N-acetylcysteine prevents pulmonary edema and acute kidney injury in rats with sepsis submitted to mechanical ventilation

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SEPSIS IS A CLINICAL SYNDROME caused by systemic inflammation responses to infection and to various molecular mechanisms of cell injury associated with multiple organ failure (21, 41). In critically ill patients, crosstalk between the kidney and lungs is common. Among patients with the combination of acute kidney injury (AKI) and acute lung injury (ALI), the mortality rate is 80% (15).

Sepsis is recognized as a common cause of acute respiratory distress syndrome (ARDS) and ALI (20). In the lungs, the initial alterations are increases in vascular permeability, congestion, and inflammation, together with intense activity of endogenous mediators and interstitial swelling (31). Although a positive water balance is considered a major predictor of outcome in critically ill patients, pulmonary edema can occur even when the water balance is normal or negative. Rabb et al. (28) showed that, in rats without lung injury and submitted to bilateral nephrectomy, there is a decrease in sodium cotransporter expression, followed by increases in vascular permeability and interstitial edema, providing evidence of the crosstalk between the lungs and kidneys. Resolution of pulmonary edema occurs as the result of active sodium transport across the alveolar epithelium via apical and basolateral sodium channels, a process known as alveolar fluid clearance, which is impaired in some models of ALI, as well as in some models of ARDS (18, 33). The α-subunit of the epithelial sodium channel (α-ENaC) and Na-K-2Cl (NKCC1) are sodium cotransporters that play key roles in this process.

Sepsis is a well-established risk factor for AKI. The pathophysiology of sepsis-associated AKI is complex and multifactorial. Traditionally, AKI in sepsis was thought to result from renal ischemia secondary to vasoconstriction and low renal blood flow. However, in hyperdynamic states, such as septic shock, the hemodynamic alterations in the kidney appear to be heterogeneous and renal blood flow remains high or normal (23). Therefore, renal alterations can occur regardless of the level of renal blood flow.

Oxidative stress plays an important role in the pathogenesis of sepsis. Sepsis-induced inflammation and infection release reactive oxygen species (ROS), leading to the production of free radicals that are harmful to all organs (32). ROS inactivate nitric oxide (NO), thus increasing inflammation and endothelial dysfunction by stimulating polymorphonuclear cells.

The plasma level of thiobarbituric acid reactive substances (TBARS) is a marker of oxidative stress. In a rat model of cecal ligation and puncture (CLP)-induced sepsis, Ritter et al. (30) reported that the levels of oxidative stress (plasma TBARS) were significantly higher in nonsurvivors than in survivors.

It has been suggested that antioxidants would be useful in treating cases of sepsis in which the lungs and kidneys are the organs most affected. To date, N-acetylcysteine (NAC) has been the most widely studied antioxidant (26). NAC can restore the antioxidant potential of cells, decrease cytokine production, downregulate the expression adhesion molecules, restore NO production, decrease bacterial translocation, and reduce ROS production, thus improving survival (30, 35, 42).

Mechanical ventilation is an important tool for the treatment of patients with sepsis because it can normalize arterial blood...
gases and unload the respiratory muscles. Despite such benefits, the use of positive pressure in mechanical ventilation can impair renal function by decreasing the glomerular filtration rate (GFR), as well as reducing cardiac and urinary output. In patients on mechanical ventilation, alveolar recruitment/derecruitment, shear forces, and ventilator-associated pneumonia can all cause lung injury.

The objective of the present study was to evaluate the effects of NAC on renal and pulmonary function in rats with sepsis submitted to mechanical ventilation.

MATERIALS AND METHODS

Animals. Male Wistar rats, weighing 200–250 g, were obtained from the animal facilities of the University of São Paulo School of Medicine, housed in standard cages, and given ad libitum access to rodent chow and water. Rats were randomly allocated to the following groups: control, control + NAC, CLP, and CLP + NAC. We used separate sets of animals to evaluate pulmonary and renal function, determining the following parameters: airway resistance, tissue resistance, lung elastance, peak pressure, wet-to-dry lung weight ratio, the arterial oxygen tension/fraction of inspired oxygen (PaO2/FIO2) ratio, as well as again receiving 25 ml/kg BW of saline solution at 6 and 12 h post-CLP.

NAC administration. NAC was administered orally (in water) for 2 days before the surgical procedure and was maintained until the day of study. The NAC dose was 4.8 g/l of drinking water. This dose of NAC has been used in other animal studies, in which it has been shown to provide benefits (5, 34). The effective daily dose of NAC was determined on the day of the surgical procedure.

Experimental model of sepsis. Rats were anesthetized by intraperitoneal injection of 2.5% tribromoethanol [1 ml/100 g body weight (BW)]. Via laparotomy, the cecal tip was ligated at 1.5 cm below the ileocecal valve. The cecum was punctured twice with a 16-gauge needle and gently squeezed to express feces, after which the abdomen was closed with sutures. In the immediate post-CLP period, animals were given saline solution (25 ml/kg BW ip) to replace the fluid losses due to the surgical procedure. All animals received a broad spectrum antibiotic (imipenem/cilastatin; 14 mg/kg BW, administered sc), as well as again receiving 25 ml/kg BW of saline solution at 6 and 12 h post-CLP. Animals were studied at 26 h post-CLP. The CLP procedure is diagramed in Fig. 1.

Mechanical ventilation and respiratory mechanics. All animals were submitted to low-tidal-volume mechanical ventilation (LV T-MV) for 2 h. At 24 h post-CLP, animals were anesthetized with thiopental (50 mg/kg BW ip) and underwent tracheotomy after which they were connected to a small-animal ventilator (flexiVent; SCIREQ Scientific Respiratory Equipment, Montreal, QC, Canada). Pancuronium (1 ml/kg BW ip) was then administered. We defined LV T-MV as a positive end-expiratory pressure (PEEP) of 6 cmH2O, an FIO2 of 50%, a respiratory rate of 90 breaths/min, and a tidal volume of 8 ml/kg. These parameters were maintained throughout the ventilation period. To determine the magnitude of the effects of LV T-MV on renal function, other animals were evaluated during spontaneous breathing post-CLP.

At 30 min after the onset of LV T-MV and at the end of the 2-h ventilation period, respiratory mechanics were evaluated. We estimated respiratory system impedance by the forced oscillation technique, using 16-s perturbation volume signals with frequency components from 0.25 to 19.625 Hz. To separate airway resistance and tissue mechanics, we employed a constant phase tissue model, evaluating parenchymal damping and tissue elastance (3).

Lung histology. After 2 h on LV T-MV, animals were anesthetized and killed after which lung tissue was perfused under controlled pressure with PBS (0.15 M NaCl and 0.01 M phosphate buffer, pH 7.4) at room temperature. The right lung (basal region) was cut into small fragments and fixed in 10% buffered formalin for 24 h. The tissue was then transferred to 70% ethanol for subsequent embedding in paraffin. The lung fragments were cut into 4-μm sections and stained with hematoxylin and eosin (H&E). The edema index and polymorphonuclear neutrophils were evaluated as described below.

Edema index and wet-to-dry lung weight ratio. The edema index, which is determined by microscopy, can provide evidence of pulmonary lesion, such as alveolar edema, alveolar septal thickening, and perivascular cuffing (27).

We photographed lung tissue slides stained with H&E, specifically the areas containing vessels, using a light microscope at a magnification of ×40. To minimize the confounding effects of distorted and collapsed vessels, the circularity of each vessel was also measured and those with a circularity of <0.6 were excluded. Subsequently, the ratio between the perivascular cuff area and the total area of the vessel was calculated, which provided the edema index (4).

To quantify the water content in lung tissue, we evaluated the wet-to-dry lung weight ratio. To that end, we weighed the lungs immediately after their removal (obtaining the wet lung weight). We then dried the lungs in an oven at 60°C for 2 days, after which we weighed them again (obtaining the dry weight). By determining the relationship between those two measurements, we calculated the wet-to-dry lung weight ratio.

Polymorphonuclear neutrophils. Slides stained with H&E were evaluated under microscopy at a magnification of ×100. Using a 100-point grid with a known area (10,000 μm2 at a magnification of ×1,000) attached to the microscope objective, we calculated the number of points touching the lung periphery. The lung area in each field was calculated, according to the number of points touching the lung, as a proportion of the total grid area. We counted the number of neutrophils in 20 fields per slide. Cell density was determined by dividing the number of stained cells in each field by the total tissue area.
Control

200,000 cells were centrifuged at low speed (2,000 g) for 15 min at 4°C to remove nuclei and cells debris. Subsequently, the supernatants were spun at 200,000 g for 1 h at 4°C (70 Ti rotor; Beckman Coulter, Fullerton, CA), producing pellets containing membrane fractions. The pellets were resuspended in the isolation solution containing protease inhibitors (protease inhibitor cocktail; Sigma, St. Louis, MO). The homogenates were centrifuged at low speed (2,000 g) for 15 min at 4°C to remove nuclei and cells debris. Subsequently, the supernatants were spun at 200,000 g for 1 h at 4°C (70 Ti rotor; Beckman Coulter, Fullerton, CA), producing pellets containing membrane fractions. The pellets were resuspended in the isolation solution containing protease inhibitors.

Preparation of membrane fractions. After 2 h of LVT-MV, lung tissue was removed, frozen in liquid nitrogen, and stored at −80°C until analysis. Using a Teflon pestle glass homogenizer (Schmidt, CA), producing pellets containing membrane fractions. The pellets were suspended in the isolation solution containing protease inhibitors (protease inhibitor cocktail; Sigma, St. Louis, MO). The homogenates were centrifuged at low speed (2,000 g) for 15 min at 4°C to remove nuclei and cells debris. Subsequently, the supernatants were spun at 200,000 g for 1 h at 4°C (70 Ti rotor; Beckman Coulter, Fullerton, CA), producing pellets containing membrane fractions. The pellets were resuspended in the isolation solution containing protease inhibitors.

Electrophoresis and immunoblotting. For the determination of NKCC1 and α-ENaC expression, membrane fractions were electrophoresed on 10% polyacrylamide gels. After transfer by electroblotting to the nitrocellulose membrane (PolyScreen, PVDF Transfer; Life Science Products, Boston, MA), blots were blocked for 1 h with 5% skim milk (w/v) and 0.1% Tween-20 (v/v) in 0.1 mol/l Tris-buffered saline (TBS, pH 7.5). Blots were then incubated with NKCC1 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) or α-ENaC antibody (1:250; Santa Cruz Biotechnology). Labeling was visualized with horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG, diluted 1:1,000 or anti-goat, diluted 1:5,000; Sigma) in an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

Enhanced chemiluminescence films presenting bands within the linear range were scanned, and quantitative analysis of antibodies was performed by densitometry with ImageJ software (National Institutes of Health, Bethesda, MD). The bands were normalized to actin protein abundance.

Immunohistochemical study. Four-micrometer sections of lungs and kidneys were fixed in 10% buffered formalin or methacarn and processed for paraffin embedding. Primary antibodies included polyclonal 8-isoprostane (anti-8-epi-PGF2α; Oxford Biomedical Research, Oxford, MI), polyclonal anti-neutrophil elastase (Santa Cruz Biotechnology), and monoclonal anti-rat CD68 (ED1; AbD Serotec, Oxford, UK). The sections were incubated overnight at 4°C, and the reaction product was detected with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). The color reaction was developed with 3,3-diaminobenzidine (Sigma), after which the material was counterstained with methyl green, dehydrated, and mounted. To obtain the mean numbers of ED1-positive macrophages infiltrating the renal cortical tubulointerstitium, we evaluated 50–60 grid fields (measuring 0.087 mm² each) and calculated the mean counts per kidney (38). To evaluate the immunostaining for 8-isoprostane, the proportions of positive areas in the tissue sections were determined by image analysis with Image-Pro Plus software, version 4.1 for Windows (Media Cybernetics, Silver Spring, MD) on a computer connected to a digital camera and coupled to a microscope (Axiioskop 40; Carl Zeiss, Göttingen, Germany). Volume proportion is expressed as the area of the tissue section tested positive for 8-isoprostane divided by the total area of the section (13).

Inulin clearance. To determine GFR, inulin clearance studies were conducted. On the day of experiment, the animals were anesthetized by intraperitoneal injection of thiopental (50 mg/kg BW). The trachea was cannulated with a PE-240 catheter. Animals were connected to LVT-MV, and pancerulon was administered. To monitor MAP and allow arterial blood sampling during the LVT-MV, a PE-60 catheter was inserted into the right carotid artery. For the infusion of inulin and fluids, another PE-60 catheter was inserted into the left jugular vein. To collect urine samples, a suprapubic incision was made, and the urinary bladder was cannulated with a PE-240 catheter. After the surgical procedure had been completed, a loading dose of inulin (100 mg/kg BW diluted in 0.9% saline) was administered through the jugular vein. Constant infusion of inulin (10 mg/kg BW diluted in 0.9% saline) was then started and was continued at 0.04 ml/min throughout the experiment. Urine samples were collected at 30-min intervals (total, 4 samples/animal). Blood and urine inulin were determined using the anthrone method. GFR is expressed as milliliters per minute per 100 g BW. The animals were on LVT-MV throughout the inulin clearance procedure.

Arterial blood sampling and hemodynamic evaluation. Blood was obtained from the carotid artery. Arterial blood gases were analyzed with a blood gas analyzer (ABL800 FLEX; Radiometer, Copenhagen, Denmark). In addition, heart rate and MAP were continually monitored with an invasive constant monitoring probe (MP100; Biopac Systems, Goleta, CA) inserted into the carotid artery. Reactive oxygen metabolites. Plasma levels of TBARS, which constitute a marker of lipid peroxidation, were determined using a thiobarbituric acid assay. In brief, a 0.2-ml plasma sample was diluted in 0.8 ml of distilled water. Immediately thereafter, 1 ml of 17.5% trichloroacetic acid was added. Subsequently, 1 ml of 0.6% thiobarbituric acid, pH 2, was added, and the sample was placed in a boiling water bath for 15 min, after which it was allowed to cool. We then added 1 ml of 70% trichloroacetic acid and incubated the mixture for 20 min. The sample was then centrifuged for 15 min at 2,000 rpm. The optical density of the supernatant was read at 534 nm against a reagent blank using a spectrophotometer. The quantity of TBARS was calculated using a molar extinction coefficient of 1.56 × 10^5·M⁻¹·cm⁻¹.

Table 1. Respiratory mechanics and renal function in control animals

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Control + NAC</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cin, ml·kg⁻¹·100 g BW⁻¹</td>
<td>0.83 ± 0.04</td>
<td>0.99 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>120.7 ± 5.0</td>
<td>120.0 ± 4.0</td>
<td>NS</td>
</tr>
<tr>
<td>Raw, cmH₂O·s/ml</td>
<td>0.01 ± 0.001</td>
<td>0.01 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Rti, cmH₂O/ml</td>
<td>0.33 ± 0.05</td>
<td>0.32 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>EL, cmH₂O/ml</td>
<td>1.22 ± 0.07</td>
<td>1.34 ± 0.17</td>
<td>NS</td>
</tr>
<tr>
<td>Peak pressure, cmH₂O</td>
<td>9.0 ± 0.25</td>
<td>8.5 ± 0.22</td>
<td>NS</td>
</tr>
<tr>
<td>W/D, g·H₂O⁻¹·1·g⁻¹</td>
<td>4.89 ± 0.08</td>
<td>4.81 ± 0.09</td>
<td>NS</td>
</tr>
<tr>
<td>TBARS, mmol/ml</td>
<td>2.04 ± 0.20</td>
<td>1.26 ± 0.38</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE. NAC, N-acetylcysteine; Cin, inulin clearance; BW, body weight; MAP, mean arterial pressure; Raw, airway resistance; Rti, tissue resistance; EL, lung elastance; W/D, wet-to-dry (lung weight) ratio; TBARS, thiobarbituric acid reactive substances; NS, not significant.

Table 2. Respiratory mechanics at the onset of (30 min into) a 2-h session of low-tidal-volume mechanical ventilation

<table>
<thead>
<tr>
<th>Group</th>
<th>Raw, cmH₂O·s/ml</th>
<th>EL, cmH₂O/ml</th>
<th>Rti, cmH₂O/ml</th>
<th>PP, cmH₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 7)</td>
<td>0.014 ± 0.001</td>
<td>1.73 ± 0.20</td>
<td>0.39 ± 0.02</td>
<td>9.40 ± 0.20</td>
</tr>
<tr>
<td>Control + NAC (n = 6)</td>
<td>0.01 ± 0.003</td>
<td>1.34 ± 0.17a</td>
<td>0.32 ± 0.01</td>
<td>8.00 ± 0.10</td>
</tr>
<tr>
<td>CLP (n = 7)</td>
<td>0.028 ± 0.001b</td>
<td>2.71 ± 0.14ed</td>
<td>0.53 ± 0.02a</td>
<td>10.20 ± 0.19g</td>
</tr>
<tr>
<td>CLP + NAC (n = 6)</td>
<td>0.015 ± 0.005</td>
<td>2.08 ± 0.15</td>
<td>0.40 ± 0.01</td>
<td>9.10 ± 0.18</td>
</tr>
</tbody>
</table>

Values are means ± SE. PP, peak pressure; CLP, cecal ligation and puncture. *P < 0.05 vs. CLP+NAC; **P < 0.001 vs. control + NAC, control, and CLP + NAC; †P < 0.001 vs. control + NAC + control; ‡P < 0.05 vs. CLP + NAC + control; §P < 0.001 vs. control + NAC, control, and CLP + NAC; ††P < 0.001 vs. control + NAC + control + CLP + NAC; ‡‡P < 0.05 vs. control.
Table 3. Respiratory mechanics at the end of a 2-h session of low-tidal-volume mechanical ventilation

<table>
<thead>
<tr>
<th>Group</th>
<th>Raw, cmH2O·s/ml</th>
<th>EL, cmH2O/ml</th>
<th>Rti, cmH2O/ml</th>
<th>PP, cmH2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 7)</td>
<td>0.01 ± 0.002</td>
<td>2.27 ± 0.23</td>
<td>0.49 ± 0.06</td>
<td>10.2 ± 0.20</td>
</tr>
<tr>
<td>Control + NAC (n = 6)</td>
<td>0.01 ± 0.003</td>
<td>2.03 ± 0.13</td>
<td>0.46 ± 0.04</td>
<td>9.66 ± 0.33</td>
</tr>
<tr>
<td>CLP (n = 7)</td>
<td>0.03 ± 0.005b</td>
<td>3.53 ± 0.50b,c</td>
<td>0.52 ± 0.16</td>
<td>10.2 ± 0.25</td>
</tr>
<tr>
<td>CLP + NAC (n = 6)</td>
<td>0.01 ± 0.003</td>
<td>2.14 ± 0.14</td>
<td>0.41 ± 0.05</td>
<td>9.7 ± 0.25</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.001 vs. control, control + NAC, and CLP + NAC; †P < 0.05 vs. control + NAC and CLP + NAC; ‡P < 0.01 vs. control.

Plasma levels of TBARS are expressed as nanomoles per milliliters (25).

Survival analysis. For the analysis of the survival curve, we used four groups: control (n = 10), control + NAC (n = 10), CLP (n = 25), and CLP + NAC (n = 15). Animals were monitored for 72 h post-CLP. Imipenem-cilastatin (14 mg/kg BW) and saline solution (25 ml/kg BW) were administered subcutaneously in the first 6 h after CLP and every 12 h thereafter until the end of the follow-up period.

Statistical analysis. All quantitative data are expressed as means ± SE. Differences among multiple parameters were analyzed by one-way ANOVA followed by the Student-Newman-Keuls post test. The level of statistical significance was set at P < 0.05.

RESULTS

NAC dose. The ingestion of water was similar between the two groups. The mean effective daily dose of NAC was 126 mg in the CLP + NAC group and 123 mg in the control + NAC group. No adverse effects were observed in any of the NAC-treated rats.

Initial effects of NAC on renal and pulmonary function. To evaluate the effects of NAC in the lung and kidneys, we studied inulin clearance, MAP, respiratory mechanics, wet-to-dry lung weight ratio, and oxidative stress (plasma level of TBARS) in the control + NAC group. However, as can be seen in Table 1, the levels of oxidative stress were lower in the control + NAC group than in the control group (P < 0.05).

With the exception of plasma TBARS, the biochemical parameters in the control + NAC group did not differ significantly from those observed in the other groups. Therefore, the remaining parameters were not determined in the control + NAC group.

Respiratory mechanics. Respiratory mechanics were evaluated at the onset and at the end of LV7-MV (Tables 2 and 3, respectively). We found that respiratory mechanics were most severely impaired in the CLP + NAC rats, which showed increases in airway resistance and lung elastance throughout the ventilation period. In addition, at the onset of LV7-MV, peak pressure was elevated in the CLP group. In contrast, there were no alterations in respiratory mechanics in the CLP + NAC group.

Biochemical and physiological parameters. Lactate, pH, and the PaO2/FIO2 ratio were evaluated in arterial blood samples collected after 30 min of LV7-MV (Table 4). In the CLP group, we observed the development of ALI, acidosis, and higher lactate levels, all of which occurred to a lesser degree in the CLP + NAC group. For the PaO2/FIO2 ratio, the difference between the CLP group and the CLP + NAC group was significant (P < 0.05).

As expected, CLP-induced peritonitis resulted in severe hypotension in the CLP group. However, no hypotension was observed in the CLP + NAC group. Nevertheless, heart rates were higher in the CLP and CLP + NAC groups than in the control group (Table 4).

Edema index and wet-to-dry lung weight ratio. The quantification of edema was shown in Fig. 2A. The degree of edema (edema index) was greater in the CLP group than in the control group (18.94 ± 1.71 vs. 13.9 ± 0.75 μm; P < 0.05) and was actually lower in the CLP + NAC group (12.47 ± 0.73 μm; P < 0.001 vs. CLP). The histological analysis (Fig. 2B) showed that there was more perivascular cuffing in the CLP group than in the control and CLP + NAC groups.

The water content in lung tissue (wet-to-dry lung weight ratio) was significantly lower in the CLP + NAC group than in the control group (4.50 ± 0.07 vs. 4.89 ± 0.08 g H2O/g dry weight; P = 0.02). The wet-to-dry lung weight ratio in the control group was comparable to that observed in the control + NAC group (4.81 ± 0.09 g H2O/g dry weight).

Polymorphonuclear neutrophils. In the CLP group, inflammatory cell counts were high (764.4 ± 39.3 cells/mm2), and the difference compared with the control group was significant (P = 0.01). In the CLP + NAC group, the number of polymorphonuclear cells (654.9 ± 96.08 cells/mm2) was lower than that observed in the CLP group, although the difference was not significant (Fig. 3).

Renal tissue inflammation. Renal inflammation was evaluated by quantifying ED1-positive macrophages. There was significantly greater inflammation in the CLP group than in the control group (13.26 ± 1.25 vs. 4.70 ± 1.10 ED1-positive cells; P < 0.001). Compared with that observed for the CLP

Table 4. Biochemical and hemodynamic parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n = 6)</th>
<th>Control + NAC (n = 6)</th>
<th>CLP (n = 8)</th>
<th>CLP + NAC (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate, nmol/l</td>
<td>1.50 ± 0.26</td>
<td>1.22 ± 0.23</td>
<td>2.70 ± 0.34c</td>
<td>1.65 ± 0.40</td>
</tr>
<tr>
<td>pH</td>
<td>7.41 ± 0.06</td>
<td>7.40 ± 0.02</td>
<td>7.30 ± 0.02b</td>
<td>7.33 ± 0.05</td>
</tr>
<tr>
<td>PaO2/FIO2</td>
<td>320.3 ± 2.1</td>
<td>330.5 ± 11.1</td>
<td>170.3 ± 28.7c</td>
<td>287.6 ± 10.5</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>120.0 ± 5.0</td>
<td>120.0 ± 4.0</td>
<td>70.0 ± 4.0d</td>
<td>122.3 ± 4.0</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>370.0 ± 104</td>
<td>348.0 ± 2.0</td>
<td>482.3 ± 23.4e</td>
<td>528.0 ± 21.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. PaO2/FIO2, arterial oxygen tension/fraction of inspired oxygen ratio; HR, heart rate. *P < 0.05 vs. control + NAC, control, and CLP + NAC; †P < 0.05 vs. control and control + NAC; ‡P < 0.001 vs. control, control + NAC, and CLP + NAC; ‡P < 0.05 vs. control, control + NAC, and CLP + NAC; ‡P < 0.001 vs. control, control + NAC, and CLP + NAC; ‡P < 0.01 vs. CLP and CLP + NAC.
The degree of renal inflammation was significantly lower in the CLP + NAC group (8.0 ± 0.50; *P < 0.001), as can be seen in Fig. 4.

**Sodium cotransporters in the lung.** The protein expression of α-ENaC was significantly upregulated in the CLP + NAC group (120.3 ± 6.4%), whereas it was downregulated in the CLP group (86.50 ± 7.94%; *P = 0.04 vs. CLP + NAC; control group, 100.0 ± 7.43%). Densitometry and immunoblots are shown in Fig. 5, A and B, respectively.

The protein expression of NKCC1 in the lungs was significantly higher in the CLP group rats than in the control group rats (167.6 ± 16.3 vs. 100.0 ± 5.0%; *P < 0.01). Although that expression was lower in the CLP + NAC group (117.0 ± 22.3%), the difference compared with the CLP group was not significant. The densitometric analysis and immunoblots can be seen in Fig. 6, A and B, respectively.

**GFR.** At 24 h after CLP, GFR was significantly lower in the CLP group than in the control group (0.45 ± 0.08 vs. 0.83 ± 0.04 ml·kg⁻¹·100 g BW⁻¹; *P = 0.0006). At the same time point, GFR in the CLP + NAC group was 0.78 ± 0.12 ml·kg⁻¹·100 g BW⁻¹, significantly higher than that observed for the CLP group (*P < 0.001) and indicative of normal renal function. The GFR in the control + NAC group (0.99 ± 0.1 ml·kg⁻¹·100 g BW⁻¹) was comparable to that observed in the control group and differed significantly only compared with that observed in the CLP group (*P < 0.001).
To evaluate the effect of LV_{T}-MV on renal function, we studied an additional group of rats during spontaneous breathing. We found no statistical differences between the LV_{T}-MV and spontaneous breathing groups in terms of GFR (Table 5).

**Oxidative stress.** After 2 h of LV_{T}-MV, the level of oxidative stress (plasma TBARS) was elevated in the CLP group, when compared with that determined for the control group (4.77 ± 0.13 vs. 2.04 ± 0.20 nmol/ml; P < 0.001). As can be seen in Fig. 7, plasma levels of TBARS in the CLP + NAC group (3.7 ± 0.21 nmol/ml) were significantly lower than those determined for the CLP group (P < 0.01). It is of note that oxidative stress was lowest in the control + NAC group (1.26 ± 0.38 nmol/ml; P < 0.001 vs. CLP and P < 0.05 vs. control).

**Isoprostane in lung and renal tissue.** To confirm the presence of oxidative stress, we evaluated 8-isoprostane in lung tissue (Fig. 8A). As expected, the level of 8-isoprostane was higher in the CLP group than in the control group (0.310 ± 0.02 vs. 0.190 ± 0.001; P = 0.02). The level of 8-isoprostane seen in the CLP + NAC group (0.204 ± 0.03) was intermediate between the two values observed for the other groups, and it was lower than that observed for the CLP group (P < 0.05). The immunohistochemical profile of 8-isoprostane was obtained in renal tissue (Fig. 8B), and oxidative stress was found to be significantly greater in the CLP group than in the control and CLP + NAC groups.

**Survival analysis.** The mortality rate was significantly higher in the CLP group than in the CLP + NAC group (P < 0.01). However, mortality in both groups (CLP and CLP + NAC) was significantly higher than that observed in the control and control + NAC groups (Fig. 9).

In the control and control + NAC groups, survival was 100% at 12, 24, 48, and 72 h. In the CLP group, survival was 100% at 12 h, 48% at 24 h, 32% at 48 h, and 24% at 72 h. In the CLP + NAC group, survival was 86.6% at 12 h, 86.6% at 24 h, 73.3% at 48 h, and 66.6% at 72 h.

**DISCUSSION**

The CLP model is widely used to study the pathogenesis of sepsis (8). In sepsis, the degree of severity in the various organs can vary among animals (29), and crosstalk among organs is common. Experimental models of CLP can cause severe AKI and signs of ARDS.

In the present study, untreated animals submitted to CLP showed significant impairment of respiratory mechanics. This might be due to endotoxin release from the bacterial cell wall, triggering the physiological and biochemical alterations that can activate various cells, such as macrophages and neutrophils, resulting in the release of certain mediators, including cytokines, leukotrienes, and proteases. This sequence of events leads to neutrophil activation, together with the release of oxygen free radicals, resulting in oxidative stress (43). In addition, the collapse of small airways and pulmonary edema seen in sepsis decreases the tidal volume, causing surfactant dysfunction, which contributes to alterations in respiratory mechanics (17).

We found that pretreatment with NAC protected the respiratory mechanics of animals submitted to CLP from the onset to the end of a 2-h period of LV_{T}-MV. This finding is likely attributable to a reduction in cytokine release and free radical production, as well as to the restoration of adequate NO production (11). In experimental models of ALI, NAC has been shown to decrease pulmonary edema and improve oxygenation (14), indicating that NAC has a positive influence on respiratory mechanics.

Another factor that can affect respiratory mechanics is pulmonary edema. The failure of the alveolar capillary membrane, the structure responsible for preserving a fluid free alveolar space (vital for appropriate gas exchange), has been the focus of increasing attention in attempts to understand the mechanisms of pulmonary edema, a widely recognized complication of sepsis (17). Sodium transport plays a significant role in the development of edema in the lung. The transport of sodium across the alveolar epithelium via apical amiloride-sensitive sodium channels, such as α-ENAC, produces an osmotic gradient that results in the passive movement of water from the air spaces into the alveolar interstitium. This process (alveolar fluid clearance) is crucial for the maintenance of efficient gas exchange in the normal lungs. In some models of acute lung injury, the ability of the lungs to handle edema is impaired (1). In a pioneering study, Ware and Matthay (40) clearly demonstrated that ALI/ARDS patients with intact alveolar fluid clearance had lower morbidity and mortality than those with impaired sodium transport.

In the present study, animals with sepsis developed pulmonary edema, as demonstrated by the edema index and the wet-to-dry lung weight ratio. However, NAC prevented that...
edema. This finding, as well as the better respiratory mechanics observed in the CLP + NAC group rats, might be explained by the NAC-induced upregulation of α-ENaC expression.

The role of NAC in sodium transport was described by Dickie et al. (7), who observed an improvement in sodium transport when NAC was added to the culture of distal lung cells exposed to LPS-stimulated alveolar macrophages. This effect (NAC-induced improvement in alveolar fluid clearance) was also described by Modelska et al. (22). The sodium cotransporter NKCC1 plays an important role in maintaining cell volume. In the present study, NKCC1 expression was highest in the CLP group. Ware and Matthay (40) found NKCC1 expression to be higher in patients with ARDS than in those without. In addition, Nguyen et al. (24) showed that NKCC1 knockout mice with lung injury were at a lower risk of developing sepsis and bacteremia than were normal mice with lung injury, as well as that the knockout mice had less hypothermia and showed an increase in their activity level. This indicates that NKCC1 influences the function of the endothelial barrier, possibly controlling cell volume and shape. The study of Nguyen et al. generated considerable discussion about this newly discovered role of NKCC1 expression as a deleterious factor in the regulation of pulmonary response (19). It is noteworthy that, in our CLP + NAC group, the lower NKCC1 expression was accompanied by decreases in plasma TBARS and lung tissue 8-isoprostane. However, the mechanisms of those NAC-induced effects remain unclear.

Oxidative stress is considered a major mediator of cell damage, contributing to the development of sepsis due to endothelial cell damage, formation of chemotactic factors, recruitment of neutrophils, lipid peroxidation, and the release of TNF-α (30). Oxidative stress can also affect alveolar fluid clearance. In the present study, we used the plasma level of TBARS as a marker of lipid peroxidation. Pretreatment with NAC proved effective in maintaining relatively low levels of oxidative stress in sepsis. An interesting study conducted by Andrades et al. (2) in an animal model of CLP-induced sepsis showed that TBARS can modulate organ failure. The authors demonstrated that animals with higher levels of TBARS also had elevated protein concentrations in bronchoalveolar lavage fluid, as well as higher levels of urea.

In sepsis, the use of antioxidants can decrease the activation of NF-κB and minimize the injury to various organs (30). In the CLP model of sepsis, higher levels of plasma lipid peroxidation have been found at 24 h after CLP (26, 30, 32). The administration of NAC (150 mg/kg BW per day ip) has been shown to increase the levels of glutathione at 72 h after CLP (10). A lower dose of NAC (20 mg/kg BW), administered at different time points and in association with deferoxamine, has also been found to decrease lung and kidney injury in a CLP model of sepsis (2). To confirm the presence of oxidative stress in lung tissue, we performed an immunohistochemical assay of 8-isoprostane. Isoprostanes are produced primarily by free radical-induced peroxidation of arachidonic acid and are reli-

Fig. 5. Densitometry and immunoblots for the groups CLP (n = 4), CLP + NAC (n = 4), and control (n = 3). A: densitometric analysis shows decreased protein abundance of the alpha subunit of the epithelial sodium channel (α-ENaC) in the lung tissue of the CLP group animals. B: immunoblots reacted with anti-α-ENaC reveal 90-kDa bands. *P = 0.04, CLP vs. CLP + NAC.
able markers of oxidative stress (36). It is well established that isoprostanes have potent biological activity and are released from tissues in a number of diseases, such as ischemic injury (9). In the present study, oxidative stress was clearly present in the CLP group rats, and, again, NAC was protective against free radical-induced peroxidation.

When ROS are generated, there is neutrophil recruitment to the lungs. A decrease in extravascular plasma equivalents has been observed in animals with neutrophil depletion (22). In our CLP group, there was an increase in neutrophils, as evaluated by lung histology. Although the neutrophil counts were lower in the CLP + NAC group than in the CLP group, the difference was not significant. In the kidneys, we found that the levels of inflammation (macrophage counts) were lower in CLP + NAC rats than in CLP rats. This indicates that NAC plays a role in preventing the renal dysfunction that leads to AKI-induced sepsis.

It has been reported that positive pressure alters GFR, renal blood flow, and free water clearance (12, 29). To assess the

Table 5. Inulin clearance in rats with sepsis during spontaneous breathing or during mechanical ventilation

<table>
<thead>
<tr>
<th>Group</th>
<th>SB, ml·kg⁻¹·100 g BW⁻¹</th>
<th>LV₇-MV, ml·kg⁻¹·100 g BW⁻¹</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.84 ± 0.02 (n = 10)</td>
<td>0.83 ± 0.04 (n = 6)</td>
<td>NS</td>
</tr>
<tr>
<td>Control + NAC</td>
<td>1.00 ± 0.08 (n = 6)</td>
<td>0.99 ± 0.03 (n = 6)</td>
<td>NS</td>
</tr>
<tr>
<td>CLP</td>
<td>0.51 ± 0.04 (n = 12)</td>
<td>0.45 ± 0.08 (n = 10)</td>
<td>NS</td>
</tr>
<tr>
<td>CLP + NAC</td>
<td>0.80 ± 0.05 (n = 12)</td>
<td>0.78 ± 0.12 (n = 6)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE. SB, spontaneous breathing; LV₇-MV, low-tidal-volume mechanical ventilation.

Fig. 6. Na-K-2Cl (NKCC1) protein expression in the groups CLP (n = 9), CLP + NAC (n = 8), and control (n = 5). A: densitometry of NKCC1 in lung tissue; B: specific bands were detected in all lanes.

Fig. 7. Oxidative stress. Plasma levels of thiobarbituric acid reactive substances (TBARS) in the groups CLP (n = 10), CLP + NAC (n = 6), control (n = 6), and control + NAC (n = 6). *P < 0.001 vs. control and vs. control + NAC; #P < 0.01 vs. CLP + NAC; &P < 0.05 vs. control.
effect of LV₇-MV on renal function, we also evaluated GFR during 2 h of spontaneous breathing in animals with sepsis. We found no difference in GFR in between those on LV₇-MV and those evaluated during spontaneous breathing. There are two reasons for this finding. The first is the short (2-h) time on LV₇-MV, which is insufficient to produce changes in GFR. The second is the use of a low tidal volume. In a recent study (16) conducted in our laboratory with healthy Wistar rats, we demonstrated that, after 120 min of mechanical ventilation at a low tidal volume (8 ml/kg BW), GFR remained unchanged in relation to baseline values. However, when we used a high tidal volume (27 ml/kg BW), GFR decreased after 60 min on mechanical ventilation, the reduction becoming more marked after 90 min (16). It is well established that mechanical ventilation can worsen renal hemodynamics and function (15), mainly when high volumes and high PEEP are used. However, marked hormonal and functional renal effects occur only when a PEEP ≥15 cmH₂O is used (6). Therefore, we believe that the low tidal volume and low PEEP used can explain the results obtained in relation to GFR in animals with sepsis submitted to mechanical ventilation in the present study.

Sepsis remains the leading cause of AKI in critically ill patients, being implicated in 50% of cases (39). In the present study, the untreated animals with sepsis developed AKI at 24 h post-CLP, whereas those pretreated with NAC did not.

Sepsis also induces hemodynamic changes. In experimental models of sepsis, systemic hypotension and tachycardia are seen. As expected, peritonitis resulted in severe hypotension in our CLP group. The animals pretreated with NAC did not show the drop in MAP seen in the untreated animals. This effect can be explained by the NAC-induced reduction in NO formation. Therefore, NAC, as an antioxidant, can decrease the production of NO and cytokines. The maintenance of hemodynamic balance and of lower levels of oxidative stress can explain the better GFR found in our CLP + NAC group.

It is known that mortality is quite high in experimental models of sepsis. In one study, 43% of the mice died within the first 6 h after LPS administration (11). When sepsis is accompanied by AKI or multiple organ dysfunction, mortality is significantly higher (8). Post-CLP support with antibiotics and saline solution plays an important role in reducing mortality. When antibiotics and fluid resuscitation are used, post-CLP survival rates can reach 100% at 24 h, 43% at 48 h, and 14% at 72 h (21). In the present study, we also used antibiotics and fluid resuscitation, obtaining
NAC PREVENTS LUNG AND KIDNEY INJURY IN SEPSIS


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