Detection of protein-bound 3-nitrotyrosine in the plasma of pediatric patients with severe ARDS and avian influenza virus infection

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Abstract

Nitric oxide (NO) and reactive oxygen species (ROS) may be involved in the pathogenesis of various diseases, including microbial infections, inflammatory diseases, and cancer. 3-Nitrotyrosine (3-NT) produced by NO and ROS is considered a biomarker of oxidative stress. Acute respiratory distress syndrome (ARDS) is an inflammatory lung disease and is associated with the excessive production of NO and ROS. Immunohistochemical analyses showed that 3-NT may be produced in the lungs of patients with ARDS. We have identified the extensive and NO-dependent formation of 3-NT in the lungs of mice with ARDS caused by the influenza virus (IFV). However, the biochemical and quantitative aspects of 3-NT formation in patients with ARDS remain poorly understood. Thus, we investigated the levels of plasma protein-bound 3-NT in pediatric patients with severe ARDS using a reverse-phase high performance liquid chromatography (HPLC) coupled with electrochemical detector (ECD). The plasma samples of 40 patients with influenza-negative ARDS (non-IFV-ARDS group) and of 7 patients with influenza-positive ARDS (IFV-ARDS group) were analyzed. IFV-ARDS group consisted of two patients with highly pathogenic avian influenza (A/H5N1) and 5 patients with seasonal influenza (A/H1N1 and A/H3N2). Twenty-five patients without ARDS were used as control (non-ARDS group). Patients in the IFV-ARDS group had significantly higher 3-NT levels (median: 0.350 μmol/mol) than those in the non-ARDS group (median: 0.210; $p = 0.046$). Moreover, the 3-NT levels were significantly higher in the non-IFV-ARDS group (median: 0.270; $p = 0.039$) than in the non-ARDS group. However, the difference was not significant, the survivors had higher 3-NT levels than non-survivors, and the 3-NT levels were higher in patients without multiple organ failure (MOF) than those with MOF. Moreover, the survival rate was more likely higher in the high 3-NT level group than in the low 3-NT level group, indicating the protective role of NO/ROS in the pathogenesis of ARDS. Using this method, we could successfully detect 3-NT from the plasma of patients with ARDS. This method is convenient, specific, and sensitive for 3-NT quantification that is applicable on clinical specimens; hence, it may help in the further understanding of the pathological roles of NO/ROS formation in ARDS.

Keywords: 3-nitrotyrosine, ARDS, highly pathogenic avian influenza, nitric oxide, reactive oxygen species

Introduction

Acute respiratory distress syndrome (ARDS) is one of the most serious inflammatory lung diseases in the intensive care field, and the rate of mortality from ARDS is still high to date[1,2]. Excessive production of inflammatory cytokines is triggered by several biological stresses, such as pneumonia, sepsis, and trauma, which damage alveolar epithelial and endothelial cells, and this phenomenon leads to respiratory failure via non-cardiogenic pulmonary edema[3,4]. ARDS triggered by an infection caused by a highly pathogenic avian influenza (H5N1) virus infection is particularly fatal, with an extremely high mortality rate of 60%[5-8]. Oxidative stress is greatly involved in the molecular pathology of fatal ARDS[9]. Nitric oxide (NO) and reactive oxygen species (ROS) may be involved in the pathogenesis of different diseases, including various microbial infections, inflammatory and neurodegenerative diseases, and cancer[10]. Particularly, in inflammatory conditions, NO is excessively produced by the inducible isoform of NO synthase (iNOS) from inflammatory cells, such as macrophages or neutrophils[11]. Accumulated evidence indicates that NO- and ROS-derived reactive nitrogen oxide species (RNS), such as peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2), also have a pathogenic potential in various diseases[12]. Based on a previous analysis that used a murine model of ARDS caused by the influenza virus (IFV), 3-nitrotyrosine (3-NT) and 8-nitroguanine-related compounds, which are the nitration products of amino acids and nucleobases, are shown to be produced in the infected locus depending on excessive NO production associated with the induction of iNOS using immunohistochemical staining and high-performance liquid chromatography (HPLC)-electrochemical detection (ECD) method[13,14]. 3-NT is a chemically stable substance produced from the nitration of tyrosine residues of proteins, which are produced by RNS that are generated by the reaction of NO and ROS[15] or by NO_2 produced from nitrite (NO_2^-) by neutrophil myeloperoxidase[16]. 3-NT is used as a biomarker of oxidative stress[17]. To date, several studies have reported the use of immunohistochemical staining in locally producing 3-NT in the lungs of individuals with severe cases of pneumonia and ARDS[18,19]. However, analytical methods that use such antibodies are problematic in terms of specificity and have poor quantitativity, and such issues have been frequently observed[20]. Moreover, numerous studies have analyzed the lung tissues of deceased individuals during autopsy, and only few studies have analyzed the lung tissues of survivors because ARDS itself is a serious health condition and it is challenging to obtain lung tissues via biopsy due to the high invasiveness of the procedure, particularly in children and elderly individuals. On the other hand, blood is a biologic sample that can be obtained with relatively minimal invasiveness and can be collected at multiple points during the clinical course of the disease. However, reports about

successful detection of 3-NT using plasma are extremely limited. This may be attributed not only to the low level of 3-NT in the plasma but also to various contaminants in the plasma that lower the signal-to-noise (S/N) ratio, which causes a major barrier to the clinical application of plasma as a 3-NT biomarker. Therefore, in this study, we established a method for treating plasma proteins and optimized elution conditions of HPLC to develop an HPLC-ECD measurement system with high sensitivity and S/N ratio. Moreover, a quantitative analysis of 3-NT in the plasma proteins of patients with fulminant ARDS was performed, and the correlation between 3-NT levels and pathologic conditions was assessed.

Experimental procedures

Collection of plasma samples

The stored plasma samples of pediatric ARDS patients (aged ≥ 1 month) with or without IFV infection who were admitted at the National Hospital of Pediatrics, Hanoi, Vietnam (NHP), from December 2007 to December 2009 were retrospectively assessed in this study. Twenty-five plasma samples obtained from patients without ARDS were used as controls. The clinical and laboratory data of patients were also collected by reviewing the hospital records. The diagnosis of ARDS was made according to the Berlin definition, which includes acute onset within 1 week; bilateral opacities on chest imaging that is not fully explained by effusion, atelectasis, or the presence of nodules; respiratory failure not fully explained by cardiac failure or fluid overlord; and hypoxia (PaO_2/FiO_2 [P/F] ratio ≤ 300) treated with mechanical ventilation with PEEP ≥ 5 cmH_2O[21]. We enrolled patients with severe ARDS whose P/F ratio is ≤ 100 during the clinical course of the disease. A/H5N1 and seasonal IFV infection were confirmed with throat and/or nasal swab tested by reverse-transcriptase polymerase chain reaction at the hospital laboratory. The study was approved by the ethical committee of the National Center for Global Health and Medicine Japan on September 28, 2007 (approved number; NCGM-G-000449-00).

Sample preparation

Plasma (0.1 mL, which is equivalent to approximately 5 mg protein) was diluted with 0.1 M acetate buffer (pH: 7.2, 0.4 mL), and then plasma protein was precipitated by adding 0.8 mL of ice-cold ethanol and centrifuged at 3,000 x g for 10 min. The pellet was washed with ethanol/0.1 M acetate buffer (8:5 v/v, 1 mL) to remove nitrite and nitrate from the plasma and then dried. The dried pellet was resuspended in 0.1 M acetate buffer with 10 mM dithiothreitol and 1% SDS (pH: 7.2, 0.4 mL) and solubilized using a constant temperature incubator shaker (Microtube Maximizer Model MBR-024, Taitec, Japan) (160 rpm, 50ºC) for 24 h. Half of the solubilized protein (0.2 mL) was digested with 0.5 mg pronase (Calbiochem), which was dialyzed against nitrite- and nitrate-free 0.1 M acetate buffer (pH: 7.2), using the microtube maximizer shaker (160 rpm, 50ºC) for 24 h. The digested product was ultrafiltrated using Microcon YM-3 (molecular cut-off of 3,000 Da, Millipore, Billerica, MA) to remove undigested materials.

HPLC-ECD

A total of 20 µL of the ultrafiltrated sample was fractionated with an SC-50ODS column (3 mm x 150 mm, Eicom, Kyoto, Japan) using the HPLC-ECD system (PEC-510/HTEC-500, Eicom). The mobile phase was 200 mM phosphate buffer (pH: 3.0) containing 2% acetonitrile and 5 µg/mL EDTA, and the flow rate was 0.4 mL/min. The sensitivity and linearity of the HPLC-ECD system to 3-NT were verified by analyzing the serial dilutions of the 3-NT standard (Sigma), which ranged from 1 nM to 100 nM. The applied potentials for the dual-mode ECD were adjusted to achieve the highest S/N ratio between 3-NT and other components in the digested plasma proteins. The applied potentials were -800 mV for the reduction cell and +200 mV for the oxidation/detection cell. The detection limit of this system was approximately 1 nM (20 fmol) for 3-NT. The identification criteria for 3-NT were as follows: 1) identical elution time with authentic 3-NT, 2) increased peak by the addition of 3-NT standard to the sample, 3) and disappearance of the peak by reducing the reduction cell potential to -400 mV. The 3-NT levels were standardized by tyrosine levels, which were determined using an ultraviolet detector (SPD-10A, Shimadzu, Japan) connected just after ECD, and they were expressed as µmol 3-NT/mol tyrosine.

NO_2^-/NO_3^- levels in the plasma

To exclude the potential artificial formation of 3-NT by NO_2^-, plasma NO_2^- and NO_3^- concentrations were analyzed using the Griess reaction-based flow reactor system (ENO10, Eicom, Kyoto).

Statistics

PASW Statistics version 18 (SPSS, Chicago, IL, USA) was used for statistical analysis. A p value < 0.05 was considered significant.

Results

Characteristics of the patients

The clinical and laboratory data of the patients enrolled in the study were collected by reviewing hospital records and are summarized in Table 1. We analyzed the plasma samples of 40 patients with IFV-negative ARDS (non-IFV-ARDS group; group A) and of 7 patients with IFV-positive ARDS (IFV-ARDS group; group B). In group A, ARDS was caused by pneumonia in 22 patients and by sepsis in 6 patients,

Table 1. Summary of Clinical and Laboratory Data of the patients

Characteristics	Non-IFV-ARDS Group A	IFV-ARDS Group B	Non-ARDS (disease control)* Group C
Number	40	7	15
Age, year	0.32 (0.20-0.76)	7.00 (3.00-9.50)	1.33 (0.23-2.70)
Male, number (%)	16 (40.0)	5 (71.4)	11 (73.3)
Prognosis			
Death, number (%)	12 (30.0)	5 (71.4)	0 (0)
MOF, number (%)	21 (52.5)	2 (28.6)	0 (0)
Inflammation			
Body temperature onset, ºC	38.0 (36.8-38.5)	38.5 (37.5-39.0)	n/a
CRP, mg/dl	1.5 (0.6-4.9)	1.6 (0.5-3.2)	6.3 (6.1-15.4)
Respiratory parameters			
PaO_2, mmHg	59.5 (42.8-66.3)	58.6 (55.7-65.1)	62.6 (52.2-72.9)
$PaCO_2$, mmHg	43.5 (37.8-53.1)	41.0 (29.4-45.3)	34.5 (27.0-42.1)
Lowest P/F	44 (33-64)	38 (29-71)	n/a
Liver function			
AST, IU/L	83 (61-145)	209 (135-618)	123 (35-204)
ALT, IU/L	37 (26-72)	72 (40-194)	41 (37-141)
LDH, IU/L	932 (673-1249)	1276 (815-1914)	n/a
Blood cell count			
WBC, cells/µL	12000 (9500-19200)	4100 (3400-6700)	13800 (8660-18500)
RBC, x 10^3 cells/µL	3680 (3275-4315)	3980 (3925-4345)	4040 (3700-4810)
Plts, x 10^3 cells/µL	322 (213-499)	131 (117-211)	267 (29-382)
Days from onset to			
admission	5.0 (2.8-6.3)	5.0 (3.5-12.0)	n/a
sampling	7.0 (4.0-12.3)	4.0 (2.0-6.5)	n/a

Data are shown in median value (IQR) unless otherwise specified. n/a: not available.
*Group C included 15 patients with various diseases and 10 healthy individuals for a medical checkup.

whereas the etiology of ARDS was unknown in 12 patients. Group B consisted of two patients with highly pathogenic avian IFV (A/H5N1), 4 patients with seasonal IFV (A/H1N1), and 1 patient with seasonal IFV (A/H3N2). The median days (interquartile range, IQR) from onset of ARDS to sampling was 7.0 (4.0–12.3) for group A and 4.0 (2.0–6.5) for group B. In total, 25 patients without ARDS were considered as controls (non-ARDS group; group C). Group C included 15 patients with various diseases, such as anemia, idiopathic thrombocytopenic purpura, myocarditis, and fever of unknown origin, and the remaining 10 patients were healthy individuals who attended NHP for a regular medical checkup.

Detection of 3-NT in the plasma samples of H5N1-infected patients

A representative elution profile of plasma protein-bound 3-NT in patients with ARDS (H5N1-positive ARDS case) detected using HPLC-ECD are shown in Figure 1. Peak was observed at an elution time (24 min) identical with authentic 3-NT, and the peak disappeared by decreasing the reduction cell potential from -800 to -400 mV, indicating the specific detection of 3-NT. The lower panel shows the corresponding peak of tyrosine (9 min) determined using an ultraviolet detector.

The 3-NT levels of ARDS patients with or without IFV infection and controls

The 3-NT levels of each individual in all 3 groups are shown in Scattered and box plots (Figure 2). The mean levels of 3-NT in group A (0.54 ± 0.11 μmol/mol) and group B (0.98 ± 0.38 μmol/mol) were significantly higher than that of group C (0.21 ± 0.02 μmol/mol). Moreover, 5/40 (12.5%) patients in group A and 3/7 (42.9%) patients in group B showed high levels of 3-NT (≥ 1.0 μmol/mol). By contrast, none of the patients in group C showed 3-NT levels greater than 1.0 μmol/mol. Patients with ARDS had significantly higher 3-NT levels (median: 0.270 μmol/mol, IQR: 0.125-0.640, $p = 0.039$ in group A; median: 0.350 μmol/mol, IQR: 0.182-1.675, $p = 0.046$ in group B) than patients without ARDS (median: 0.210 μmol/mol, IQR: 0.122-0.278 in group C) (Mann-Whitney U test, vs group C). Although the median 3-NT level was slightly higher in group B than in group A, the difference was not significant ($p = 0.166$).

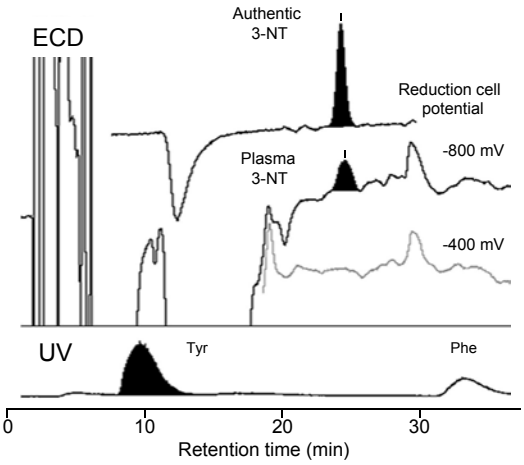

Figure 1. Typical elution profile of plasma protein-bound 3-NT in patients with ARDS detected using HPLC-ECD
Peak was observed at an identical elution time with authentic 3-NT, and disappeared by decreasing the reduction cell potential, indicating the specific detection of 3-NT. The lower panel shows the corresponding peak of tyrosine.

Logistic regression analysis for plasma 3-NT levels in patients with ARDS and controls

Compared to the control group (group C), the group with ARDS (group A+B) had significantly higher odds ratio (OR: 3.30, 95% confidence interval [CI]: 1.12-9.74, $p = 0.027$) for high levels of plasma protein 3-NT (≥ 0.3 μmol/mol, median of all 72 cases). Between the two groups with ARDS, group B had higher OR (OR: 4.42, 95% CI: 0.73-24.44, $p = 0.094$) for 3-NT than group A (OR: 3.17, 95% CI: 1.05-9.59, $p = 0.037$) (Figure 3). Next, groups A and B were compared via logistic regression analysis (Figure 4A). Results suggested that, compared with group A, group B had a higher 3-NT level with an OR of 2.26 ($p = 0.330$) where the level of 3-NT increases from the mean value (0.6 μmol/mol) of groups A and B. The risk of mortality in group B was 5.83 (0.99-34.38), which indicate a significantly worse prognosis than that in group A ($p = 0.035$). The ORs at which CRP and LDH had values greater than the median are more likely to be high (2.50 [0.43-14.51] and 2.71 [0.43-16.96], respectively). Although these results suggested that group B had higher inflammatory response and cytotoxicity than group A, the differences were

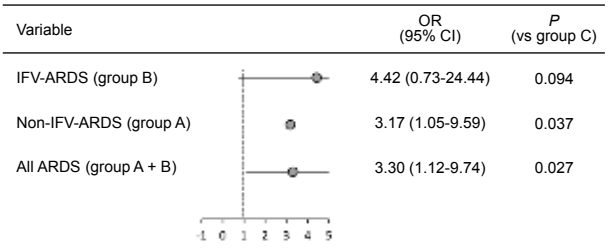

Figure 3. Logistic regression analysis for plasma 3-NT levels in patients with ARDS and controls
Compared to the control group (group C), the group with ARDS (group A+B) had significantly higher OR for high levels of plasma protein 3-NT (≥ 0.3 μmol/mol). Between the two groups with ARDS, group B had higher OR for 3-NT than group A.

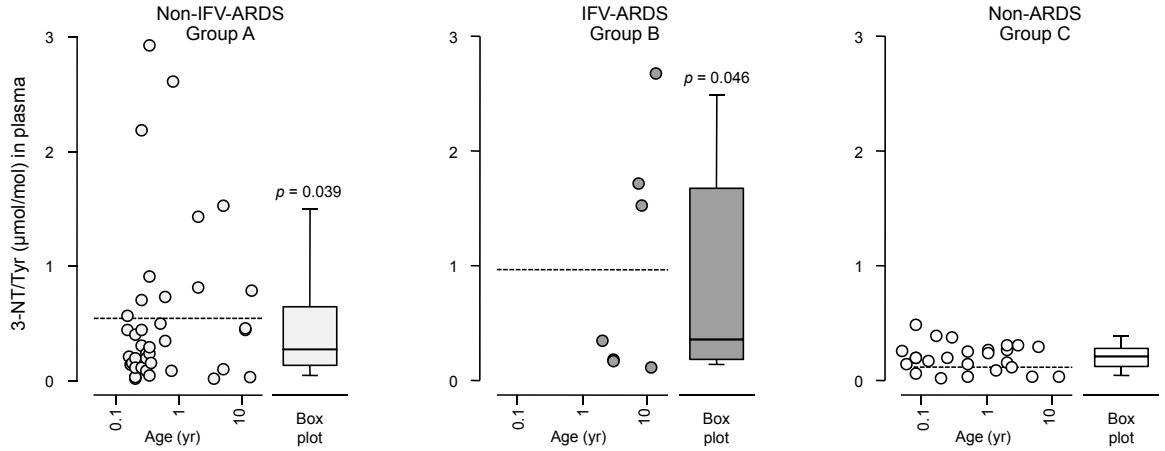

Figure 2. The 3-NT levels of ARDS patients with or without IFV infection and controls
The 3-NT levels in all groups are shown in Scattered and box plots. Patients with ARDS (Group A, B) had significantly higher 3-NT levels than patients without ARDS (Group C) (Mann-Whitney U test). Although the difference was not significant, the median 3-NT level was higher in group B than in group A. Dot lines show mean 3-NT level.

not significant (p = 0.296, 0.273, respectively). The risk of multiple organ failure (MOF) in group B was not significantly different from that of group A (p = 0.243), however, it was more likely lower than that of group A (OR: 0.36 [0.06-2.09]). This suggests that IFV infection is not systematic, rather localized in the lungs. The OR at which the white blood cell (WBC) count exceeded the median was 0.08 (0.01-0.77), thus showing a significantly decreasing trend (p = 0.010). This result suggests the presence of leukopenia, which is often observed in individuals with IFV infection. The examination of the survival curve revealed that the mean survival time (MST) was 35.9 ± 2.3 days (group A) and 14.3 ± 2.9 days (group B), and the survival rate of the group B was significantly lower than that of the group A (p < 0.001, Log-Rank test, Figure 4B).

NO_2^-/ NO_3^- levels in the plasma

The formation of 3-NT is possible via the nitration of tyrosine by NO_2^- in acidic conditions[22]. Therefore, to exclude the potential artificial formation of 3-NT by NO_2^-, plasma NO_2^- and NO_3^- concentrations were analyzed using the Griess reaction-based flow reactor system. Both NO_2^- and NO_3^- concentrations did not significantly correlate to 3-NT levels, indicating the exclusion of artificial 3-NT formation in the plasma (data not shown).

Correlation between 3-NT levels as well as prognosis and clinical parameters

To examine the clinical significance of 3-NT levels, we divided the data of patients with ARDS into two groups according to clinical parameters, and the 3-NT levels were compared. We found that plasma 3-NT levels in the non-survivors (median: 0.24, IQR: 0.19-0.36) was lower than survivors (median: 0.43, IQR: 0.11-0.79) (Figure 5A). Similarly, plasma 3-NT levels in ARDS patients without MOF (median: 0.38, IQR: 0.13-0.94) are more likely higher than those with MOF (median: 0.24, IQR: 0.17-0.64). However, the differences were not statistically significant (p = 0.474, 0.235, respectively, Figure 5B). Interestingly, the comparison of the survival curves of ARDS patients who were divided into groups (those with a 3-NT level higher and lower than the mean 3-NT level [0.6 μmol/mol]) revealed that the survival rate was more likely higher in the high 3-NT level group (n = 13) than in the low 3-NT level group (n = 34). However, the difference was not significant (p = 0.232, Log-Rank test, Figure 5C).

Discussion

3-NT is produced by the nitration of tyrosine residues of proteins by RNS such as $ONOO^-$ and NO_2 produced by the reaction of NO and ROS[12,17], or NO_2 produced from NO_2^- by neutrophil myeloperoxidase[16]. 3-NT is used as a biomarker of oxidative stress in numerous infectious and inflammatory conditions, such as ARDS[15]. HPLC-ECD is a quantitative and specific detection method independent of antibodies[20]. Although the use of HPLC-ECD has been reported in animal experiments, reports about its efficiency in detecting 3-NT in human plasma proteins are extremely limited. In this study, we precipitated plasma proteins with ethanol. As a result, the infectious virus particles potentially contained in the plasma could be completely inactivated, which enable a safe transport by air (from Vietnam to Japan). Moreover, NO_2^-, which is the cause of artificial 3-NT formation[22], and the low molecular weight contaminants, which inhibited the performance of HPLC-ECD analysis, were successfully removed. Consequently, 3-NT was quantified with high sensitivity and excellent S/N ratio. In this study, high levels (≥ 1 μmol/mol) of 3-NT were observed in 8 patients, of which 6 survived and 2 died (Figure 2). Plasma 3-NT levels in the survivors was higher than non-survivors, and the survival rate was higher in the high 3-NT level group than in the low

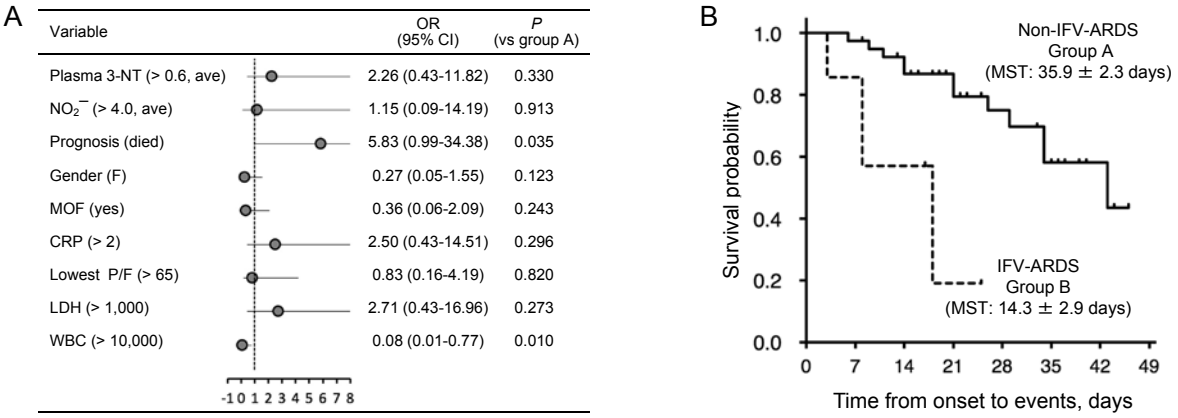

Figure 4. Comparison of Groups A and B via logistic regression analysis and Kaplan Meyer's plot
Compared with non-IFV-ARDS group (group A), IFV-ARDS group (group B) had a higher 3-NT (OR: 2.26). The risk of mortality in group B was 5.83, which indicate a significantly worse prognosis than that in group A. Kaplan Meyer's plot showed significantly lower survival rate of the group B than that of the group A.

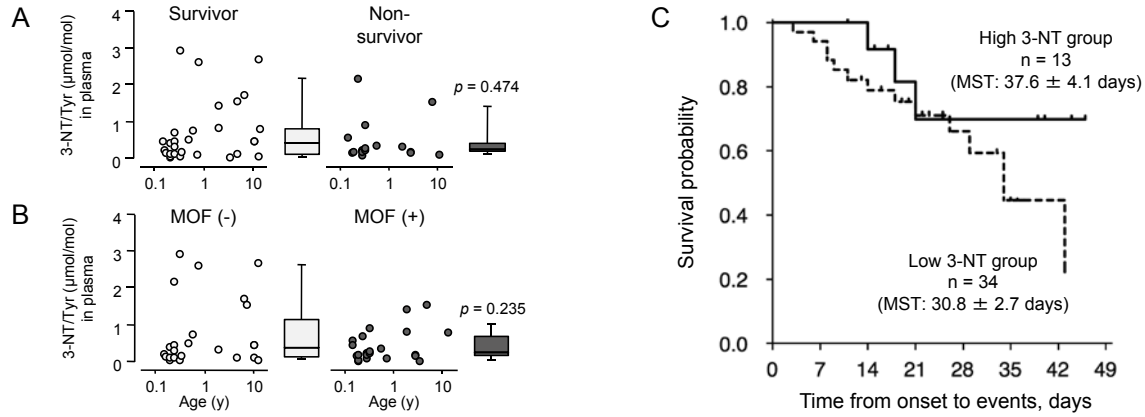

Figure 5. 3-NT levels and prognosis in ARDS patients
3-NT levels in the non-survivors was lower than survivors (A). Similarly, 3-NT levels in ARDS patients without MOF are more likely higher than those with MOF (B). The survival rate was higher in the high 3-NT level group than in the low 3-NT level group (C).

3-NT level group (Figure 5). These results indicates that 3-NT level is not necessarily an indicator of poor prognosis. Rather, the 3-NT level is more likely inversely correlated with prognosis and disease state. The results suggested that the in vivo production of 3-NT by NO/ROS has a direct or indirect biological defense function. NO and ROS are harmful substances that cause pulmonary cell injury and extracellular matrix destruction in ARDS, and they had been considered important effector molecules in ARDS. However, in recent years, NO and ROS have various physiological functions in various cells. We previously found that cGMP, the second messenger of NO signaling, was nitrated to form its unique nitrated derivative 8-nitroguanosine 3', 5'-cyclic monophosphate (8-nitro-cGMP) in cells that depended on the production of NO/ROS[23]. The formation of protein Cys-cGMP adducts by 8-nitro-cGMP was identified as a new post-translational modification, which referred to as protein S-guanylation[23]. Importantly, 8-nitro-cGMP strongly induced antioxidant enzyme heme oxygenase-1 (HO-1) in cultured cells and experimental animal models, indicating the potent signaling functions of 8-nitro-cGMP for HO-1 induction[24,25]. Guanine nitration forming 8-nitro-cGMP may be involved in a unique signal transduction, which contributes to the oxidative stress responses during IFV infection and ARDS[14]. Furthermore, 8-nitro-cGMP and a variety of unidentified nitrated biomolecules may be simultaneously produced in the inflammation site where 3-NT is generated. To understand the entirety of such biological reactions, more studies and case analyses must be assessed. In the future, 3-NT may be further clinically used as a biomarker of oxidative stress in various infectious and inflammatory conditions, such as severe pneumonia and ARDS.

Conflict of interest statement

The authors declare that they have no conflict of interests.

Acknowledgements

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