

## Oxidative Stress Markers Correlate with Renal Dysfunction and Thrombocytopenia in Severe Leptospirosis

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**Abstract.** Leptospirosis is a zoonotic disease that causes severe manifestations such as Weil's disease and pulmonary hemorrhage syndrome. The aim of this study was to evaluate whether reactive oxygen species (ROS) production and antioxidant reduced glutathione (GSH) levels are related to complications in patients hospitalized with leptospirosis. The ROS production and GSH levels were measured in blood samples of 12 patients and nine healthy controls using chemiluminescence and absorbance assays. We found that ROS production was higher and GSH levels were lower in leptospirosis patients compared with healthy individuals. Among patients, GSH depletion was correlated with thrombocytopenia and elevated serum creatinine, whereas a strong positive correlation was observed between ROS production and elevated serum potassium. Additional investigation of the biological significance of ROS production and GSH levels is warranted as they may guide the development of novel adjuvant therapies for leptospirosis targeting oxidative stress.

### INTRODUCTION

Leptospirosis is a widespread zoonosis caused by pathogenic leptospires. The disease occurs in different settings including rural and/or occupational settings, large urban areas with poor sanitation, and recreational exposure.<sup>1</sup> Human infections are mostly asymptomatic or manifest as a mild febrile illness that is clinically indistinguishable from disease caused by other infectious agents. On the other hand, 5–10% of human infections will evolve to severe forms, such as Weil's disease, characterized by acute renal failure (ARF), hemorrhage, and jaundice with 5–30% related case fatality, and severe pulmonary hemorrhagic syndrome, with  $\geq 50\%$  related case fatality.<sup>2–5</sup>

Additional tools for the treatment of severe forms are urgently needed because a major issue in the management of patients is the limited effect of antibiotics when initiated late in the course of the disease.<sup>4</sup> The pathophysiology of the serious life-threatening complications of leptospirosis, such as ARF, thrombocytopenia, and pulmonary hemorrhages, are poorly understood.<sup>6,7</sup> The comprehension of underlying disease mechanisms is a critical step for the development of adjuvant and supportive therapies that may improve leptospirosis outcomes.

The association between markers of oxidative stress and target organ dysfunction in clinical leptospirosis has not been investigated. It is well established that *Leptospira* membrane components may enhance expression of pro-inflammatory markers from renal tubular cells *in vitro*, including inducible nitric oxide synthase.<sup>8–10</sup> We have previously reported a positive correlation between serum levels of nitric oxide and serum creatinine levels in patients with severe leptospirosis.<sup>11</sup> Only a single study has previously investigated the effect of *N*-acetylcysteine (NAC) as an adjuvant antioxidant therapy, using a hamster model treated with antibiotics (ampicillin) late in the course of infection. The study found that the adjuvant antioxidant therapy did not reduce the level of leptospiral

antigens or increase the expression of renal tubule transporters in the experimental model.<sup>12</sup> In other infectious diseases, and notably in sepsis, reactive oxygen species (ROS) production and antioxidant depletion are associated with tissue damage and disease severity.<sup>13–18</sup> Severe leptospirosis has clinical features similar to sepsis syndromes from other Gram-negative organisms, such as a systemic inflammatory response<sup>7,19</sup> and hemodynamic changes (combined high cardiac index/low systemic vascular resistance).<sup>20</sup> In this study, we evaluated whether ROS production and antioxidant depletion are related to clinical complications in severe leptospirosis.

### MATERIALS AND METHODS

**Patients.** Between May 11 and May 31, 2011, we performed active hospital-based surveillance for severe leptospirosis in the state reference hospital for infectious diseases in Salvador, Brazil. A total of 18 patients met clinical criteria for suspected leptospirosis, and 12 patients with laboratory-confirmed leptospirosis were included in this study. Clinical data related to disease presentation and clinical outcome were extracted by review of patient records using a standardized questionnaire and entered into a database. The primary outcome of interest was ARF on admission, defined as serum creatinine concentration above 1.5 mg/dL. Subjects were grouped based on clinical status: subjects who developed ARF ( $N = 7$ ), and those who did not ( $N = 5$ ). Blood samples were collected in 2 mL Vacutainer tubes with EDTA (Becton Dickinson Vacutainer System San Jose, CA) at hospital admission for ROS and glutathione (GSH) analysis and 14–30 days later for laboratory confirmation of leptospirosis. An additional tube of 8 mL without anticoagulant was collected for biochemical analysis (alanine aminotransferase, aspartate aminotransferase, urea, and creatinine) at hospital admission. Healthy, leptospirosis-negative individuals ( $N = 9$ ) from our laboratory were included as control subjects.

**Ethical approval.** The study was approved by the Ethics Committee of the Oswaldo Cruz Foundation, Salvador, Bahia, Brazil; the Ethics Committee of the Brazilian National Research (CONEP) and the ethics review committees at

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Hospital Couto Maia and Yale University. Written informed consent was obtained from all participants.

**Laboratory diagnosis.** Laboratory confirmation of leptospirosis was made by the microscopic agglutination test (MAT), enzyme-linked immunosorbent assay (ELISA) (Molecular Devices Corporation, CA) (Bio-Manguinhos, Rio de Janeiro, Brazil), or blood culture. The MAT confirmation criteria included seroconversion or a 4-fold rise in titer between acute and convalescent sera obtained on the day of hospital admission and after 14–30 days of convalescence, or a single titer of  $\geq 1:800$ .<sup>21</sup> The MAT panel included 10 reference strains and a local isolate.

*Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130, representing nine serovars and nine serogroups. This panel effectively identifies most locally circulating *Leptospira*, 90% of which are *L. interrogans* serovar Copenhageni.<sup>21</sup>

**Assay for ROS detection in peripheral blood samples.** Reactive oxygen species were evaluated in venous blood from severe leptospirosis patients and normal healthy individuals within 60 minutes after sample collection. The ROS were determined as described previously.<sup>22</sup> Briefly, the blood samples (50  $\mu$ L) were incubated in 0.5 mL  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ -free Hank's balanced salt solution (HBSS) (Invitrogen, Auckland, NZ) containing 6 mmol/L KCl, and 6 mmol/L  $\text{MgCl}_2$  in the presence of 90  $\mu$ mol/L L-012 (Sigma, St. Louis, MO). After incubation for 3 min at 37°C, chemiluminescence intensity was recorded continuously for 20 min using the Photon Counter<sup>23</sup>; the ROS production was evaluated by the maximal chemiluminescence intensity.

**Determination of total glutathione.** Total glutathione (GSH + glutathione disulfide) levels were measured in red blood cells (RBCs) using the Glutathione Assay kit (Sigma CS0260) and following the manufacturer's instructions. Peripheral blood samples were collected in EDTA tubes, aliquots of 50  $\mu$ L of erythrocytes were washed in HBSS, and the samples were centrifuged at 600  $\times$  g for 1 min (4°C), and then

stored at  $-70^\circ\text{C}$  until use. To perform the assay, erythrocytes were deproteinated in 5% 5-sulfosalicylic acid, and centrifuged at 10,000  $\times$  g for 10 min at 25°C. The supernatant was collected and used for spectrophotometric analysis at 412 nm in a UV Sofmax automated plate reader. Each assay was performed in triplicate. The GSH levels were normalized as a ratio of total GSH per 1,000,000 RBCs.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA) and Epi Info 3.5.4 software (Centers for Disease Control and Prevention, Atlanta, GA). Clinical characteristics between patients with and without ARF were compared using Fisher's exact test or Mann-Whitney/Wilcoxon test for qualitative and quantitative data, respectively. As the ROS and GSH data were not normally distributed, they were plotted using box and whiskers plots, and statistical differences between groups were determined using Mann-Whitney-Wilcoxon test. Correlation analysis was performed using Spearman's test. Differences were considered significant at  $P \leq 0.05$ .

## RESULTS

**Patient characteristics.** The age range and sex distribution for patients with ARF, patients without ARF, and for the control group were 24–51 years (100% male), 17–61 years (80% male), and 21–39 years, (56% male), respectively (Table 1). Clinical characteristics of inpatients with and without ARF were similar, with the exception of elevated serum creatinine and urea in patients with ARF ( $P < 0.05$ ). (Table 1). The median days of symptoms at hospital admission was 6 (range: 5–10) days for patients with ARF compared with 4 (range: 1–15) days for patients without ARF. All patients survived. Patients with ARF had a higher frequency of oliguria (43% versus 20%), lower hematocrits (median of 27.9% versus 37.4%), and fewer platelets (median of 53,000/ $\mu$ L versus 142,000) at initial presentation, when compared with

TABLE 1  
Characteristics of studied patients

Characteristics	Total patients (N = 12)	Patients with ARF (N = 07)	Patients without ARF (N = 05)
<b>Demographics</b>			
Age	40.5 (17.0–61.0)	38.0 (24.0–51.0)	43.0 (17.0–61.0)
Male gender	11 (91.7)	7 (100.0)	4 (80.0)
<b>Clinical presentation</b>			
Days of symptoms before presentation	5.5 (1.0–15.0)	6.0 (5.0–10.0)	4.0 (1.0–15.0)
Jaundice	11 (91.7)	7 (100.0)	4 (80.0)
Oliguria	4 (33.3)	3 (42.9)	1 (20.0)
Serum creatinine (mg/dL)*	1.6 (0.5–6.2)	2.1 (1.6–6.2)	1.0 (0.5–1.4)
Urea (mg/dL)*	57.0 (30.0–290.0)	76.0 (57.0–290.0)	34.0 (30.0–53.0)
Potassium (mEq/L)	3.6 (2.8–4.9)	3.2 (2.8–4.9)	3.9 (3.2–4.8)
AST (U/L)	122.5 (30.0–567.0)	167.0 (50.0–567.0)	100.0 (30.0–201.0)
ALT (U/L)	69.0 (31.0–238.0)	73.0 (31.0–238.0)	65.0 (45.0–101.0)
Total WBC ( $\times 1,000/\mu\text{L}$ )	13.3 (7.3–21.2)	16.0 (7.3–21.2)	10.9 (8.1–19.0)
Hematocrit (%)	37.3 (22.5–46.7)	27.9 (22.5–46.7)	37.4 (34.7–42.6)
Hemoglobin (g/dL)	11.7 (7.0–14.8)	10.7 (7.0–14.8)	11.8 (11.2–13.9)
Platelets ( $\times 1,000/\mu\text{L}$ )	89.5 (10.0–549.0)	53.0 (10.0–297.0)	142.0 (32.0–549.0)
<b>Clinical course and outcomes</b>			
Treatment with antibiotics before hospitalization	0 (0.0)	0 (0.0)	0 (0.0)
Hemodialysis	3 (25.0)	3 (42.9)	0 (0.0)
Intensive care unit admission	2 (16.7)	1 (14.4)	1 (20.0)
Severe pulmonary hemorrhage syndrome	4 (33.3)	3 (42.9)	1 (20.0)

\*P-value (Mann-Whitney/Wilcoxon Test) for the comparison between patients with and without acute renal failure (ARF) was 0.004.  
N = number; ARF = acute renal failure; AST = aspartate aminotransferase; ALT = alanine aminotransferase; WBC = white blood cell.

hospitalized patients without ARF. In addition, patients with ARF had a higher frequency of severe pulmonary hemorrhage syndrome (42.9% versus 20%) and more frequently needed hemodialysis (42.9% versus 0%), when compared with hospitalized patients without ARF.

**High production of ROS and low level of GSH in severe leptospirosis patients.** The ROS production was significantly higher ( $P = 0.0003$ ) and GSH levels were significantly lower ( $P = 0.0002$ ) in leptospirosis patients compared with healthy subjects (Figure 1A and B). Subgroup analysis showed that severe leptospirosis patients with and without ARF had ROS production significantly higher than healthy subjects ( $P = 0.0012$  and  $P = 0.0043$ , respectively) (Figure 1C). However, no significant difference was observed in the production of ROS between patients with and without ARF (Figure 1C).

In contrast, GSH levels were significantly lower in severe leptospirosis patients with and without ARF ( $P = 0.0290$  and  $P = 0.0002$ , respectively) compared with healthy subjects (Figure 1D). No significant differences were observed in the levels of GSH among severe leptospirosis patients with and without ARF (Figure 1D).

**GSH levels from patients with severe leptospirosis correlate with renal dysfunction status.**

An additional analysis was performed to determine the correlation between oxidative stress and the following parameters: serum urea, creatinine, alanine transaminase, aspartate transaminase, serum potassium, and platelet count. We found a strong positive correlation between ROS production and serum potassium ( $r = 0.7975$ ;  $P = 0.0027$ ) (Figure 2A). In addition, GSH levels positively correlated with platelet count ( $r = 0.6294$ ;  $P = 0.0323$ ) (Figure 2B). Finally, a moderate negative correlation between serum creatinine and GSH levels ( $r = -0.6070$ ;  $P = 0.0385$ ) was also observed, meaning that higher serum creatinine levels are associated with lower GSH levels (Figure 2C). No significant differences were observed for the other parameters (data not shown).

## DISCUSSION

In this study, we investigated the association between markers of oxidative stress and target organ dysfunction in clinical leptospirosis. The GSH depletion has been reported

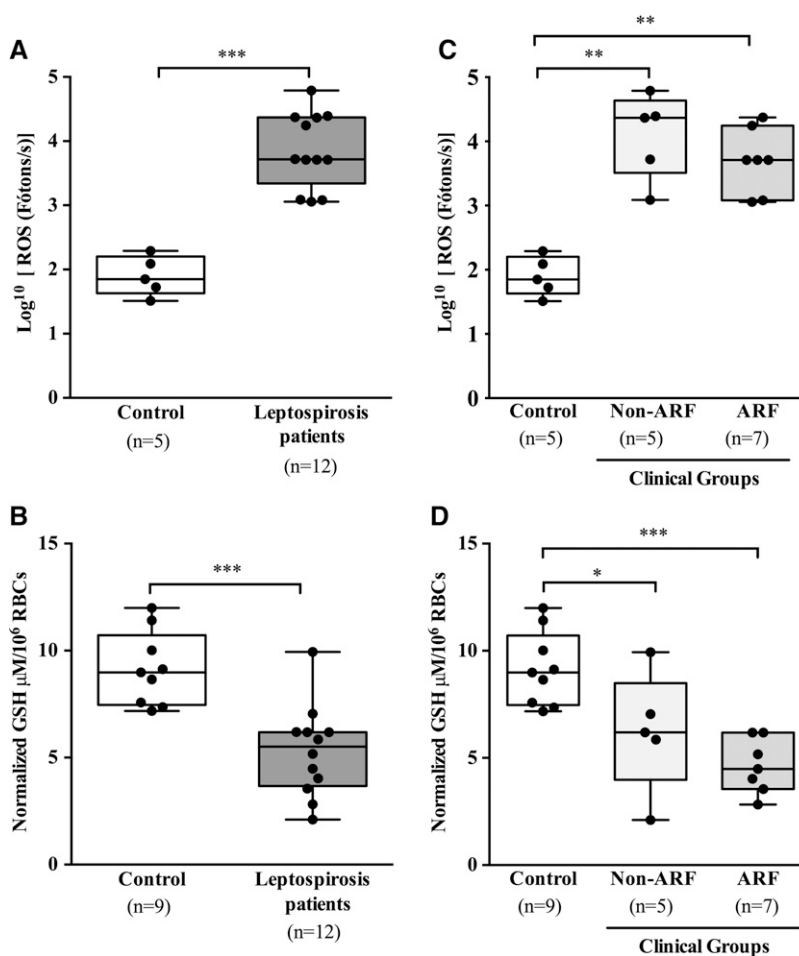


FIGURE 1. Oxidative stress in leptospirosis patients. (A) Reactive oxygen species (ROS) detection in peripheral blood samples of patients with severe leptospirosis at the day of admission and in five healthy control subjects (ROS detection was not possible for all controls caused by technical problems), and (C) clinical groups non-acute renal failure (non-ARF), acute renal failure (ARF), and control. (B) Glutathione (GSH) levels in erythrocytes samples of patients with severe leptospirosis at the day of admission and nine normal control individuals, and (D) clinical groups non-ARF, ARF, and control. The results are expressed as box plot format. The bottom, median, and top lines of the box mark the 25th, 50th, and 75th percentiles, respectively. The vertical line with whiskers shows the range of values. Significant differences at  $P < 0.05$ , Spearman's test are identified by the “\*” compared with the normal group. The GSH data were normalized per  $10^6$  red blood cells (RBCs).

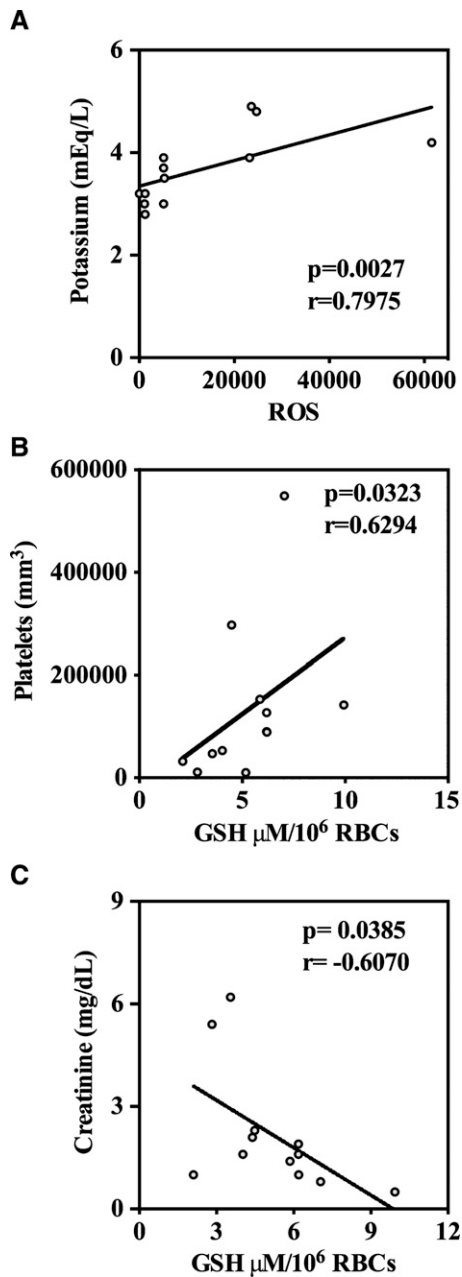


FIGURE 2. Correlation between serum potassium and reactive oxygen species (ROS) concentration in whole blood of leptospirosis patients (A); platelet count and glutathione (GSH) levels in erythrocyte of leptospirosis patients (B); and serum creatinine and GSH levels (C). The GSH data were normalized per  $10^6$  red blood cells (RBCs). Spearman's correlation indices ( $r$  and  $P$  values) are shown in the figure.

in cattle with clinical signs of leptospirosis<sup>24</sup>; the relationship between oxidative stress and clinical markers of severity has not been investigated in human leptospirosis. The present results agree with previous data from studies of sepsis from other Gram-negative infections. In these previous reports, oxidative stress was associated with clinical severity and the onset of multiple organ failure.<sup>15–18</sup> Oxidative stress in leptospirosis is probably a non-specific feature shared with many severe diseases with tissue damage from different etiologies. We did not test alternative control groups to test in which

extent oxidative stress in leptospirosis differs from other severe infectious diseases (e.g., Sepsis by gram negatives). Anyway, the associations identified in this study may be of relevance to seek molecular targets for adjuvant therapies.

The correlations between ROS and serum potassium, and GSH and serum creatinine are consistent with a previous report, where we described that serum nitric oxide level (another free radical) correlates with serum creatinine levels in patients with severe leptospirosis.<sup>11</sup> Although renal involvement in leptospirosis typically presents as hypokalemia without oliguria, if the disease progresses to oliguric renal failure, hyperkalemia is a strong predictor of unfavorable outcome.<sup>6,21</sup> Thrombocytopenia is a consistent feature of severe leptospirosis; however, its pathogenesis remains poorly understood. Diverse factors involved in host homeostasis such as excessive platelet activation, uremia, and disseminated intravascular coagulation have been implicated<sup>7</sup>; in this study, we identified oxidative stress as a potential contributory factor for platelet depletion in severe leptospirosis.

The proposed relation between oxidative stress and target organ dysfunction in leptospirosis, specifically renal failure and thrombocytopenia, opens the possibility of therapies focused on antioxidant defenses. As mentioned previously, our group previously tested the antioxidant NAC as an adjuvant treatment in leptospirosis. No objective effect on outcome was added from NAC treatment when compared with ampicillin treatment alone.<sup>12</sup> Antioxidant therapies have been more exhaustively tested in sepsis models and patients with no proven benefit.<sup>25–27</sup> More recently, NAC proved to be beneficial in the model of cecal ligation and puncture in rats preventing kidney and lung injuries, and leading to higher survival rates. In that study, NAC was administered in a higher dose (126 mg/day) and as a pre-treatment (2 days before induction of sepsis). Thus, in light of our findings of significant associations between markers of oxidative stress and clinical complications in patients, we proposed that further experimental studies (with different study designs) are needed to test adjuvant therapies for severe leptospirosis.<sup>28</sup>

In conclusion, we report that oxidative stress occurs in patients with severe leptospirosis and correlates with renal dysfunction and thrombocytopenia. These results encourage further studies in experimental models to explore the underlying physiopathology of leptospirosis. In addition, evaluation of new antioxidant strategies as adjuvant therapies in leptospirosis is urgently needed.

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