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Short communication

High serum nitric oxide levels in patients with severe leptospirosis

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Abstract

Leptospirosis is a globally distributed zoonosis of major public health importance and is associated with severe disease manifestations such as acute renal failure and pulmonary haemorrhage syndrome. However, the extent to which the pathogenesis of leptospirosis mimics sepsis caused by Gram-negative bacteria remains unknown. The aim of this study was to evaluate serum levels of nitric oxide (NO) in patients diagnosed with severe leptospirosis. Sera from 35 confirmed cases of severe leptospirosis and 13 healthy subjects were analysed. Patients with severe leptospirosis had significantly higher NO levels compared to healthy individuals (30.82 ± 10.90 μM versus 3.86 ± 1.34 μM, $P < 0.001$), indicating that this immune mediator plays a role in the underlying systemic inflammatory response.

Keywords: Leptospirosis; Nitric oxide; Acute renal failure; Pulmonary haemorrhage

Leptospirosis is a widespread zoonosis caused by pathogenic spirochetes from the *Leptospira* genus. Transmission to humans is accidental and typically occurs through contact with mud or water contaminated with urine of infected animals, principally rodents. Traditionally a sporadic disease confined to a rural setting, leptospirosis now causes yearly epidemics in urban living conditions associated with extreme poverty (Johnson et al., 2004; Karande et al., 2003; Ko et al., 1999; LaRocque et al., 2005; Sarkar et al., 2002). Outbreaks are also associated with water sports and recreational activities that result in exposure to pathogenic *Leptospira* (Haake et al., 2002). The clinical spectrum ranges from a mild anicteric disease to the more severe forms such as Weil's syndrome (jaundice, haemorrhagic diathesis and acute renal failure), associated with a 10% mortality and severe pulmonary haemorrhage syndrome (SPHS), for which the case fatality rate can be >50% (McBride et al., 2005).

The underlying pathogenic mechanisms associated with the severe manifestations of leptospirosis are poorly understood but it is assumed that a generalized endothelial dysfunction, as observed in experimental and human leptospirosis, is the main pathogenic mechanism of tissue damage (De Brito et al., 1979; Nicodemo et al., 1997). A similar process occurs in sepsis and is mediated

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by pro-inflammatory cytokines, including interleukins IL-1, IL-6 and tumour necrosis factor alpha (TNF-α) (Cohen, 2002). These pro-inflammatory cytokines lead to increased expression of inducible nitric oxide synthase (iNOS) and increased production of nitric oxide (NO) in patients with sepsis. *Leptospira* components, such as lipopolysaccharide and glycolipoprotein, have been reported to activate leukocytes and stimulate the production of pro-inflammatory cytokines such as TNF-α and/or IL-8 (Cinco et al., 1996; Diament et al., 2002; Werts et al., 2001; Yang et al., 2002). Furthermore, it has been reported that high levels of TNF-α were associated with lethal outcomes in hospitalised patients (Tajiki and Salomao, 1996), modulated, to some degree, by IL-10 (Tajiki et al., 1997). Nevertheless, it remains unclear as to the extent, if any, that sepsis and leptospirosis share similar mechanisms. As NO is considered to be a major mediator of endothelial dysfunction in sepsis, we determined NO levels in serum from patients with severe leptospirosis and compared them to those obtained from healthy individuals.

Acute phase serum samples were collected from 35 patients with confirmed severe leptospirosis from Couto Maia Hospital, the infectious disease referral hospital in Salvador, Bahia, Brazil during the period April 2000 to September 2002. Patients who fulfilled the criteria for severe leptospirosis (clinical symptoms compatible with leptospirosis, jaundice, renal insufficiency (oliguria, creatinine >1.5 mg/dl, urea >150 mg/dl), acute fever and haemorrhagic manifestations) were eligible for inclusion in the study. Non-hospitalized patients or those who did not fulfill the selection criteria were excluded from the study. Diagnosis was confirmed by the microagglutination test (MAT) with the following criteria: a four-fold or greater rise in titre between paired serum samples; seroconversion (initial MAT titre of <100 increasing to ≥200); or a single serum titre ≥800 were considered positive. Demographic, clinical and laboratory data were available from hospital charts. Control serum samples were collected from 13 healthy individuals. Informed consent was obtained from each participant prior to the collection of blood according to protocols approved by the Ethical Committee of the Oswaldo Cruz Foundation.

Serum NO levels were determined by the measurement of NOx (nitrite and nitrate) after enzymatic reduction of nitrate with nitrate reductase, as previously described (Tavares-Murta et al., 2002). Briefly, 50 μl of undiluted serum was incubated with the same volume of reductase buffer (0.1 M potassium phosphate, pH 7.5, containing 1 mM nicotinamide adenine dinucleotide phosphate, 10 mM flavin adenine dinucleotide and four units of nitrate reductase/ml) for 20 h at 37 °C. The nitrite (NO_2^-) concentration was determined using the Griess method (Green et al., 1981).

Comparison of the NO levels between cases and control subjects was assessed using the non-parametric Mann–Whitney test. The correlation between the NO levels and serum creatinine was evaluated using the Spearman correlation co-efficient. SPSS 10.0 and GraphPad Prism 4.03 software packages were used for all statistical analyses. A $P < 0.05$ was considered significant. Standard deviations were included where appropriate in the text as ±values.

Among the 35 leptospirosis patients, 33 were male and the mean age was 35.5 ± 13.7 (range of 14–71 years). The most frequent clinical manifestations on hospital admission were jaundice (33/35) and fever (30/35). Respiratory insufficiency (respiratory rate greater than 28 per min) occurred in four patients. Shock, defined by a systolic blood pressure less than 90 mmHg, was observed in five patients. Serum creatinine levels on admission were >1.5 mg/dl in 10 patients. The mean maximum creatinine level measured during hospitalization was 4.92 ± 2.41 mg/dl and 97.1% (32/35) of patients had levels >1.5 mg/dl. Eleven patients were subject to intraperitoneal dialysis for, on average, 3 days (range 1–6 days). All patients survived and the average time of hospitalization was 8 days (range 3–27 days). The control individual group included 10 males, 3 females with an average age of 24.7 ± 3.2 (range 19–30 years of age). The mean NO level in patients with acute leptospirosis was 30.82 ± 10.90 μM compared to 3.86 ± 1.34 μM among control subjects ($P < 0.001$). Using a cut-off based on the mean NO level of the control group plus two standard deviations (6.54 μM), resulted in all patients having detectable NO levels above the cut-off (Fig. 1).

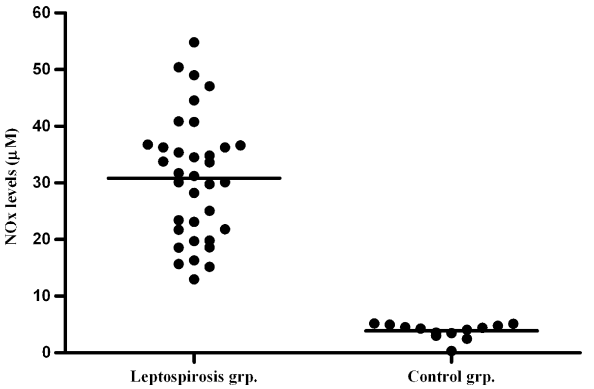

Fig. 1. Serum nitric oxide levels in patients with leptospirosis and in healthy adult individuals. A non-parametric Mann–Whitney test comparing NO levels was highly significant ($P < 0.001$), horizontal bars represent the mean value for each group.