Edaravone, a Hydroxyl Radical Scavenger, Ameliorates the Severity of Pulmonary Hypertension in a Porcine Model of Neonatal Sepsis

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Systemic infection in the newborn (neonatal sepsis) is the most common cause of neonatal mortality. Neonatal sepsis is complicated by pulmonary hypertension. In this study, we analyzed the effect of edaravone, a free radical scavenger that is known to reduce the production of inflammatory mediators, such as tumor necrosis factor α (TNFα), on pulmonary hypertension. Experimental and sham groups were drawn from 19 three-day-old piglets; 5 underwent a modified procedure of cecal ligation and perforation (CLP) (CLP group), 8 underwent CLP followed 30 min later by edaravone intravenous administration (edaravone group), and 6 did not undergo CLP and did not receive edaravone (sham group). To evaluate the pulmonary blood pressure despite the sepsis-induced low cardiac output, mean arterial blood pressure (mABP), mean pulmonary arterial pressure (mPAP), and comparative pulmonary hypertension ratio (mPAP/mABP) were determined. Serum TNFα levels were measured before the procedure and at 1, 3, and 6 h after. The mPAP levels were higher in the CLP group at 9 h compared to the edaravone group. The mPAP/mABP ratio was lower in the edaravone and sham groups compared to the CLP group at 6 and 9 h. TNFα in the edaravone and sham groups were lower at 1 and 3 h compared to that in the CLP group. In all animals, mPAP/mABP at 6 h correlated with serum levels of TNFα at 1, 3, and 6 h. These findings suggest that edaravone ameliorates the severity of pulmonary hypertension in a neonatal sepsis model by reducing serum TNFα levels.

Keywords: newborn; infection; septic shock; oxidative stress; circulation


Neonatal sepsis, which results in significant morbidity and mortality, is the most serious problem in neonatal intensive care. Neonatal sepsis or septicemia is a clinical syndrome caused by bacterial invasion of the bloodstream in the first month of life. The World Health Organization (WHO) estimates that 1 million deaths per year (10% of all under-five mortalities) are due to neonatal sepsis and that 42% of these deaths occur in the first week of life (Lawn et al. 2005). The hemodynamic features of neonatal sepsis differ from those of adult sepsis, as there is more severe circulatory impairment and more evident pulmonary hypertension. The time course of septic hemodynamic responses typically consists of two phases: a hyperdynamic phase and a hypodynamic phase. The hyperdynamic phase is characterized by an early decrease in systemic resistance and an increase in pulmonary resistance, cardiac output, and heart rate. The hypodynamic phase is characterized by a decrease in mean arterial blood pressure accompanied by a significant fall in cardiac output (Yang et al. 1999). Hemodynamic states are highly influenced by myocardial function, including both diastolic and systolic states, as well as systemic and pulmonary vascular resistance during the different phases of neonatal sepsis and septic shock.

In sepsis, the infectious stimulus induces the release of local and systemic inflammatory mediators, tumor necrosis factor α (TNFα), and interleukin (IL)-1β from monocytes,
macrophages, and other cells. These cytokines stimulate polymorphonuclear leukocytes, macrophages, and endothelial cells to release a cascade of cytokines and free radicals (Hack et al. 1997; Silveira and Procianoy 1999; Stoclet et al. 1999; Victor et al. 2004). The excessive production and release of cytokines and free radicals during neonatal sepsis provoke hemodynamic dysfunction and multiple organ failure (Kumar et al. 1996; Seema et al. 1999).

We designed a modified porcine neonatal cecal ligation and perforation (CLP) sepsis model, which yields immunological and hemodynamic changes similar to those of human neonatal sepsis. These changes include the elevation of cytokines such as TNFα and IL-6, and free radicals, and each has a different peak, along with progressive circulatory dysfunction, including a collapse of the mean arterial blood pressure (mABP) and elevation of comparative pulmonary blood pressure (PAP) (Kato et al. 2004). Recently, research into the treatment of septic shock has focused on targeting nitric oxide (NO), endothelin, and inflammatory mediators (Schilling et al. 1993; Iskit et al. 1999; Konrad et al. 2004). Edaravone, a hydroxyl radical scavenging agent, exhibits beneficial free radical scavenging and antioxidant characteristics. It has been used for protection against ischemia reperfusion injury in patients with cerebral infarction (Kawai et al. 1997). It has been reported that edaravone decreased the serum levels of TNFα and IL-6 after lipopolysaccharide (LPS) administration in rats (Kono et al. 2003). In a previous study, we found that edaravone maintained the mABP, heart rate, and cardiac output and prevented the sepsis-induced elevation of NO and total hydroperoxide in our porcine neonatal sepsis model, which in turn delayed the pathophysiological events of sepsis, resulting in prolonged survival time and improved survival rates (Kato et al. 2009).

In this article, we evaluated the effects of edaravone on sepsis-induced pulmonary hypertension and examined the relation of the latter to serum TNFα and free radical levels at different time points in the neonatal sepsis model.

Materials and methods

Animal preparation

Experiments were performed in adherence with the National Institutes of Health guidelines on the use of experimental animals, and the protocol was approved by the Ethics Committee of Nagoya City University Graduate School of Medical Sciences. Subjects were 19 newborn mixed-strain piglets obtained on their third day of life from a local farmer. The piglets were subsequently divided into three groups: a CLP group (n = 5), which underwent a modified CLP procedure as previously described (Kato et al. 2004), an edaravone group (n = 8), which underwent the same modified CLP procedure, but which also received edaravone treatment 30 min after CLP, and a sham group (n = 6), which received only a sham operation. Piglets were kept with their mothers and were transported only on the day of their procedures.

Piglets were premedicated with an intramuscular injection of ketamine chloride (10 mg/kg), and anesthesia was then induced using pentobarbital sodium (5 mg/kg/h) in 5% glucose solution via a peripheral line at a rate of 5 mL/kg/h throughout the study to avoid hypovolemia. All surgical procedures were performed under sterile conditions.

Each piglet underwent a tracheotomy and was intubated with an endotracheal tube (internal diameter 3.5 mm) and ventilated with an infant ventilator (IV-100, Sechrist Industries, Anaheim, CA, USA). Inspiration/expiration pressures were initially set at 14/4 cmH2O, with an inspiration time of 0.5 s using room air. Pressures were then adjusted to maintain PaCO2 at 30–50 mmHg throughout the experiments. A cut-down procedure was used to insert a 3F polyvinyl catheter into the left femoral artery to measure the mean arterial blood pressure (mABP) and to withdraw blood samples. A 4-Fr Berman angiographic catheter (American Edwards Laboratories, Irvine, CA) was inserted under fluoroscopy via the right external jugular vein and placed in the main pulmonary artery to measure the mPAP. The mABP and mPAP were measured using a neonatal monitor (model 78801 B, Hewlett Packard, Andover, MA, USA), and the data was collected with a MacLab/8s system (ADI Instruments, Mountain View, CA, USA). Relative pulmonary hypertension was evaluated using the ratio mPAP/mABP to evaluate the pulmonary blood pressure despite the sepsis-induced low cardiac output. To prevent hypothermia, body temperature was maintained using a thermal pad and a polyvinyl cover and was monitored by a rectal probe.

Modified CLP was performed on piglets in the CLP group and on those in the edaravone group. Briefly, a paramedian incision approximately 4 cm long, which was sufficient to expose the cecum and terminal ileum, was made. The ileocecal artery was identified and ligated near the cecum, resulting in devascularization of the distal end of the cecum. A 1-cm incision was made on the antimesenteric side. The cecum was gently milked to extrude feces into the peritoneal cavity. The abdominal incision was then closed. In the sham group, the cecum was exposed for 2 min, and the abdomen was then closed in two layers. The study was continued to the time of spontaneous death of the CLP animals around 9 h. The sham animals survived longer and were sacrificed with a lethal dose of phenobarbital sodium.

Edaravone treatment

Edaravone was supplied by Mitsubishi Tanabe Pharma (Osaka, Japan). In the edaravone group, edaravone treatment was started 30 min after the CLP procedure at a speed of 3 mg/kg/h (2.5 mL/kg/h) in 5% glucose solution via the central venous line; total IV fluids were adjusted to maintain a volume of 5 mL/kg/h throughout the study. The edaravone solution was prepared by dissolving 120 mg of edaravone in 1 mol/L NaOH solution and adding the resulting mixture to 100 mL of 5% glucose. To restore the edaravone solution to a pH of 7.4, 1 mol/L HCl was added, as previously described (Watanabe et al. 1994).

Experimental protocol

In each group, blood samples were aseptically collected from the femoral arterial catheter to measure arterial blood oxygen saturation (SaO2), arterial serum TNFα, NO metabolites, and hydroperoxide. The samples were taken before beginning the procedure, and at 0, 1, 3, and 6 h after. All samples were placed in pyrogen-free sterilized tubes.

Measurements

Arterial blood SaO2 levels were analyzed using a standard ana-
lyzer (Model 1248; CIBA Corning, Medfield, MA, USA). Serum TNFα was measured using immunoassay kits specific for porcine TNFα (R&D Systems, Minneapolis, MN, USA). NO metabolites were evaluated by measuring the serum concentration of NO2- + NO3- (NOx). NOx concentration was measured using total nitrite and nitrate ELISA kits (R&D Systems).

Total hydroperoxide was measured with a free radical analytic system using the derivatives of a reactive oxygen metabolites kit (Diacron srl, Grosseto, Italy) as previously described (Hussein et al. 2010a). Briefly, in the presence of iron (which is released from the proteins by an acidic buffer), free radicals are able to generate alkoxyl and peroxyl radicals, according to Fenton’s reaction. Such radicals, in turn, are able to oxidize an alkyl-substituted aromatic amine (A-NH2, dissolved in a chromogenic mixture), which transforms them into a pink-colored derivative. Finally, this colored derivative was photometrically quantified. The intensity of the developed color is directly proportional to the concentration of total hydroperoxide.

Statistical analysis
The distributions of data were tested using the Shapiro-Wilk test. The means of the measurements from all three groups (inter-group means) at particular time points were compared using analysis of variance (ANOVA), followed by the Bonferroni post hoc test. If the data were not normally distributed, the Kruskal-Wallis test was used, and when significances were detected, the Mann-Whitney test was used. The coefficient of relation was studied using the Pearson two-tailed correlation coefficient, and if data were nonparametric, the Spearman two-tailed test was used. Data are reported as mean ± standard error of the mean (SEM). Probability values of less than 0.05 were considered significant. All data analyses were performed with the commercially available statistical analysis software package SPSS (Statistical Package for Social Sciences, Chicago, Illinois, USA).

Results
The mPAP was higher in the CLP group compared to the edaravone and sham groups and became significantly higher than that in the edaravone group at 9 h 19.74 ± 1.7 mmHg versus 15.2 ± 2.8 mmHg, \( p = 0.04 \) (Fig. 1A).

The mPAP/mABP ratios in both the edaravone and the sham groups were significantly lower than those in the CLP group at 6 and 9 h (0.54 ± 0.07 and 0.35 ± 0.04 versus 0.79 ± 0.05, \( p = 0.02 \) and 0.001) and (0.55 ± 0.09 and 0.34 ± 0.01 versus 0.85 ± 0.12, \( p = 0.04 \) and 0.003), respectively (Fig. 1B).

The mPAP/mABP ratios in the edaravone group were higher than those in the sham group at 6 and 9 h, but this difference only reached significance at 6 h (\( p = 0.03 \)) (Fig. 1B).

The arterial blood SaO2 levels in both the edaravone and CLP groups were significantly lower than those in the sham group at 1, 3, and 6 h: \( p = 0.03 \) at 1 h, \( p = 0.01 \) at 3 h, and \( p = 0.003 \) at 6 h in the edaravone group and \( p = 0.04 \) at 1 h, \( p = 0.02 \) at 3 h, and \( p = 0.03 \) at 6 h in the sham group (Table 1).

The arterial blood SaO2 levels in the edaravone group were significantly higher than those in the CLP group at 3 and 6 h (\( p = 0.03 \) and 0.04), respectively (Table 1).

Serum levels of TNFα in the edaravone and the sham groups were lower than those in the CLP group at 1 and 3 h: \( p = 0.03 \) at 1 h and \( p = 0.04 \) at 3 h in the edaravone group and \( p = 0.01 \) at 1 h and \( p = 0.007 \) at 3 h in the sham group (Table 1).

At 6 h, serum levels of TNFα in the CLP group were higher than those in the sham group (\( p = 0.016 \)) (Table 1).

Serum levels of NOx in the edaravone and the sham groups did not differ and were lower than those in the CLP group at 3 and 6 h: \( p = 0.004 \) at 1 h and \( p = 0.003 \) at 3 h in
the edaravone group and $p = 0.009$ at 1 h and $p = 0.002$ at 3 h in the sham group (Table 1).

Serum levels of TH in the edaravone and the sham groups did not differ and were lower than those in the CLP group at 1 and 3 h, but these differences did not reach significance.

In all animals, mPAP/mABP at 6 h correlated with the serum levels of TNFα at 1, 3, and 6 h ($r = 0.93$, 0.89, and 0.77, $p < 0.001$, 0.001, and 0.005, respectively), and with their NO at 3 and 6 h ($r = 0.6$ and 0.63, $p < 0.05$ and 0.005, respectively) (Fig. 2A, B). However, mPAP/mABP at 6 h did not correlate with serum levels of NO and TH at 1 h, nor with serum TH at 3 and 6 h (Fig. 2B, C).

**Discussion**

Neonatal septic shock is associated with serious progressive circulatory disturbances, microvascular injuries, disseminated intravascular coagulation, and multiple organ failure (Despond et al. 2001). The pulmonary hypertension that often accompanies neonatal sepsis is a serious matter that carries with it a high risk of mortality in preterm infants (Kumar et al. 2007).

Newborn animal models of group B streptococcal and endotoxin shock have documented reduced cardiac output and increased pulmonary vascular resistance (Meadow and Meus 1986). The vascular endothelium is an important target for the modulators of inflammatory response and proinflammatory cytokines. Dysfunction of the vascular endothelium is an early finding in septic shock (Iskit and Guc 2003). Endothelium-derived substances such as NO and endothelin are regarded as key mediators during septic shock (Stoclet et al. 1999; Figueras-Aloy et al. 2004; Vo et al. 2005). Another study reported on the role of endothelin, nitric oxide, and cyclooxygenase products in pulmonary hypertension during septic shock (Yamamoto et al. 1997). During septic shock, these mediators play roles in circulatory impairment by inducing systemic hypotension and pulmonary hypertension. The circulatory changes complicating neonatal sepsis are associated with elevated levels of cytokines and inflammatory mediators such as TNFα, IL-1β, NO, and free radicals (Silveira and Procianoy 1999).

Pulmonary vascular tone during the neonatal period exhibits a great deal of instability (Haworth 2006). This natural instability, along with the effect of the cytokine storm that occurs during sepsis, severely impairs pulmonary vascular tone and pulmonary blood flow with elevation of pulmonary blood pressure, causing pulmonary hypertension (Yamamoto et al. 1997). This is associated with severe left ventricular dysfunction and respiratory impairment during neonatal septic shock (Pollack et al. 1984).

### Table 1. Arterial blood oxygen saturation and carbon dioxide partial pressure (pCO₂), serum TNFα, and NO metabolites (NOx) in the sham, cecal ligation and perforation (CLP), and edaravone groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Before</th>
<th>1 h</th>
<th>3 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen saturation %</td>
<td>Sham ($n = 6$)</td>
<td>98.7 ± 0.1</td>
<td>98.6 ± 0.2</td>
<td>98.7 ± 0.3</td>
<td>98.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>CLP ($n = 6$)</td>
<td>98.7 ± 0.3</td>
<td>93.0 ± 1.8</td>
<td>87.3 ± 4.4</td>
<td>92.1 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>Edaravone ($n = 6$)</td>
<td>99.3 ± 0.16</td>
<td>94.73 ± 2.3</td>
<td>96.5 ± 0.5</td>
<td>96.9 ± 0.5</td>
</tr>
<tr>
<td>pCO₂ (mmHg)</td>
<td>Sham ($n = 6$)</td>
<td>31 ± 2.1</td>
<td>31.3 ± 2.6</td>
<td>33.2 ± 4.2</td>
<td>33.7 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>CLP ($n = 6$)</td>
<td>28.2 ± 3.6</td>
<td>42.2 ± 2.8</td>
<td>41.0 ± 4.6</td>
<td>39.2 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>Edaravone ($n = 6$)</td>
<td>29.8 ± 2.8</td>
<td>38.1 ± 2.6</td>
<td>32.7 ± 2.6</td>
<td>31.9 ± 1.0</td>
</tr>
<tr>
<td>TNFα (pg/ml)</td>
<td>Sham ($n = 5$)</td>
<td>28.7 ± 16.5</td>
<td>4.6 ± 4.6</td>
<td>1.8 ± 1.8</td>
<td>10.2 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>CLP ($n = 5$)</td>
<td>40.2 ± 34.4</td>
<td>236.9 ± 38.3</td>
<td>235.5 ± 36.7</td>
<td>206 ± 46.7</td>
</tr>
<tr>
<td></td>
<td>Edaravone ($n = 5$)</td>
<td>8.7 ± 6.1</td>
<td>45.1 ± 25.1</td>
<td>85.7 ± 38.3</td>
<td>166.5 ± 79.1</td>
</tr>
<tr>
<td>NOx (µmol/L)</td>
<td>Sham ($n = 6$)</td>
<td>1.0 ± 0.4</td>
<td>2.1 ± 1.0</td>
<td>1.4 ± 0.3</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>CLP ($n = 5$)</td>
<td>3.0 ± 2.2</td>
<td>4.6 ± 1.9</td>
<td>12.76 ± 6.3</td>
<td>46.7 ± 30.1</td>
</tr>
<tr>
<td></td>
<td>Edaravone ($n = 5$)</td>
<td>1.0 ± 0.02</td>
<td>0.9 ± 0.06</td>
<td>1.0 ± 0.05</td>
<td>0.9 ± 0.7</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. $^p < 0.05$ edaravone or sham group compared with CLP group, $^*p < 0.05$ sham group compared with edaravone group at that time point.
Fig. 2. Correlation of the relative pulmonary blood pressure to serum TNFα.
Correlation between mPAP/mABP at 6 h and (A) serum TNFα at 1, 3, and 6 h, (B) serum NO at 1, 3, and 6 h, and (C) serum TH at 1, 3, and 6 h. The CLP group (open circles: n = 5), edaravone group (closed circles: n = 5), and sham group (triangles: n = 5 in TNFα, and n = 6 in NO and TH).
The coefficient of correlation was tested using the Pearson two-tailed correlation coefficient, and if data were nonparametric, the Spearman two-tailed test was used.
TNFα, IL-1β, and overabundant NO directly induce myocardial depression during sepsis (Kumar et al. 1996; Stoclet et al. 1999). TNFα administered to human and animal myocardial tissue in vitro yield a concentration-dependent depression of contractility (Kumar et al. 1996). Moreover, the removal of TNFα from the serum or the administration of an anti-TNFα monoclonal antibody reverses this myocardial depressant effect (Kumar et al. 1996).

Several treatments have proven beneficial for treating pulmonary hypertension in experimental septic models. For example, polymyxin-B direct hemoperfusion (PMX-DHP) has been demonstrated to be effective in the clearance of LPS and has improved pulmonary hypertension and respiratory impairment in a neonatal sepsis model (Hussein et al. 2005; Hussein et al. 2010b).

Edaravone suppresses the production of TNFα, free radicals, and NO, thereby suppressing the effect of these inflammatory mediators in different body organs and systems during sepsis and preventing sepsis-induced multiple organ failure.

In severe neonatal septic shock, the decreases in systemic blood pressure and myocardium contractility, and the increase in pulmonary pressure, are often resistant to catecholamines, and the progression to acute hemodynamic dysfunction is difficult to prevent, with dire consequences in terms of risk of mortality (Kermorvant-Duchemin et al. 2008). In a previous study, we found that edaravone prolonged survival in our neonatal sepsis model (Kato et al. 2009).

In the present study, we found that comparative pulmonary hypertension, as assessed by the mPAP/mABP ratio to evaluate the pulmonary blood pressure despite the sepsis-induced low cardiac output, was significantly lower in the edaravone group than in the CLP group at 6 and 9 h. Edaravone therapy prevented the elevation of mPAP and prevented the decline of mABP at 6 and 9 h post-CLP, improved the respiratory function, as assessed by higher arterial blood SaO2, and prevented the elevation of TNFα at 1 and 3 h, and NO at 3 and 6 h compared to the CLP group.

The correlation of mPAP/mABP at 6 h with the serum levels of TNFα at 1, 3, and 6 h, and its correlation with the serum levels of NO at 3 and 6 h, indicate that edaravone’s suppression of TNFα and the cytokine storm was likely responsible for the improved mPAP/mABP values at 6 and 9 h in the treated animals. The suppressive effect of edaravone on TNFα prevented TNFα’s effect on the vascular system and the myocardium, thereby preventing pulmonary hypertension and systemic hypotension in our neonatal sepsis model.

In neonatal sepsis, preventing systemic hypotension, pulmonary hypertension, and cardiac failure is important for reducing sepsis-induced morbidity and mortality (Carcillo and Fields 2002). Circulatory system competency is essential for the perfusion of multiple organs in the newborn, particularly the brain, which is vulnerable to morbidity during septic shock.

Conclusion

In this study, we have shown that edaravone is useful in ameliorating the sepsis-induced pulmonary hypertension and improving the respiratory function in a porcine model of neonatal sepsis. This warrants further clinical studies.

Conflict of Interest

The authors declare no conflict of interest.

References


