infection. Endothelial function seems to play a role in the pathogenesis of dengue infection. Further studies are required to see whether serum NO levels could play a role in the

course of the disease and could help predict the severity of dengue infection.

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REFERENCES

1. WHO. "Dengue hemorrhagic fever. Diagnosis, treatment, prevention and control." 1997. <http://www.who.int/emc/diseases/ebola/Denguepublication/012-23.pdf> (December 20, 2003).
2. Kalayanarooj S, Vaughn DW, Nimmannitya S, *et al.* Early clinical and laboratory indicators of acute dengue illness. *J Infect Dis* 1997; 176: 313-21.
3. Marianneau P, Steffan AM, Royer C, *et al.* Infection of primary cultures of human Kupffer cells by Dengue virus: no viral progeny synthesis, but cytokine production is evident. *J Virol* 1999; 73: 5201-6.
4. Mukerjee R, Misra A, Chaturvedi UC. Dengue virus-induced cytotoxin releases nitrite by spleen cells. *Int J Exp Pathol* 1996; 77: 45-51.
5. Valero N, Espina LM, Anez G, *et al.* Short report: increased level of serum nitric oxide in patients with dengue. *Am J Trop Med Hyg* 2002; 66: 762-4.
6. Aisaka K, Gross SS, Griffith OW, Levi R. NG-methylarginine, an inhibitor of endothelium-derived nitric oxide synthesis, is a potent pressor agent in the guinea pig: does nitric oxide regulate blood pressure *in vivo*? *Biochem Biophys Res Commun* 1989; 160: 881-6.
7. Shah V, Haddad F, Garcia-Cardena G, *et al.* Liver sinusoidal endothelial cells are responsible for nitric oxide modulation of resistance in the hepatic sinusoids. *J Clin Invest* 1997; 100: 2923-30.
8. Stuehr DJ, Gross SS, Sakuma I, *et al.* Activated murine macrophages secrete a metabolite of arginine with the bioactivity of endothelium-derived relaxing factor and the chemical reactivity of nitric oxide. *J Exp Med* 1989; 169: 1011-20.
9. Shah V, Kamath PS. Nitric oxide in liver transplantation: pathobiology and clinical implications. *Liver Transpl* 2003; 9: 1-11.
10. Gopalakrishna R, Chen ZH, Gundimeda U. Nitric oxide and nitric oxide-generating agents induce a reversible inactivation of protein kinase C activity and phorbol ester binding. *J Biol Chem* 1993; 268: 27180-5.

11. Ignarro LJ. Signal transduction mechanisms involving nitric oxide. *Biochem Pharmacol* 1991; 41: 485-90.
12. Halstead SB. Antibody, macrophages, dengue virus isolation, shock and haemorrhage: a pathogenetic cascade. *Rev Infect Dis* 1989; 11: 830-9.
13. Chaturvedi UC, Agarwal R, Elbishbishi EA, Mustafa AS. Cytokine cascade in dengue hemorrhagic fever: implications for pathogenesis. *FEMS Immunol Med Microbiol* 2000; 28: 183-8.
14. Srichaikul T, Nimmannitya S. Haematology in dengue and dengue haemorrhagic fever. *Baillieres Best Pract Res Clin Haematol* 2000; 13: 261-76.
15. Avirutnan P, Malasit P, Seliger B, Bhakdi S, Husmann M. Dengue virus infection of human endothelial cells leads to chemokine production, complement activation, and apoptosis. *J Immunol* 1998; 161: 6338-46.
16. Marianneau P, Flamand M, Deubel V, Despres P. Apoptotic cell death in response to dengue virus infection: the pathogenesis of dengue haemorrhagic fever revisited. *Clin Diagn Virol* 1998; 10: 113-9.
17. Bunyaratvej A, Butthep P, Yoksan S, Bhamarapravati N. Dengue viruses induce cell proliferation and morphological changes of endothelial cells. *Southeast Asian J Trop Med Public Health* 1997; 28 (Suppl 3): 32-7.
18. Lin YS, Lin CF, Lei HY, *et al.* Antibody-mediated endothelial cell damage *via* nitric oxide. *Curr Pharm Des* 2004; 10: 213-21.
19. Lin CF, Lei HY, Shiau AL, *et al.* Antibodies from dengue patient sera cross-react with endothelial cells and induce damage. *J Med Virol* 2003; 69: 82-90.
20. Nathan C, Xie QW. Nitric oxide synthases: roles, tolls, and controls. *Cell* 1994; 78: 915-8.
21. Kittigul L, Temprom W, Sujirarat D, Kittigul C. Determination of tumor necrosis factor-alpha levels in dengue virus infected patients by sensitive biotin-streptavidin enzyme-linked immunosorbent assay. *J Virol Methods* 2000; 90: 51-7.
22. Endo S, Inada K, Nakae H, *et al.* Nitrite/nitrate oxide (NOx) and cytokine levels in patients with septic shock. *Res Commun Mol Pathol Pharmacol* 1996; 91: 347-56.
23. Peralta C, Hotter G, Closa D, *et al.* The protective role of adenosine in inducing nitric oxide synthesis in rat liver ischemia preconditioning is mediated by activation of adenosine A2 receptors. *Hepatology* 1999; 29: 126-32.
24. Peralta C, Hotter G, Closa D, Gelpi E, Bulbena O, Rosello-Catafau J. Protective effect of preconditioning on the injury associated to hepatic ischemia-reperfusion in the rat: role of nitric oxide and adenosine. *Hepatology* 1997; 25: 934-7.
25. Nicotera P, Brune B, Bagetta G. Nitric oxide: inducer or suppressor of apoptosis? *Trends Pharmacol Sci* 1997; 18: 189-90.
26. Brune B, von Knethen A, Sandau KB. Nitric oxide and its role in apoptosis. *Eur J Pharmacol* 1998; 351: 261-72.
27. Chung HT, Pae HO, Choi BM, Billiar TR, Kim YM. Nitric oxide as a bioregulator of apoptosis. *Biochem Biophys Res Commun* 2001; 282: 1075-9.
28. Shen YH, Wang XL, Wilcken DEL. Nitric oxide induces and inhibits apoptosis through different pathways. *FEBS Lett* 1998; 433: 125-31.
29. Li J, Bombeck CA, Yang S, Kim YM, Billiar TR. Nitric oxide suppresses apoptosis via interrupting caspase activation and mitochondrial dysfunction in cultured hepatocytes. *J Biol Chem* 1999; 274: 17325-33.
30. Delikouras A, Hayes M, Malde P, Lechler RI, Dorling A. Nitric oxide-mediated expression of Bcl-2 and Bcl-xL and protection from tumor necrosis factor-a-mediated apoptosis in porcine endothelial cells after exposure to low concentrations of xenoreactive natural antibody. *Transplantation* 2001; 71: 599-605.