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Serum nitrite levels in Sri Lankan patients with leptospirosis

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ABSTRACT

Objective: To determine whether blood nitrite levels are elevated in patients with leptospirosis. **Methods:** Male patients fulfilling clinical and epidemiological criteria for a diagnosis of leptospirosis were recruited. Those with MAT titre of $\geqslant$400 together with those seroconverting to a titer of $\geqslant$200 were included in the analysis. Serum nitrite levels were measured in these patients and age, sex matched healthy controls. **Results:** Patients from 3 hospitals (n=75) were screened during a 3 month period from 28th June to 3rd September 2009, of whom 20 were eligible for the study. Serum nitrite levels were found to be significantly higher in patients with acute leptospirosis [n=20, (0.359±0.229) μ M] compared to controls [(n=13,(0.216±0.051) μ M] (P=0.014). A significant correlation was also observed between the MAT titre and the day of illness (r = 0.547; P<0.0001). **Conclusions:** Serum nitrite levels are higher in patients with acute leptospirosis compared to age and sex matched controls. No correlation could be assessed with severity of illness, as sample size was inadequate to determine this.

1. Introduction

Over the past decade leptospirosis remains endemic in Sri Lanka with outbreaks once every four to five years. Beginning from the latter part of the year 2007, Sri Lanka experienced the largest ever recorded outbreak of leptospirosis. The incidence of leptospirosis in Sri Lanka in 2008 was 35.7 per 100 000 population. Many infections are asymptomatic or pass off as a mild febrile episode. However, a small proportion develops a severe form of the disease with multiple organ dysfunction. Weil's disease, the most severe form of leptospirosis, is characterized by jaundice, hemorrhage and renal failure. Pulmonary haemorrhage, myocarditis, meningitis and multiorgan failure are known complications, with an overall mortality rate of 5%-15%[1]. Ten percent of ICU admissions in Sri Lanka in 2007 were due to leptospirosis and 65% of these patients died due to complications of leptospirosis[2].

Endothelial dysfunction is considered one of the mechanisms by which organ damage takes place in severe leptospirosis[3,4]. Nitric oxide (NO) is considered to be an important mediator of endothelial dysfunction in sepsis. Pro-inflammatory cytokines such as interleukins 1 and 6, and tumour necrosis factor alpha are known to increase the expression of inducible endothelial nitric oxide synthase (iNOS), leading to increased levels of NO in sepsis[5]. The clinical manifestations of severe sepsis and severe leptospirosis are similar, however little is known of the similarities in pathogenesis between the two conditions. Nonetheless, leptospirosis is known to stimulate the production of pro-inflammatory cytokines[6] which could

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result in elevated NO levels[7]. High serum nitrate and nitrite levels have been previously demonstrated in severe leptospirosis[8]. Expression of iNOS and NO has been shown to have important roles in eliminating the leptospires[9] as well as leading to severe pathology[10].

The aim of this prospective case control study was to determine whether serum nitrite levels are significantly elevated in patients with leptospirosis compared to healthy controls.

2. Materials and methods

The study was conducted in three centres in Sri Lanka - the Colombo North Teaching Hospital (Ragama), Base Hospital (Gampaha) and Base Hospital (Homagama). Ethics approval was obtained from the Ethics Review Committee of the Faculty of Medicine, Colombo.

Patients with fever, myalgia, and conjunctival injection, with a history of working in paddy fields or other contact areas of rat infestation, presenting within 7 days of onset of symptoms were recruited for the study over a period of approximately 3 months, *i.e.*, from 31st June to 3rd September 2009. After the initial assessment for eligibility criteria, the cases were screened using a symptom checklist and questionnaire to diagnose leptospirosis according to the leptospirosis case definition. The surveillance case definition for diagnosis of leptospirosis published by the Epidemiology Unit of the Ministry of Health Sri Lanka in 2005 was used for this study[11]. Diagnosis was confirmed by the microagglutination test (MAT) with the following criteria: those with a titre $\geqslant$400, along with those seroconverting to a titre of $\geqslant$200[12,13]. Demographic data and clinical data were obtained from the patients themselves whilst results of investigations done were obtained from patient records.

Patients without complications were followed-up for two weeks or until they were discharged from hospital. Patients who developed complications/severe disease were followed-up until discharge/death in the respective hospitals.

Out of the 20 in whom the first MAT test was performed, thirteen patients were sampled for a repeat MAT approximately 3 weeks from the onset of illness. Thirteen age, sex matched healthy individuals who tested MAT negative were taken as control subjects. Informed written consent was obtained from patients prior to sampling. A total of 4 mL of blood was collected from each patient, 2 mL for the determination of nitrite levels and the other 2 mL for the MAT test. Sera were separated and frozen at -20 ℃. MAT tests were performed at the Medical Research Institute (MRI), Colombo, Sri Lanka.

Serum nitrite levels were measured in these patients and age, sex matched healthy controls using the Griess method[14]. The serum samples were first thawed then deproteinized by adding Zinc Sulfate. Deproteinization is a necessary step in the measurement of serum nitrite concentrations[15]. Twenty microliters of 300 mg/mL Zinc sulphate solution was added to 400 μL of serum, vortexed for 1 minute, and centrifuged at 10 000 g for 10 minutes at room temperature. The supernatant was pipetted out and centrifuged again for 8 minutes. The clear supernatant was distributed equally (100 μL each) amongst 3 wells in the 96 well ELISA plate. An equal volume of 5% phosphoric acid was added to the first well, to determine the reading obtained by the background colour of the serum and an equal volume of Griess reagent (equal mixture of 1% Sulphanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine hydrochloride in distilled water) was added to the second and third wells. The plates were kept for 15 minutes at room temperature and the optical density was measured at 540 nm
using the ELISA reader (Bio-Tek Instruments INC, USA). A dilution series (0.193, 0.391, 0.781, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 μM) of $NaNO_2$ was prepared from 100 μM $NaNO_2$ solution using distilled water. Each dilution (100 μL) was mixed with an equal volume of Griess reagent and the optical density was measured at 540 nm. A standard curve was plotted against optical density and $NaNO_2$ concentration. For each serum, OD reading of the Griess reaction was corrected by subtracting the OD value of the phosphoric acid control. The amount of nitrite in the human serum was calculated using this standard curve for $NaNO_2$.

Statistical analysis was performed using SPSS version 16.0. Data was analyzed to determine if a correlation existed between nitrite levels and hematological indices and reciprocal MAT titre using Pearson's correlation. The nitrite levels were compared between the patient group and the control group using the Student *t* test. The reciprocal MAT titre was compared to the day of illness (Spearman's correlation). Statistical significance defined as $P<0.05$ was evaluated using the Student *t* test.

3. Results

Twenty of the 75 patients tested were diagnosed as having leptospirosis. Because of the high prevalence of other infectious disease in the patient population from which the sample was drawn, a MAT results cut off titre of 400 together with patients seroconverting to 200 or more were selected as this would give a higher specificity for leptospirosis. Of the 18 controls, 4 had positive MAT titres of 100 for leptospirosis, and one patient had an overt abscess; these were excluded.

Thus, thirteen controls were included in the final analysis. There was a significant positive correlation between the MAT titre and the day of illness (r = 0.547; P<0.0001)(Figure 1).

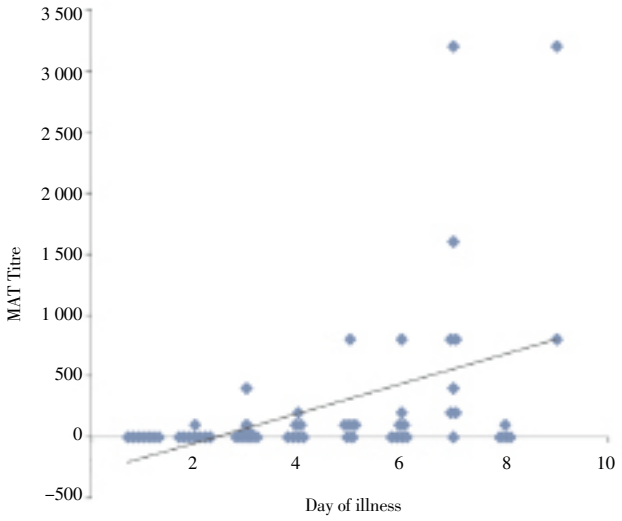

Figure 1. Association between MAT titer and day of illness. (r = 0.547; P<0.0001 Spearmann correlation, n=61).

3.1. Serum nitrite levels

The standard curve obtained with a dilution series of $NaNO_2$ is shown in Figure 2. Serum nitrite levels of patients and controls were calculated as described above. According to the standard curve used for the calculation of nitrite levels, the lowest detectable value was 0.193 μM; therefore levels falling below this were assigned this value. The values ranged from 0.193 to 1.00 μM, and the mean value was 0.359 μM. This was statistically, significantly higher compared to the nitrite levels in the control group (P = 0.014) (Figure 3).

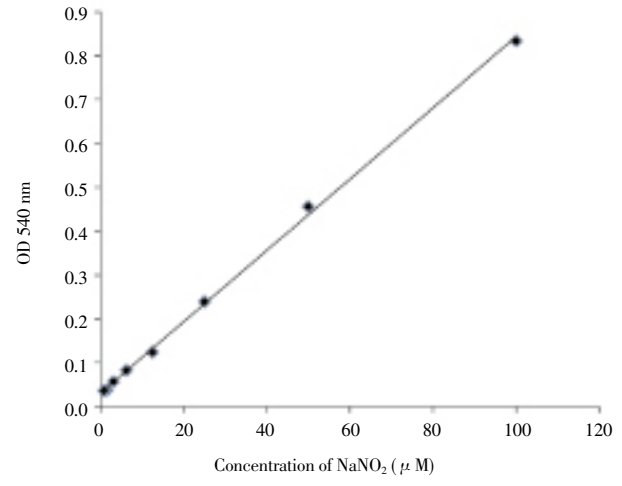

Figure 2. The standard curve for $NaNO_2$.

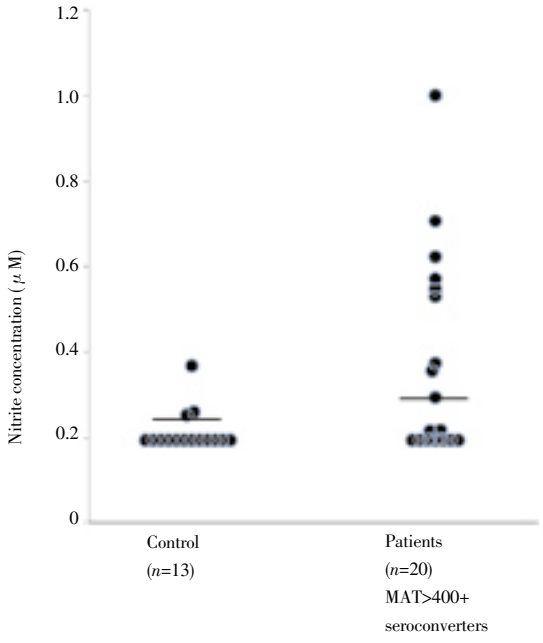

Figure 3. Nitrite levels in patients and controls.
[Controls (n=13): (0.216±0.051) μM; mean±SD]; Patients (n=20): 0.193 to 1.00 μM, mean 0.359 μM, P<0.014.

3.2. Correlation of nitrite values with disease parameters.

Nitrtite levels were correlated with haematological indices (white blood cell count, neutrophil count, lowest platelet count) and MAT, but there was no significant correlation. The sample size was inadequate to compare nitrite levels with clinical outcome, severity, or the occurrence of organ dysfunction.

4. Discussion

The main aim of this study was to see whether blood nitrite levels are increased in patients with leptospirosis. Our study showed that serum nitrite levels are increased in patients with confirmed leptospirosis compared to healthy controls. This finding is in keeping with previous studies[8]. Elevated serum nitrite levels have been reported in other infectious diseases such as dengue[16] and malaria[17]. It is possible that elevated serum nitrite levels reflect a non-specific response to certain infections; nonetheless the demonstration of elevated serum nitrite levels in leptospirosis supports the hypothesis that endothelial dysfunction plays a role in the pathogenesis of the disease.

The need for predictors of mortality and morbidity in leptospirosis has been highlighted[18]. Novel Biomarkers such as Copeptin and Pentraxin have been investigated as potential predictors of severity in leptospirosis[19-21]. Serum nitrite levels may be one of the potential biomarkers

of severity in leptospirosis although our small sample size precluded such analysis. Total NO levels, *i.e.*, nitrate and nitrite together, may be a better indicator of endothelial dysfunction and severity than nitrite alone, and we suggest the need for further studies in this area.

Limitations of our study included small sample size and the difficulty in differentiating other infectious diseases with similar clinical syndromes when identifying the cases. For logistic reason and due to economic constrains, serology for other infectious disease could not be performed on the 20 patients included in our sample. Another potential limitation is that only male patients were enrolled in our study.

In conclusion, patients with leptospirosis have higher serum nitrite levels compared to matched controls. We were unable to demonstrate a correlation between nitrite levels and markers of disease severity due to small sample size.

Conflict of interest statement

We declare that we have no conflict of interest.

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