岩手医科大学
審査学位論文
（博士）
Relationship between serum nitrite/nitrate (NOx) levels in the early phase of septic acute lung injury and the prognosis

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Abstract
Serum nitrite/nitrate (NOx) levels in the early phase of septic acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) were investigated. Both NOx and tumor necrosis factor-α (TNF-α) levels were significantly higher in the ARDS group than in the ALI group. A negative correlation was found between the PaO₂/FIO₂ (P/F) ratio and serum NOx levels. In addition, a positive correlation was found between the TNF-α and serum NOx levels. The 30-, 60-, and 90-day mortality rates were 8.7%, 15.2%, and 19.6%, respectively, in the patients with ALI/ARDS. There were no differences in the P/F ratio, serum NOx levels or TNF-α levels in the early phase of ALI/ARDS between the 30-day survival and death groups. On the other hand, the P/F ratio, serum NOx levels and TNF-α levels in the early phase of ALI/ARDS were significantly higher in the 60- and 90-day death groups than in the corresponding survival groups. There were no significant differences in the 90-day mortality rates between the ALI and ARDS groups. Our findings suggested that NOx may be involved in the pathogenesis of ALI/ARDS.

Key words : sepsis, ARDS, ALI, NO, TNF-α, endothelin, P/F ratio, mortality

1. Introduction
Septic acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) occurs in 25 to 42% of patients with sepsis syndrome, and this frequency further increases when shock persists. Acute respiratory failure (ARF) associated with sepsis is often a systemic process leading to multiple organ dysfunction syndrome (MODS).

The pathogenesis of ALI/ARDS is complex, but we have reported some humoral mediators that may be involved in the development of septic ALI/ARDS.

It has been successively reported that endothelium-dependent relaxing factor (EDRF), which has a vasodilatory effect and reduces blood pressure, is very similar in characteristics to nitric oxide (NO). Several studies have reported strong involvement of NO in the pathogenesis of septic shock. We have reported increases of the serum nitrite/nitrate (NOx) levels and their correlations with those of tumor necrosis factor-α (TNF-α) and interleukin 8 (IL-8) in septic shock and also increase of the serum NOx levels in septic MODS.
On the other hand, attention has recently been paid to the involvement of NO and a reactive nitrogen species, peroxynitrite (ONOO⁻), in the pathogenesis of ARDS. We have also reported the existence of a relationship between ALI/ARDS and serum NOx levels. In the present study, we investigated the relationship between the serum NOx levels in the early phase of ALI/ARDS and the survival prognosis.

II. Subjects and methods

Consent for participation in this study was obtained from the patients or their families. This study was approved by the Ethics Committee of Iwate Medical University.

Diagnosis of sepsis was made according to the criteria of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference Committee. Diagnosis of ALI/ARDS was made according to the criteria reported by Bernard et al.; in this study, patients with a PaO₂/FIO₂ ratio (P/F ratio) of ≥200 but <300 were diagnosed as having ALI, and those with a P/F ratio of <200 were diagnosed as having ARDS.

The Acute Physiology and Chronic Health Evaluation score (APACHE II score) and Sequential Organ Failure Assessment score (SOFA score) were used as the indicators of severity. The severity was assessed by 2 or more emergency physicians certified as infection control doctors.

The study involved 46 patients with an APACHE II score of ≥15 seen over the 3-year period from April 2008 to March 2011, who had undergone endotracheal intubation and from whom blood samples could be collected within approximately 3 hours of the diagnosis of ALI/ARDS. Fourteen patients had ALI and 32 had ARDS.

The mean age of the patients was 68 ± 16 years. The mean age of the male patients was 65 ± 17 years (n=26) and that of the female patients was 71 ± 13 years (n=20), with no significant difference in age between the male and female patients. The mean age of the ALI patients was 67 ± 15 years and that of the ARDS patients was 68 ± 16 years, with no significant difference in age between the two groups.

The underlying diseases were generalized peritonitis (n=33), pneumonia (n=4), burn (n=4), multiple trauma (n=3), and drug intoxication (n=2).

Sepsis and the associated septic shock and disseminated intravascular coagulation (DIC) were treated by the usual measures. Similarly, MODS was treated with regimens that have been commonly used for various states of MODS. ALI/ARDS was controlled by mechanical ventilation at a tidal volume of 8 to 10 ml/kg and positive end-expiratory pressure (PEEP) of 5 to 12 cmH₂O. Furthermore, 0.2 mg/kg/h of sivelestat sodium hydrate was administered while the patient had an endotracheal tube in place and for 1 to 2 days after extubation. The maximum administration period was 14 days.

Samples were collected using endotoxin-free heparin syringes within approximately 3 hours of the diagnosis of ALI/ARDS. The samples were immediately centrifuged at 3000 rpm for 40 seconds to separate platelet-rich plasma (PRP), and endotoxin levels were measured immediately. The PRP samples were stored at −80°C until
measurement of the serum NOx and TNF-α levels.

NOx levels were measured by an autoanalyser (TCI-NOX 1000; Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan), based on the Griess reaction\(^9\). The normal range of NOx is 38.3 ± 19.1 μmol/L.

TNF-α levels were measured by enzyme-linked immunosorbent assay (ELISA) (TFB, Inc., Tokyo, Japan), the detection limit of which was 3 pg/ml.

Endotoxin levels were measured by an endotoxin-specific assay using Limulus HS-T Single Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and the Toxinometer (Wako Pure Chemical Industries, Ltd., Osaka, Japan)\(^9\). The cutoff value for the diagnosis of endotoxemia was 1.1 pg/ml\(^2\).

The unpaired t-test was used to analyze the data for significant differences, and Pearson’s formula was used to test for correlations. The $\chi^2$ test was used for comparisons between the groups, and the log-rank method was used for survival curves. A p value < 0.05 was used as the probability value for significant differences in all of the tests.

### III. Results

On average, the APACHE II score was 30 ± 9, the SOFA score was 12 ± 5, the P/F ratio was 169 ± 63, the serum NOx level was 86 ± 47 μmol/L, the TNF-α level was 204 ± 109 pg/mL, and the endotoxin level was 5.5 ± 15.3 pg/mL in the 46 patients at the time of diagnosis of ALI/ARDS. The endotoxin positivity rate was 60.9% (21 out of 46 cases).

The P/F ratio, APACHE II score, SOFA score, serum NOx levels and TNF-α levels were significantly higher in the ARDS group than in the ALI group, but there were no significant differences in endotoxin levels between the 2 groups (Table 1). The endotoxin positivity rate was 59% (7 out of 14 cases) in the ALI group and 65.6% (21 out of 32 cases) in the ARDS group, with no significant differences between the 2 groups ($r = 0.3230$).

A significant positive correlation was found between the TNF-α and serum NOx levels ($r = 0.7613$, p < 0.0001; Fig. 1). No significant correlation was found between the endotoxin and TNF-α levels ($r = 0.1974$, p = 0.1885). A significant correlation was found between the endotoxin and serum NOx levels ($r = 0.3266$, p = 0.0268).

A significant negative correlation was
A significant positive correlation was observed between the TNF-α and serum NOx levels at the time of diagnosis of ALI/ARDS.

A significant negative correlation was observed between the P/F ratio and serum NOx levels at the time of diagnosis of ALI/ARDS.

No significant correlation was found between the P/F ratio and serum NOx levels ($r = -0.5544, p<0.0001$; Fig. 2).

A negative correlation was also found between the P/F ratio and TNF-α levels ($r = -0.5800, p<0.0001$). No significant correlation was found between the P/F ratio and endotoxin levels ($r = -0.1726, p=0.2512$).

A significant positive correlation was found between the serum NOx levels and APACHE II score, and between the serum NOx levels and SOFA score ($r=0.6698, p<0.0001$ and $r=0.6753, p<0.0001$, respectively).

A significant positive correlation was found between the TNF-α levels and APACHE II score, and between the TNF-α levels and SOFA score ($r=0.6233, p<0.0001$ and $r=0.6662, p<0.0001$, respectively).

No significant correlation was found between the endotoxin levels and APACHE II score ($r=0.1716, p=0.2542$) or between the endotoxin levels and SOFA score ($r=0.1281, p=0.3962$).
Table 3. Comparison of the P/F ratio, APACHE II score, SOFA score, serum NOx levels and TNF-α levels in patients with ALI/ARDS between the 30-day death group and the corresponding survival group

<table>
<thead>
<tr>
<th></th>
<th>Survivors (n=42)</th>
<th>Nonsurvivors (n=4)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs.)</td>
<td>56.4 ± 15.8</td>
<td>82.3 ± 38</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>APACHE II score</td>
<td>28.9 ± 7.7</td>
<td>42.5 ± 10.6</td>
<td>0.0078</td>
</tr>
<tr>
<td>SOFA score</td>
<td>10.7 ± 4.6</td>
<td>17.3 ± 6.0</td>
<td>0.0099</td>
</tr>
<tr>
<td>P/F ratio</td>
<td>173.5 ± 63.1</td>
<td>125.8 ± 43.4</td>
<td>0.1417</td>
</tr>
<tr>
<td>NOx (µmol/L)</td>
<td>80.7 ± 42.2</td>
<td>145.0 ± 57.1</td>
<td>0.1156</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>186.6 ± 91.8</td>
<td>303.3 ± 95.0</td>
<td>0.0826</td>
</tr>
<tr>
<td>Endotoxin (pg/mL)</td>
<td>5.4 ± 15.9</td>
<td>6.5 ± 5.9</td>
<td>0.7830</td>
</tr>
</tbody>
</table>

Table 4. Comparison of the P/F ratio, APACHE II score, SOFA score, serum NOx levels and TNF-α levels in patients with ALI/ARDS between the 60-day death group and the corresponding survival group

<table>
<thead>
<tr>
<th></th>
<th>Survivors (n=39)</th>
<th>Nonsurvivors (n=7)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs.)</td>
<td>66.2 ± 16.3</td>
<td>77.0 ± 7.5</td>
<td>0.0118</td>
</tr>
<tr>
<td>APACHE II score</td>
<td>28.1 ± 7.3</td>
<td>41.4 ± 7.7</td>
<td>0.0037</td>
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<tr>
<td>SOFA score</td>
<td>10.4 ± 4.5</td>
<td>16.6 ± 4.0</td>
<td>0.0062</td>
</tr>
<tr>
<td>P/F ratio</td>
<td>177.2 ± 61.8</td>
<td>125.3 ± 63.6</td>
<td>0.0469</td>
</tr>
<tr>
<td>NOx (µmol/L)</td>
<td>75.9 ± 37.3</td>
<td>144.1 ± 53.9</td>
<td>0.0148</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>175.8 ± 75.7</td>
<td>389.1 ± 179.5</td>
<td>0.0212</td>
</tr>
<tr>
<td>Endotoxin (pg/mL)</td>
<td>3.0 ± 4.4</td>
<td>18.3 ± 37.0</td>
<td>0.2898</td>
</tr>
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</table>

Table 5. Comparison of the P/F ratio, APACHE II score, SOFA score, serum NOx levels and TNF-α levels in patients with ALI/ARDS between the 90-day death group and the corresponding survival group

<table>
<thead>
<tr>
<th></th>
<th>Survivors (n=37)</th>
<th>Nonsurvivors (n=9)</th>
<th>p value</th>
</tr>
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<tr>
<td>Age (yrs.)</td>
<td>66.3 ± 16.5</td>
<td>74.1 ± 10.9</td>
<td>0.0002</td>
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<tr>
<td>APACHE II score</td>
<td>27.5 ± 7.0</td>
<td>40.9 ± 6.8</td>
<td>0.0031</td>
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<tr>
<td>SOFA score</td>
<td>10.0 ± 4.2</td>
<td>16.8 ± 37</td>
<td>0.0004</td>
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<tr>
<td>P/F ratio</td>
<td>181.6 ± 60.4</td>
<td>118.7 ± 48.4</td>
<td>0.0050</td>
</tr>
<tr>
<td>NOx (µmol/L)</td>
<td>72.8 ± 35.5</td>
<td>141.9 ± 47.3</td>
<td>0.0021</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>171.1 ± 73.6</td>
<td>327.4 ± 117.7</td>
<td>0.0107</td>
</tr>
<tr>
<td>Endotoxin (pg/mL)</td>
<td>3.1 ± 4.5</td>
<td>15.4 ± 32.9</td>
<td>0.2965</td>
</tr>
</tbody>
</table>

The 30-, 60- and 90-day mortality rates were 8.7%, 15.2% and 19.6%, respectively, in the 46 patients with ALI/ARDS (Table 2). The 30-, 60- and 90-day mortality rates tended to be higher in the ARDS group than in the ALI group, but there were no significant differences.

The Kaplan-Meier survival curve from the onset of ALI/ARDS to day 90 is shown in Fig. 3. There were no significant differences in mortality rates between the ALI and ARDS groups.

There were no significant differences in the P/F ratio, APACHE II score, SOFA score, serum NOx levels, TNF-α levels, or endotoxin levels between the 30-day death and survival groups (Table 3).

The APACHE II score, SOFA score,
serum NOx levels and TNF-α levels were significantly higher in the 60- and 90-day death groups than in the corresponding survival groups (Tables 4, 5). There were no significant differences in the endotoxin levels between the 60-day death and survival groups or between the 90-day death and survival groups.

IV. Discussion

Inducible nitric oxide synthase (iNOS) is induced by the stimulation of inflammatory cytokines and endotoxin in macrophages, neutrophils, vascular endothelial cells, airway epithelial cells and alveolar epithelial cells, and produces a large amount of NO. NO reacts with superoxide (O₂⁻) to produce peroxynitrite (ONOO⁻). Peroxynitrite itself is toxic, and hydroxyl radicals (•OH) generated from peroxynitrite have even higher toxicity and are known to damage not only bacteria and tumor cells, but also normal pneumocytes, thereby acting against living bodies.

Administration of steroids has also been reported to inhibit the induction of iNOS, thereby suppressing lung damage. Also, administration of the iNOS antagonists, aminoguanidine (AG) and S-methylisothiourea sulfate, has also been reported to inhibit ARDS expression as well as iNOS activity, in a canine model of ARDS. Because NOS inhibitors suppress the formation of edema induced by various stimuli in lung tissue, NO is speculated to be involved in the formation of inflammatory edema.

The relationship between iNOS and other NOS isoforms, i.e., endothelial NOS (eNOS) and neuronal NOS (nNOS), has not been well understood, but Jeremy et al. have reported that AG administration inhibited iNOS and induced recovery of reduced eNOS and nNOS in a rat model of sepsis. The regulatory mechanisms of the 3 NOS isoforms, mainly by NO, is expected to be elucidated in the future.

To date, many clinical reports have shown the involvement of iNOS and NO in the pathogenesis of ALI and ARDS, and their relationship with various humoral mediators has also been suggested. Cells circulating in blood vessels, such as neutrophils, platelets and monocytes, and cells in the lung tissue, such as macrophages, vascular endothelial cells and alveolar endothelial cells, are known to be involved in the development of lung injury seen in ALI/ARDS. In sepsis, endotoxin and other mediators are released into the blood, which activate complement and macrophages, producing TNF, interleukin-1 (IL-1), IL-8, etc., which, in turn, activate neutrophils. Activated neutrophils produce reactive oxygen species and macrophages also produce NO. A strong negative correlation was found between the P/F ratio and serum NOx levels in the present study. Namely, if the P/F ratio is low, serum NOx levels are high, suggesting the involvement of NO in the development of lung injury. In addition, a positive correlation was found between the TNF-α and serum NOx levels, as in a previous study, suggesting that TNF-α may be involved in NO production. A significant negative correlation was found between the serum NOx levels and the P/F ratio, consistent with previously reported results from animal experiments. However, it is unclear whether NO is directly involved.
in the development of acute lung injury. It has been reported that synergistic or additive effects of endotoxin and cytokines are important for the development of septic shock\(^{28}\), and it is possible that endotoxin, together with cytokines, whose production it induces, may be involved in NO production. Also in the present study, there were no significant differences in the endotoxin positivity rate, i.e., the rate of endotoxemia, or in the endotoxin levels between the ALI and ARDS groups.

The mortality rate of ALI/ARDS was previously said to be 30 to 40\%\(^{29,30}\). However, a recent study has reported that the mortality has declined to the range of 20-29\%\(^{30}\). Recently, we reported that the 30-day mortality rates of patients with ARDS and ALI were 19.5\% and 23.0\%, respectively, in 158 patients with ALI/ARDS in the Tohoku district, Japan\(^{30}\). In addition, Oda et al. have reported, based on a study of 79 patients with ALI/ARDS, that the 28-day mortality rate of ALI/ARDS was 31.6\%\(^{30}\).

In the present study, the 30-, 60- and 90-day mortality rates in the 46 patients with ALI/ARDS were 8.7\%, 15.2\% and 19.6\%, respectively, which were almost the same as the mortality rates reported in our recent study on the relationship between ALI/ARDS and high mobility group box 1 (HMGB1) and between ALI/ARDS and interleukin-18\(^{31,32}\). Considering the APACHE II score of 30 ± 9 and SOFA score of 11 ± 5, the mortality rates of the patients seemed to be very low in the present study. We aimed at collecting blood samples within approximately 3 hours of the diagnosis of ALI/ARDS, that is to say, the treatment was initiated at an early stage, and good results were considered to be obtained as a result. In addition, there were no significant differences in the 90-day mortality rates between the ALI and ARDS groups. Namely, the findings suggest that the P/F ratio alone at the time of diagnosis of ALI/ARDS does not allow precise prediction of the prognosis.

There were no significant differences in the P/F ratio, APACHE II score, SOFA score, serum NOx levels or TNF-\(\alpha\) levels between the 30-day survival and death groups. The P/F ratio, APACHE II score, SOFA score, serum NOx levels and TNF-\(\alpha\) levels were significantly higher in the 60- and 90-day death groups than in the corresponding survival groups. It appears that if the early NOx values are high, the tissue and cell damage caused by NOx is severe. The NOx values gradually declined even in the group that died, but they declined more slowly than in the group that survived. That is why death can be prevented by multidisciplinary treatment in the first 30 days, if the early NOx values are high. However, as one might expect, there is a limit, and when 30 days have passed, there appears to be a higher likelihood that organ dysfunction attributable to the tissue and cell damage caused by NOx will develop, and death will ensue.

Thus, our findings suggest that the P/F ratio, serum NOx levels and TNF-\(\alpha\) levels in the early phase of septic ALI may be only weakly related to death up to day 30, but more strongly related to death after day 60. However, the endotoxin levels in the early phase of ALI were not related to the mortality up to day 90.

In the present study, 9 out of 46 patients
with septic ALI/ARDS (19.6%) died, and the causes of death were MODS in 7 cases and heart failure in 2. As reported previously \cite{32,34,35}, it was confirmed that very few patients died of ARF alone. Therefore, it would be important to prevent the development of MODS associated with ALI/ARDS for reducing mortality rates, as pointed out by Suchyta et al.\cite{36}. For this purpose, it is considered necessary to initiate multidisciplinary treatments from as early a stage as possible, considering that ALI/ARDS occurs acutely and progresses rapidly.

Serum NOx levels at the onset of septic ALI were strongly correlated with the APACHE II and SOFA scores, suggesting that NO may be strongly involved in the pathogenesis of sepsis. NO appears to be involved in the pulmonary oxygenation capacity in ALI/ARDS. There were no significant differences in the mortality rates between the ALI and ARDS groups. The 30-, 60- and 90-day mortality rates were 8.7%, 15.2% and 19.6%, respectively, in the 46 patients with ALI/ARDS.

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Conflict of interest statement: Takayuki Masuda and other co-authors have no conflict of interest.

References

of the relationship between the blood levels of nitrite/nitrate (NOx) and the development of ALI/ARDS in sepsis. Med Postgr 44, 61-66, 2006.


敗血症性急性呼吸不全発症早期の
nitrite/nitrate (NOx) 値と予後の検討

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要旨

septic acute lung injury (ALI) /acute respiratory distress syndrome (ARDS) 発症早期のnitrite/nitrate (NOx) 値について検討した。ALI 群に対してARDS 群ではNOx値、tumor necrosis factor α (TNF-α) 値いずれも有効に高値を示した。PaO2/ FiO2 (P/F ratio) とNOx値間には負の相関関係がみられた。また、TNF-α値とNOx値間には正の相関関係がみられた。ALI/ARDS の30日死亡率は87%、60日死亡率は152%、90日死亡率は196%であった。30日目まででは、ALI/ARDS 発症早期のP/F ratio、NOx値、およびTNF-α値はいずれも生存群と死亡群間で差はみられなかった。ALI/ARDS 発症早期のP/F ratio、NOx値、およびTNF-α値は60日目、90日目で、生存群に対して死亡群間で高値であった。ALI群とARDS群に90日まででの死亡率に有意差はみられなかった。ALI/ARDS 発症にNOxが関与している可能性が示唆された。