Leptospirosis leads to dysregulation of sodium transporters in the kidney and lung

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drosis manifests as pulmonary edema leading to acute respiratory
distress syndrome and polyuric acute renal failure (ARF). The etiol-
ogy of leptospirosis-induced pulmonary edema is unclear. Lung
edema clearance is largely affected by active sodium transport out
of the alveoli rather than by reversal of the Starling forces. The objective
of this study was to profile leptospirosis-induced ARF and pulmonary
edema. We inoculated hamsters with leptospires and collected 24-h
urine samples on postinoculation day 4. On day 5, the animals were
killed, whole blood was collected, and the kidneys and lungs were
removed. Immunoblotting was used to determine expression and
abundance of water and sodium transporters. Leptospirosis-induced
ARF resulted in natriuresis, lower creatinine clearance, and impaired
urinary concentrating ability. Renal expression of the sodium/hydro-
gen exchanger isoform 3 and of aquaporin 2 was lower in infected
animals, whereas that of the Na-K-2Cl cotransporter NKCC2 was
higher. Leptospirosis-induced lesions, predominantly in the proximal
tubule, were responsible for the polyuria and natriuresis observed. The
polyuria might also be attributed to reduced aquaporin 2 expression
and the attendant urinary concentrating defect. In the lungs, expres-
sion of the epithelial sodium channel was lower, and NKCC1 expres-
sion was upregulated. We found that leptospirosis profoundly influ-
ences the sodium transport capacity of alveolar epithelial cells and
that impaired pulmonary fluid handling can impair pulmonary func-
tion, increasing the chance of lung injury. Greater knowledge regard-
ingsodium transporter dysregulation in the lungs and kidneys can
provide new perspectives on leptospirosis treatment.

acute respiratory distress syndrome; acute renal failure

LEPTOSPIROSIS IS A SPIROCHEタル ZOONOSIS CAUSED BY PATHOGENIC Leptospira SPECIES. IN HUMANS, THE DISEASE PRESENTS AS AN ACUTE FEBRILE AND SYSTEMIC ILLNESS (15). leptospirosis is public health problem worldwide and is epidemic in some areas of Brazil during the rainy season. In 2004, the reported incidence of leptospirosis cases was 2.6/100,000 inhabitants in the city of São Paulo (5). leptospirosis typically affects young adults, mainly men, in their most economically productive years (8). The most severe form of leptospirosis (Weil’s disease) is a classic model of sepsis that includes acute respiratory distress syndrome (ARDS) and acute renal failure (ARF) (27). The mortality rates associated with Weil’s disease are still unacceptably high, reaching 55% among patients admitted to intensive care unit (11). The current treatment includes penicillin and supportive care (26). In areas where it is not epidemic, leptospirosis is occasionally misdiagnosed and managed as a pulmonary-renal syndrome. Leptospirosis can manifest as severe lung injury, characterized by diffuse alveolar hemorrhage and ARDS, accompanied by ARF, and can therefore be highly lethal (11). Leptospirosis-induced renal impairment constitutes a special form of ARF that is characterized by hypokalemia and elevated fractional excretion of potassium (25). Seguro et al. (25) showed that patients with leptospirosis frequently present polyuric ARF. In support of those findings, Magaldi et al. (10) found that, in guinea pigs infected with leptospirosis, the functional alterations in tubular cells precede a drop in the glomerular filtration rate. The same authors also showed that the inability to concentrate urine in leptospirosis-induced renal failure is due, at least in part, to inner medullary collecting duct resistance to vasopressin.

In the last two decades, there has been a marked increase in the incidence of pulmonary edema and hemorrhage as predominant features of leptospirosis. Such cases have been reported in China, Korea, Australia, Nicaragua, Argentina, Brazil, and India (3). Nevertheless, it has only recently been recognized that pulmonary edema/hemorrhage leading to ARDS constitutes the most prominent, and often fatal, manifestation of severe leptospirosis in humans. The noncardiogenic pulmonary edema seen in hamsters with leptospirosis is caused by edema of the alveolar septae and by endothelial cell swelling (19). Nally et al. (18) showed that guinea pigs infected with leptospirosis present no signs of systemic vasculitis. The etiology of leptospirosis-induced pulmonary edema and hemorrhage is still unclear.

Understanding the mechanisms that regulate the removal of sodium chloride and water from the distal air spaces of the lung is essential to comprehending the resolution of pulmonary edema in ARF. Resolution of pulmonary edema occurs as the result of active sodium transport across the alveolar epithelium via apical amiloride-sensitive sodium channels and via basolateral α-Na-K-ATPase (14). This active vectorial sodium flow produces a transepithelial osmotic gradient that results in passive movement of water from the air spaces into the alveolar interstitium. In some models of acute lung injury, as well as in patients with ARDS, the ability of the lungs to resolve edema is impaired (13, 24). Nevertheless, the effects of leptospirosis on alveolar fluid clearance, which is of potential importance in the setting of increased permeability, are unknown.

In view of the fact that ARF has repeatedly been shown to correlate strongly with ARDS in leptospirosis patients and that leptospirosis-induced ARF progresses to a unique type of
tubular dysfunction that includes potassium secretion and polyuria, we hypothesized that leptospirosis also leads to dysregulation of sodium transporters and aquaporins, potentially resulting in deleterious accumulation of sodium chloride and water in the lungs.

The objective of the present study was to analyze the exact mechanism of the urinary concentrating defect accompanying leptospirosis-induced polyuria, as well as to determine the mechanisms involved in leptospirosis-induced pulmonary edema. We test the hypothesis that reduced expression of the proteins encoding renal membrane transporters forms the molecular basis of this pulmonary-renal syndrome. To that end, we endeavored to establish whether leptospirosis-induced ARF affects the renal expression of aquaporin 2 (AQP2) or that of any of the major sodium transporters. To determine the impact of leptospirosis on the clearance mechanisms of pulmonary edema, we also investigated the effects of leptospirosis infection on pulmonary expression of the alpha subunit of the epithelial sodium channel (α-ENaC), as well as of α-Na-K-ATPase, aquaporin 5 (AQP5), and the Na-K-2Cl cotransporter NKCC1.

METHODS

Bacteria. Isolates of Leptospira pomona serovar Pomona were obtained from blood cultures of leptospirosis patients admitted to the Emílio Ribas Institute of Infectology in São Paulo, Brazil. All leptospire cultivation processes were carried out at the Adolfo Lutz Institute, also located in São Paulo. Cultures were maintained in EMJH liquid medium or on EMJH semisolid medium. To increase the virulence of the leptospires, the isolates were passaged through hamster hosts as described below.

Animals. Hamsters weighing 110–150 g were housed in conventional cages with access to standard rodent chow and fresh water ad libitum. A total of 25 hamsters were injected intraperitoneally with 0.5 mL of the leptospiral inoculum. Of those animals inoculated, only 13 lost mobility and became jaundiced. Those 13 animals were therefore classified as infected. The mortality in the hamster hosts was 22% (5/25). This was evidenced by the fact that serum levels of bilirubin and CPK were higher in infected animals than in control hamsters indicating that the infected animals had developed ARF (Table 1).

Preparation of membrane fractions. Samples of cortices, medullas, and lungs were homogenized in ice-cold isolation solution (200 mM mannitol, 80 mM HEPES, and 41 mM KOH, pH 7.5) containing protease inhibitors (protease inhibitor cocktail; Sigma, St. Louis, MO) using a Teflon pestle glass homogenizer (Schmidt, Frankfurt am Main, Germany). The homogenates were centrifuged at low speed (2,000 g) for 15 min at 4°C to remove nuclei and cell debris. Subsequently, the supernatants were spun at 100,000 g for 1 h at 4°C (70 Ti rotor; Beckman Coulter, Fullerton, CA), producing pellets containing membrane fractions enriched with plasma membranes and with intracellular vesicles. The pellets were suspended in isolation solution with protease inhibitors.

Electrophoresis and immunoblotting. Samples of membrane fractions were run either on 12.5% polyacrylamide gels (for AQP2 and AQP5), on 10% polyacrylamide minigels (for α-ENaC, α-Na-K-ATPase, and NHE3), or on 8% polyacrylamide minigels (for NKCC1, NKCC2, and NCC). After transfer by electroelution to nitrocellulose membranes (PolyScreen, PVDF Transfer; Life Science Products, Boston, MA), blots were blocked with 5% milk and 0.1% Tween 20 in PBS (8.7 g/l sodium chloride, 7.2 mM dibasic phosphate, and 2.8 mM monobasic phosphate) for 1 h. Blots were then incubated with one of the following: anti-AQP2 antibody (1:2,000), anti-AQP5 antibody (1:500); NKCC1 antibody (1:500); NKCC2 antibody (0.12 μg/ml); α-Na-K-ATPase antibody (in renal cortex and medulla, 1:1,000 in lung tissue, 1:500); α-ENaC antibody (in renal cortex, 1:1,000 in lung tissue, 1:250); NHE3 antibody (1:500). The labeling was visualized with horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG, diluted 1:2,000, or anti-goat IgG, diluted 1:10,000; Sigma) using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Piscataway, NJ). Quantitation of renal levels of AQP2, AQP5, and sodium transporters. The ECL films presenting bands within the linear range were scanned using Image Master VDS software (Pharmacia Biotech, Uppsala, Sweden). Quantitative analysis of antibodies was performed using densitometry. These bands were normalized to actin protein abundance.

Statistical analysis. All quantitative data are expressed as means ± SE. Differences among the means of multiple parameters were analyzed by ANOVA followed by the Student-Newman-Keuls test. Differences between two parameters were analyzed either by unpaired t-test or by nonparametric methods (Mann-Whitney U-test). Values of P < 0.05 were considered statistically significant.

RESULTS

Experimental infection. Over the course of the study, none of the animals lost weight (data not shown). Of the 25 animals inoculated, only 13 lost mobility and became jaundiced. Those 13 animals were therefore classified as infected. The mortality rate was 15.4% (2 of 13 infected animals died). The time to death among Leptospira-infected animals was 3–4 days.

Biochemical data on infected animals. Inoculated animals that developed leptospirosis presented infection by postinoculation day 5. This was evidenced by the fact that serum levels of bilirubin and CPK were higher in infected animals than in controls (Table 1).

Leptospirosis can result in ARF, including impaired handling of renal water, sodium, and potassium. Creatinine clearance was significantly lower in hamsters with leptospirosis than in control hamsters indicating that the infected animals had developed ARF (Table 1).
The leptospirosis seen in the infected animals was accompanied by altered renal handling of water, sodium, and potassium. Infected hamsters presented urine output that was significantly greater than that observed in the control hamsters (Table 1). The increased urine output was paralleled by significant increases in urinary sodium excretion and fractional excretion of sodium. Although infected animals presented significantly increased fractional excretion of potassium, there was no difference between infected animals and controls in terms of urinary potassium excretion (Table 1).

Infected animals presented markedly lower urine osmolality than did control animals (Table 1), indicating that urinary concentrating ability was severely impaired in the infected animals.

NHE3 protein abundance is lower in Leptospira-infected hamsters. In renal proximal tubules, transport of sodium through the proximal apical membrane is largely performed by the NHE3. We examined the expression of NHE3 protein in infected animals by isolating the protein from hamster cortices on postinoculation day 5. As shown in Fig. 1, NHE3 protein expression was significantly lower in infected animals than in controls (infected: 115.6 ± 5.46%; control: 100.1 ± 5.6%; P = 0.009).

Leptospirosis increases NKCC2 protein expression in the renal medulla. The apical Na-K-2Cl cotransporter is the major transporter for apical sodium reabsorption by the thick ascending limb. To determine whether leptospirosis alters NKCC2 protein expression, immunoblotting was performed on protein from the entire medulla. As indicated in Fig. 2, A and B, NKCC2 protein expression was significantly higher in the medullas of infected animals than in those of controls (infected: 149.2 ± 17.43%; control: 100 ± 7.2%, P = 0.04).

Abundance of NCC in the renal cortex is unchanged in leptospirosis-infected animals. The thiazide-sensitive Na-Cl cotransporter NCC, a known aldosterone target expressed in the distal convoluted tubule, was examined in infected animals. As indicated in Fig. 3, NCC protein expression was slightly, although not significantly, increased in the renal cortices of the infected animals (infected: 115.6 ± 11%; control: 99 ± 5.6%).

Abundance of α-ENaC in the renal cortex and of α-Na-K-ATPase in the renal cortex and medulla is unchanged in leptospirosis-infected animals. Figure 4, A and B, shows representative immunoblots of renal cortex homogenates probed with antibody against the α-ENaC channel. Abundance of α-ENaC was unaffected by leptospirosis infection (infected: 89.7 ± 11%; control: 99.8 ± 7.3%). Immunoblotting of the renal cortex and medulla homogenates was used to assess the relative abundance of α-Na-K-ATPase. Figure 5, A and B, displays representative immunoblots of cortical and medullary homogenates from hamsters euthanized on postinoculation day 5. Leptospirosis had no apparent affect on α-Na-K-ATPase abundance in the cortex (infected: 106 ± 5.46%; control: 99.97 ± 5.46%) or in the medulla (infected: 89.16 ± 6%; control: 100.1 ± 2.4%).

AQP2 abundance in the renal medulla is altered in response to leptospirosis infection. Semiquantitative immunoblotting, using membrane fractions prepared from the medullas harvested from control hamsters and infected hamsters, revealed a correlation between leptospirosis and reduced AQP2 expres-

**Table 1. Physiological data for control and infected animals on day 4 after inoculation with Leptospira Pomona**

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 7)</th>
<th>Lepto (n = 13)</th>
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<tbody>
<tr>
<td>CPK, U/l</td>
<td>767.3 ± 69.4</td>
<td>527.6 ± 155</td>
</tr>
<tr>
<td>Br, mg/dl</td>
<td>0.36 ± 0.04</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>Clscr, ml·min⁻¹·100 g body wt⁻¹</td>
<td>4.2 ± 1.0</td>
<td>2.5 ± 2.1</td>
</tr>
<tr>
<td>Uvol, ml/day</td>
<td>1293 ± 272</td>
<td>648 ± 117</td>
</tr>
<tr>
<td>Osm, mosmol/kg</td>
<td>358 ± 8</td>
<td>368 ± 8</td>
</tr>
<tr>
<td>FENa, %</td>
<td>0.11 ± 0.02</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>UVNa, meq/day</td>
<td>24 ± 2</td>
<td>42 ± 11</td>
</tr>
<tr>
<td>FEK, %</td>
<td>0.64 ± 0.07</td>
<td>0.71 ± 0.08</td>
</tr>
<tr>
<td>UVK, meq/day</td>
<td>0.02 ± 0.00</td>
<td>0.02 ± 0.00</td>
</tr>
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Values are means ± SE; Mann-Whitney U-test. n, Number of hamsters; Br, plasma levels of bilirubin; Clscr, creatinine clearance; Uvol, urinary volume; Osm, urinary osmolality; FENa, fractional excretion of sodium; FEK, fractional excretion of potassium; Lepto, leptospirosis; NS, not significant.
In the lungs, leptospirosis causes decreased expression of the $\text{ENaC}$ protein, and $\text{Na-K-ATPase}$ protein expression is unaffected. Active sodium transport across the alveolar epithelium creates an osmotic gradient that leads to fluid absorption from the alveolar space. Sodium ions enter the apical membranes of alveolar epithelial cells, in part, through $\text{ENaC}$ and are transported across the basolateral membrane by $\text{Na-K-ATPase}$. Western blotting of membrane preparations from the lung tissue of control and infected animals was used to assay expression of $\text{ENaC}$ and of $\text{Na-K-ATPase}$. In contrast to the results obtained for $\text{ENaC}$ protein expression in the renal cortices of infected animals, leptospirosis induced decreased protein expression of $\text{ENaC}$ in lung tissue (infected: 68.46 ± 6.2%; control: 103 ± 5.1%, $P = 0.0015$; Fig. 7, A and B). Similar to what was seen in the renal cortex and medulla, $\text{Na-K-ATPase}$ protein expression in the lung tissue of infected animals was not significantly different from that seen in controls (infected: 101.3 ± 4.53; control: 100 ± 8.12; Fig. 5C).

NKCC1 protein expression is upregulated in the lungs of infected animals. The NKCC1 cotransporter is responsible for maintaining cell volume and is activated by cell shrinkage. Immunoblotting was performed on protein from the lung. As

Fig. 3. Semiquantitative immunoblotting of membrane fractions prepared from renal cortices tissue. A: densitometric analysis revealing NCC abundance in the kidneys of infected animals. B: immunoblots reacted with anti-NCC revealing a 165-kDa band.

Fig. 4. $\alpha$-ENaC protein expression was assessed by Western blot analysis. A: results were quantitated through densitometry in kidney cortices. B: specific band was detected in all lanes.

Fig. 5. Immunoblot of $\alpha$-Na-K-ATPase in renal cortices (A), renal medullas (B), and lung tissue (C). Immunoblots reacted with $\alpha$-Na-K-ATPase revealing an 86-kDa $\alpha$-Na-K-ATPase band.

Fig. 6. Semiquantitative immunoblotting of membrane fractions prepared from renal medullas and lung tissue. A: densitometric analysis revealing decreased AQP2 abundance in the medullas of infected animals. B: immunoblots reacted with anti-AQP2 revealing 29- and 35- to 50-kDa AQP2 bands. C: densitometric analysis showing AQP5 protein expression in lung tissue. D: immunoblots reacted with anti-AQP5 revealing a 28-kDa band.
in leptospirosis is frequently followed by high urine output, as and clinical characteristics. The decreased renal function seen increases in serum CPK, a finding that is characteristic of rhabdomyolysis. The animal model used in the present study mimics the loss of mobility and jaundice that occurs in human patients with leptospirosis. The animals studied also presented significant increases in serum CPK, a finding that is characteristic of rhabdomyolysis.

Leptospirosis-induced ARF presents distinct morphological and clinical characteristics. The decreased renal function seen in leptospirosis is frequently followed by high urine output, as well as by high fractional excretion of sodium and potassium (10, 25). We demonstrated that there was a significant decrease in NHE3 protein expression, which can partially explain the polyuria and might completely explain the high fractional excretion of sodium. However, we cannot automatically assume that the NHE3 activity is decreased to the same extent in the proximal tubule, although it is a certainly a possibility. Apical NHE3 plays an important role in reabsorption of sodium and fluid by the proximal tubule (1). As a consequence of the decreased NHE3 protein expression, fluid delivery to the distal nephron might be increased. We demonstrated a marked increase in NKCC2 band density. It is possible that this might represent a compensatory response to the greater sodium chloride and water delivery. It has been shown that sodium transporter abundance is upregulated in response to chronic diuretic treatment in rats (16). However, since the glomerular filtration rate is reduced, the filtered sodium load might also be lower in infected animals. Considering the possibility that fluid delivery to the distal nephron might not be increased, the increased NKCC2 protein expression could be due to increased vasoressin levels in infected animals. The protein expression of NCC and α-ENaC, both regulated by aldosterone, was unchanged in the renal cortices of infected animals. We can speculate that the renin-angiotensin-aldosterone system is not involved in this animal model of leptospirosis, although it is equally possible that these transporters are, for whatever reason, unresponsive to aldosterone.

We also demonstrated that there was no change in α-Na-K-ATPase expression in the renal cortex or in the renal medulla. Younes-Ibrahim et al. (31) showed that the glycolipoprotein fraction extracted from L. interrogans contains an inhibitor of renal α-Na-K-ATPase. However, since those authors inoculated animals with the glycolipoprotein fraction only, the model developed was different from that used in our study, thereby precluding any direct comparisons between the two. The fact that leptospirosis, which causes structural damage and NHE3 suppression, also induced increased expression of NKCC2 without altering expression of α-ENaC or α-Na-K-ATPase suggests that the severity of cell injury per se cannot explain the differential regulation of these membrane cell transporters. This phenomenon has also been reported in other models of ARF (9).
Notably, the leptospirosis-infected animals evaluated in the present study exhibited significant increases in urinary volume and significant decreases in urinary osmolality, findings that are consistent with urinary concentrating defect. Similarly, we found that leptospirosis downregulated the expression of AQP2. In a normal state, urine is concentrated as a result of the combined functions of Henle’s loop and the collecting duct (22, 23). In the present study, we demonstrated a marked increase in the expression of NKCC2 cotransporter. We also found that AQP2 expression in the renal medulla was decreased by 50%. Since the NKCC2 cotransporter might not induce any apparent defect in the generation of medullary interstitial hypertonicity, it is possible that the decrease in water channel expression in the collecting duct is due to a direct effect of leptospirosis on the collecting duct cells.

In human patients, leptospirosis has many presentations, including the severe pulmonary (ARDS) form, which is characterized by impairment of the alveolar-capillary barrier. The etiology of pulmonary edema in ARDS is unclear. It has been reported that pulmonary edema clearance is greatly affected by active sodium transport out of the alveoli rather than by reversal of the Starling forces (14).

One of the principal findings of our study was that leptospirosis infection decreased α-ENaC protein expression. In addition, we found that basolateral NKCC1 was upregulated. Furthermore, we demonstrated that AQPS and α-Na-K-ATPase protein expression were unchanged in the lung tissue of hamsters infected with leptospirosis. These results demonstrate for the first time the effects of leptospirosis on alveolar ion transporters. Since these transporters have been shown to play a vital role in the maintenance of alveolar fluid (12), our results are of potential clinical significance. Our data show that leptospirosis has a profound influence on the sodium transport capacity of alveolar epithelial cells. Impaired pulmonary fluid handling can hinder lung function and increase the susceptibility of the lung to injury (24). Active transport by the α-Na-K-ATPase pump generates an osmotic driving force favorable to the entrance of sodium via α-ENaC. There is therefore continuous transport of sodium from the lumen into the interstitial space (2). Despite the presence of AQP5, the osmotic gradient between the lumen and the interstitial space promotes the movement of water via the paracellular pathway (4). Volume is regulated primarily by electroneutral cotransporters such as NKCC1, which is found in virtually all cells and mediates coupled influx of sodium, potassium, and chloride. The mechanism by which cell shrinkage activates NKCC1 is unknown (20). We hypothesized that leptospirosis induces a decrease in alveolar clearance by decreasing α-ENaC protein abundance, which reduces the transport of sodium from the lumen into the interstitial space, as well as decreasing the movement of water from the lumen into the interstitial space, thereby lowering the osmotic gradient. The cell shrinkage induced by this mechanism can stimulate the NKCC1 protein in the basolateral membrane. In turn, NKCC1 mediates the coupled influx of sodium, potassium, and chloride into the epithelial cells. The decreased influx of sodium from the lumen into the cells (induced by the lower levels of α-ENaC), together with the increased influx of sodium from the interstitial space into the cells (induced by the higher levels of NKCC1), can block the net influx of sodium and water from the alveoli (Fig. 9). Impaired pulmonary fluid clearance resulting from downregulated α-ENaC expression, as well as the potential derangements related to increased NKCC1 expression, might have significant deleterious effects in the context of increased pulmonary permeability such as that observed in ARDS. These effects would be expected to increase susceptibility to ventil-
tor-induced lung injury, an entity which is of increasing concern in intensive care settings (7, 21). The exact mechanism by which leptospirosis affects the expression of transporter membrane proteins has yet to be investigated. A direct effect of leptospirosis on the transporter proteins cannot be ruled out. However, leptospirosis can induce hypoxia. It has clearly been shown that, both in vitro and in vivo, hypoxia reduces the capacity of alveolar epithelial cells to actively transport sodium (29). Leptospirosis has also been shown to interfere with the NF-κB system and to induce apoptosis by triggering crosstalk between the JNK and NF-κB signaling pathways (30). Both mechanisms are also likely to play a role in the dysregulation of these sodium transporters. In multiple organ dysfunction syndrome, ARF is an additional independent risk factor for mortality (28). It has been repeatedly demonstrated that there is a strong correlation between ARF and ARDS (6).

Greater knowledge regarding the dysregulation of sodium transporters in the lungs and kidneys could provide new perspectives on corticosteroid treatment of leptospirosis (17). In summary, our results show that leptospirosis decreases expression of the principal sodium transporters in the lungs and kidneys, as well as decreasing AQP2 expression in the renal medulla. Therefore, further study of renal pulmonary interaction in Weil’s disease might prove fruitful in the effort to reduce the unacceptably high mortality rates seen among patients with leptospirosis.

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