

LACK OF ASSOCIATION BETWEEN CSF NITRATE AND SERA NITRATE IN FALCIPARUM MALARIA INFECTION

Yaowapa Maneerat¹, Polrat Wilairatana², Rachanee Udomsangpetch³,
and Sornchai Looareesuwan²

¹Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University;

²The Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University;

³Department of Pathobiology, Faculty of Science, Mahidol University

Abstract. Nitrate levels in CSF and sera from 16 coma and 19 noncoma falciparum malaria patients were determined using nitric oxide colorimetric assay. The medians (range lower, upper limits) of nitrate in sera of comatose and noncomatose patients were 0.28 (0.11, 1.24) and 0.23 (0.05, 0.87) μM , respectively. The medians of nitrate level in CSF of coma and noncoma cases were 0.09 (0.01, 0.28) and 0.15 (0, 1.18) μM , respectively. There was no difference of nitrate level in sera and CSF from comatose or noncomatose patients compared to that in normal sera and CSF. The amount of nitrate in sera and CSF of both groups was not significantly correlated with coma depth, parasitemia, parasite clearance time and time to recovery. Contrast to our *in vitro* study using immunoperoxidase staining, we found inducible nitric oxide synthase production by brain endothelial cells during 4-24 hours of coculturing with late stage of *P. falciparum* infected red blood cells. These results suggests that malaria severity can not be differentiated by nitrate level in body fluid.

INTRODUCTION

Nitric oxide is an important biological free radical produced by various mammalian cell types and plays important roles in infections including falciparum malaria (Clark *et al*, 1991). Nitric oxide is synthesized from L-arginine through the mechanism catalyzed by enzyme nitric oxide synthase (NOS) and finally oxidized to the stable end products, nitrite and nitrate. An important isoform of NOS, inducible nitric oxide synthase (iNOS), can be enhanced by many factors *eg* cytokines, chemical agents and some microorganisms (Whittle, 1995). In some previous studies, increased CSF nitrate and nitrite levels are represented as or associated with the damage in the central nervous system *eg* in cerebral systemic lupus erythematosus (Brundin *et al*, 1998), methylmercury intoxication in degenerated cerebellum (Yamashita *et al*, 1997), bacterial meningitis (Uysal *et al*, 1999), acute ischemic stroke (Castillo *et al*, 2000) and complication of HIV infection with blood-

brain-barrier dysfunction (Giovannoni *et al*, 1998). In malaria infection, nitric oxide may play a protective role. Patients with mild malaria have higher amounts of reactive nitrogen intermediates in sera, plasma, CSF and urine compared with severe malaria and cerebral malaria (Cot *et al*, 1994; Anstey *et al*, 1996; Kremsner *et al*, 1996). On the other hand, nitric oxide and its derivatives are shown to correlate with severity of falciparum malaria (Clark *et al*, 1991; Nussler *et al*, 1994; Al-Yaman *et al*, 1996; Taylor *et al*, 1998; Weiss *et al*, 1998).

Recently, we have demonstrated the presence of iNOS in the brain tissues from fatal cases of cerebral malaria patients. In contrast, brain tissues from the recovered cerebral malaria patients and normal brain tissues from trauma cases lack of iNOS (Maneerat *et al*, 2000). This finding supports the notion that nitric oxide production in comatose brain tissues may cause histopathologic changes including ring hemorrhage, demyelination and neuronal degeneration. Level

of iNOS expression in endothelial cells of sequestered vasculature was significantly correlated to the severity of the disease (Maneerat *et al.*, 2000). To investigate the effects of *P. falciparum* in nitric oxide production, in this study, we 1) determined and compared the amount of nitrate in sera and CSF of *P. falciparum* infected Thai patients having different disease severity, and 2) demonstrated nitric oxide production by endothelial cells stimulated with *P. falciparum*-infected red blood cells.

MATERIALS AND METHODS

Determination of nitrate level in sera and CSF

The sera and CSF were collected from acute falciparum malarial patients admitted at the Hospital for Tropical Diseases, Bangkok and Mae Sot Hospital, Tak Province, Thailand. Malaria severity was defined by the Glasgow coma score (Teasdale and Jennett, 1974), and the patients were grouped as coma (n=16) (score \leq 5) and without coma (n=19) (score $>$ 5). The clinical manifestations are summarized in Table 1. Normal sera were obtained from healthy Thai donors at the Blood Bank, Ramathibodi Hospital and normal CSF was kindly provided by The Childrens Hospital, Bangkok, Thailand. The use of specimens from patients and controls obtained in this study were approved by the institutional ethical review board.

Determination of nitrate in the sera and CSF was carried out using a nitric oxide colorimetric kit from Boehringer Mannheim, USA (Cat. No.1756281). Three hundred microliters of the samples were diluted in the same volume of phosphate buffered saline, pH 7.5, then ultrafiltrated through an ultrafilter membrane (Centricon, Millipore Corporation, MA, USA) by centrifugation at 2,000g for 45 minutes to deplete proteins which may interfere the assay. Nitrate level was measured in 96 well microtiter plates. The assay was completed according to the

recommendation from the manufacturer. Levels of nitrate in the specimens were determined from the standard curves drawn by using ten-fold dilution (80.0 μ M to 80.0 mM) of standard potassium nitrate.

The data was analyzed by using SPSS program for Windows 7.5.1 (No.3168930). The correlation between nitrate level in the sera or CSF and the severity of the disease, parasitemia, parasite clearance time, and recovery time was analyzed using Spearman's rank correlation coefficient test. The differences of nitrate levels between coma and noncoma patients were compared to those of normal sera and CSF using the Kruskal-Wallis test.

Stimulation of NO production *in vitro*

Human brain endothelial cells (BEC) were obtained from Dr Adisak Wongkhajornsilapa, Department of Pharmacology, Faculty of Medicine, Siriraj Hospital. BEC were cultured continuously in 25 cm² plastic flasks in medium M199 (Gibco, USA) containing 10% fetal calf serum (Gibco, USA) and 0.5 mg/ml of endothelial cell mitogen (Biomedical Technologies Inc, USA). The BEC in passage 2-6 were used in this study.

Plasmodium falciparum culture and late stage parasite preparations

A *P. falciparum* isolate selected for high binding to BEC was maintained under *in vitro* culture conditions (Trager and Jensen, 1976) at 37°C, 5% CO₂ and air mixture, in human group O erythrocytes using RPMI-1640 medium (Gibco, USA) at pH 7.4 containing 40 μ g/ml of gentamicin, supplemented with 25 mM HEPES (Sigma Chemical Co, USA), and 10% heat-inactivated human AB serum. The late stage *P. falciparum* infected erythrocytes were enriched by gradient centrifugation the mix parasites on 60% percoll (Amersham Pharmacia Biotech, Inc, USA) in 0.1M PBS at 400g for 20 minutes at room temperature (Lowry *et al.*, 1951). The late stage parasites were washed twice in RPMI 1640 and adjusted to 10% parasitemia for the study.

Stimulation of BEC with late stage *P. falciparum* infected erythrocytes

Ten thousand BEC were cultured on 13 mm diameter cover slips (Thermanox, ICN, USA) inserted in 24-well plate (Costar, USA) until growing to a confluent monolayer in a few days. The late stage parasite-infected erythrocyte culture (10^6 cell/ml of RPMI 1640 containing 10% FCS) were cocultured with BEC monolayer in a 24 well plate.

Determination of iNOS by immunoperoxidase staining

At 0, 1, 4, 6, 8, 12, 24, and 36 hours after coculturing, the cover slips with BEC were harvested and air dried overnight. The cells were fixed in methanol:acetone (1:1, V/V) for 15 minutes at -20°C . Detection of iNOS production was confirmed by immunoperoxidase technique as described previously (Maneerat *et al*, 2000). Briefly, endogenous peroxidase was blocked by incubating the cells with 3% H_2O_2 in 0.1 M PBS for 15 minutes at room temperature. After washing 3 times, the cells were incubated in 0.01% BSA (Sigma Chemical Co, USA) in 0.1 M PBS for 15 minutes at room temperature to block nonspecific protein. The cells were incubated with rabbit polyclonal antibody to human iNOS (Santa Cruz Biotechnology, Inc, USA) at dilution 1:100 in 0.01% BSA in 0.1 M PBS for 45 minutes at room temperature and 4°C overnight. The cells were washed in 0.01% BSA in 0.1 M PBS and incubated with 30 μg of biotinylated F(ab')₂ fraction of goat anti rabbit IgG (DAKO, Denmark) for 30 minutes, washed and incubated with streptavidin-biotin complex conjugated with peroxidase (Vector Laboratories, Inc, USA) for 30 minutes. After washing, peroxidase enzyme activity in BEC was detected by adding a mixture of 3,3'-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 (Vector Laboratories, Inc, USA) for 15 minutes. The cells were counterstained with Meyer's hematoxylin for 5 minutes, washed and mounted with Permount and examined under light microscope. Controls for immunostaining included omission

of primary antibodies, and a positive control for iNOS immunostaining was 24 hour LPS stimulated monocytes.

RESULTS AND DISCUSSION

The median (range lower, upper limits) of nitrate level in sera of comatose, noncomatose patients and normal controls were 0.28 (0.11, 1.24), 0.23 (0.05, 0.87), and 0.15 (0.10, 0.35) μM , respectively. The median of nitrate level (range) in CSF of the comatose, noncomatose and normal were 0.09 (0.01, 0.28), 0.15 (0, 1.18), and 0.45 (0.02, 1.55) μM , respectively. There was no significant correlation between the amount of nitrate in the serum and CSF of the acute falciparum patients ($p = 0.86$). Neither was any difference found when compared with normal sera and CSF ($p = 0.263$ and $p = 0.068$ respectively). Level of nitrate was not associated with severity of malaria infection including the coma depth, parasitemia, and time to recovery. Contrast to studies in Vietnam and Brazil, nitrite and nitrate plasma levels in *P. falciparum* and *P. vivax* infected patients were significantly higher than in controls (Nussler *et al*, 1994). However, our results were consistent with a study in Ghanaian children that the depth of coma is not related to nitrate level ($p > 0.5$) (Agbenyega *et al*, 1997). Recent study has shown that nitrate level in CSF correlated well with plasma level, but showed no correlation with coma depth and neither differ from that in the healthy control group (Dondorp *et al*, 1998). Interestingly, nitrogen oxide in plasma during acute phase of the disease is shown to inversely correlate with parasite clearance time and the duration of symptoms (Kremsner *et al*, 1996). This is supported by a finding that plasma of asymptomatic patients contained high nitrate levels suggesting the protective role of NO production against clinical symptoms (Anstey *et al*, 1996). The present finding suggested there was no association between the brain tissue localized NO in cerebral malaria and that in serum and CSF. Recent work in Wistar rat cerebellar degeneration by methylmercury intoxication showed an increase in CSF nitric

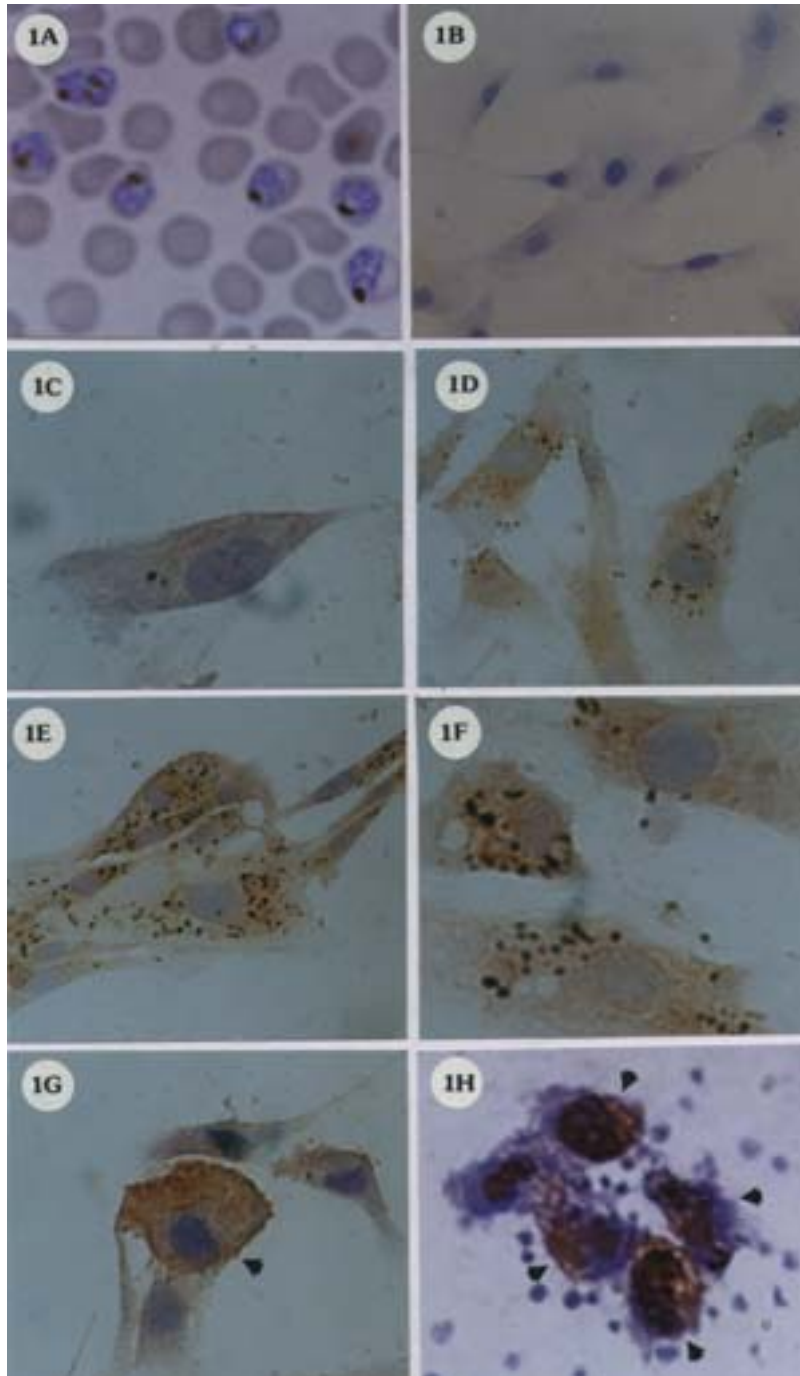


Fig 1—Determination of iNOS enzyme produced by BEC stimulated with *P. falciparum* infected red blood cells at late stages (30-36 hours), X 1,000 (1A); normal BEC cultured in RPMI 1640 + 10% FCS, X 200 (1B); BEC at 4 hours after coculture with *P. falciparum* infected red blood cells, X 1,000 (1C); BEC at 8 hours after coculture with *P. falciparum* infected red blood cells, X 400 (1D); BEC at 18 hours after coculture with *P. falciparum* infected red blood cells, X 400 (1E); BEC at 24 hours after coculture with *P. falciparum* infected red blood cell, X 1,000 (1F); BEC (1G) and monocyte (1H) 24 hours after stimulation with LPS from supernatant of *Burkholderia pseudomallei* culture X 1,000.

Table 1
Clinical manifestations of patients with acute falciparum infection on admission.

	Coma patients (n = 16)	Noncoma patients (n = 19)
Sex (male/female)	12/4	15/4
Age (years) ^a	23.5 (14, 57)	22 (15, 56)
Temperature (°C) ^b	38.4 ± 0.95	38.4 ± 1.01
Parasitemia (per µl) ^a	10,174 (520, 882,968)	95,790 (400, 486,780)
Parasite clearance time (hours) ^b	48.0 ± 23.04	59.6 ± 20.4
Glasgow coma score	≤ 5	> 5
No. of final death /recover patients	6/10	1/19
Time to recovery (hours) ^a	52 (24, 67)	30 (12, 168)
Nitrate in sera (µm) ^a	0.28 (0.11, 1.24)	0.23 (0.05, 0.87)
Nitrate in CSF (µm) ^a	9.000E-02 (0.01, 0.28)	0.15 (0, 1.18)

^a = median (range lower, upper limits); ^b = mean ± standard deviation

Median of nitrate level from 8 normal sera = 0.15 (0.10, 0.35 µm), n=8

Median of nitrate level from 10 normal CSF = 0.45 (0.02, 1.55 µm), n=10

Table 2
Production of iNOS protein by brain endothelial cell using immunoperoxidase staining.

Cell	Time after coculture					
	0 hour	4 hours	8 hours	18 hours	24 hours	36 hours
BEC + media	-	ND	ND	ND	ND	-
BEC + NRBC	-	-	-	-	-	ND
BEC + PRBC	ND	++	++	+++	+++	++
BEC + LPS	ND	ND	ND	ND	+++	ND
Monocyte+ LPS	ND	ND	ND	ND	+++	ND

BEC = brain endothelial cell; NRBC = normal red blood cell

PRBC = *Plasmodium falciparum* infected red blood cell

LPS = lipopolysaccharide from supernatant of *Burkholderia pseudomallei* culture

Interpretation

- = no staining or no enzyme iNOS production

+ = weak brown staining in cytoplasm of BEC refers to little enzyme iNOS production

++ = moderate brown staining in cytoplasm of BEC refers to moderate amount of enzyme iNOS production

+++ = strong brown staining in cytoplasm of BEC or monocyte refers to a lot of enzyme iNOS production

oxide metabolite and NOS activity in the brain tissues (Yamashita *et al*, 2000), as did our previous study in the brain tissue of fatal cerebral malaria (Maneerat *et al*, 2000). Determination of intermediate reactive nitrogen in biological fluids is complicated and there are several interfering factors including food, age, immunity of patients, the strains of falciparum malaria in various geographic regions, sensitivity of the assays and timing

of specimen collection. In this study the level of reactive nitrogen intermediates in CSF or sera cannot be represented by the nitrate level during acute falciparum infection. In contrast to our study in *P. falciparum*-stimulated BEC, we found iNOS production in the cytoplasm of the BEC 4-24 hours after coculturing with late stage *P. falciparum* infected erythrocytes compared to those stimulated with normal red blood cells or unstimulated BEC (Fig 1, Table

2). The peak of iNOS expression in the stimulated BEC appeared during 18-24 hours of coculture. The finding is consistent with endothelial cells in sequestered vessels in cerebral malaria brain tissues shown in our recent report (Maneerat *et al*, 2000). The increased iNOS in endothelial cells after coculture with the parasite may indicate the possibility of NO production in natural falciparum infection. The failure of nitrate detection in our study may be due to unsuitable time of specimen collection. However, further studies are needed to obtain better understanding of nitric oxide production by *P. falciparum*-stimulated endothelial cells producing nitric oxide. The importance of nitric oxide as protective factor or severity enhancing factor in falciparum malaria may be applicable in a restricted time frame during the disease process.

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