DR. MÜNEVVER TUGBA TEMEL (Orcid ID : 0000-0001-8636-6641)

PROF. ABDULLAH TUNCAY DEMIRYÜREK (Orcid ID : 0000-0002-9994-8541)

Article type : Original Articles

Prof. Abdullah Tuncay DEMIRYUREK (Orcid ID: 0000-0002-9994-8541)

Evaluation of dynamic thiol/disulfide homeostasis in children with community-acquired pneumonia

Short running title: Thiol/disulfide homeostasis in CAP

Münevver Tugba Temel[1], Seniz Demiryürek[2], Levent Temel[3], Ahmet Saracaloglu[4], Necmi Eke[5], Elif Baysalman[4], Azad Mammadov[4], Mehmet E. Coskun[1], Abdullah T. Demiryürek[4]

Departments of [1]Pediatrics, [2]Physiology, and [4]Medical Pharmacology, Faculty of Medicine, University of Gaziantep, Gaziantep, Turkey

[3]Pediatrics Clinic, Private Gaziantep Defa Life Hospital, Gaziantep, Turkey

[5]Medical Student, Faculty of Medicine, University of Gaziantep, Gaziantep, Turkey

Correspondence:

Abdullah T. Demiryürek, Ph.D.

Department of Medical Pharmacology, Faculty of Medicine,

University of Gaziantep, Gaziantep 27310, Turkey

Phone: 90-342-3606060 Ext.74355; Fax: 90-342-3601617

E-mail: demiryurek@gantep.edu.tr

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ped.13773

Abstract

Background: The alterations in the thiol levels under oxidative stress may contribute to the community-acquired pneumonia (CAP). The goal of this study was to evaluate whether there are changes in thiol/disulfide homeostasis and nitric oxide (NO) levels in children with CAP.

Methods: In total, 130 participants were included in the study. Among them, 65 had been diagnosed with CAP on admission, and the remaining 65 were healthy individuals. Total thiol and native thiol levels in serum of each participant were measured by a novel automated spectrophotometric method. The amount of dynamic disulfide bonds and related ratios were calculated from these values. Serum NO levels were detected using a chemiluminescence assay.

Results: The average native thiol, total thiol, and disulfide levels of patients with CAP were found to be significantly lower than those levels of healthy individuals ($P<0.0001$, $P<0.0001$, $P=0.0126$, respectively). In addition, disulfide/native thiol ($P=0.0002$), and disulfide/total thiol ratios ($P=0.0004$) were found to be significantly higher, whereas the native thiol/total thiol ratio ($P=0.0004$) was lower in the CAP group. We detected high serum NO levels ($P=0.0003$) in the CAP group, but there was no marked correlation between thiol/disulfide and NO levels.

Conclusion: Our results showed that the changes in the endogenous thiol levels under oxidative stress may be associated with the pathogenesis of CAP in pediatric patients.

Key words: Thiol, disulfide, nitric oxide, pneumonia, oxidative stress

Introduction

Community-acquired pneumonia (CAP) particularly refers to clinical signs and symptoms of pneumonia acquired outside a hospital setting.[1] Pneumonia remains one of the most common serious infections in childhood, accounting for 16% of all deaths of children younger than 5 years of age.[2] The pathophysiology of CAP includes various mechanisms such as microorganism invasion, airway damage and activation of immune defense systems. During CAP an extensive influx of activated phagocytes into the lower airways is observed, which represents the first line of defense against invading microorganisms. The production of reactive oxygen species (ROS) and nitrogen species from neutrophils and macrophages is an important component of the host defense system for the process of pathogen clearance and modulation of immunological response.[3,4] This pulmonary increase in ROS levels and proteolytic enzymes may be reflected at the systemic level as an increment in the oxidative stress in these patients.[5,6] Although oxidative stress is a crucial part of the host innate immune response to foreign pathogens, excessive amounts of oxidative stress cause cellular damage and a systemic inflammatory response.[7,8] Chen et al.[9] reported that patients with CAP showed increased ROS and DNA damage in peripheral blood mononuclear cells. It has been demonstrated that antioxidant vitamin C, vitamin E and GSH levels were diminished in children with acute pneumonia when compared to the controls.[10] A clinical trial also supports the relationship between GSH levels and CAP severity.[11] Collectively, these findings show that oxidant/antioxidant imbalance is present in CAP.

Thiol is an organic compound, and it contains an –SH group, which plays an important role in suppression of oxidative stress in cells. Plasma thiol pool is mostly formed by albumin and protein thiols and, to a lesser extent, by low-molecular weight thiols such as homocysteine, cysteine, cysteinylglycine, γ-glutamylcysteine, and glutathione.[12] In the presence of oxidative

stress, thiols groups are converted into disulfides. However, these disulfide bonds are reversible, and can subsequently turn back into thiols. So, this cycle continues in a balanced state, and is referred to the presence of a dynamic thiol–disulfide homeostasis condition.[12-14] In disease processes associated with oxidative stress, an imbalance of dynamic thiol/disulfide homeostasis has been reported.[12,14] Currently, there is only one clinical study established the association of thiol/disulfide homeostasis with CAP.[15] However, this study was performed in adults, and there is no published research in children. Thus, the purpose of this study was to determine the possible role of thiol/disulfide homeostasis in children with CAP. We also aimed to investigate the contribution of serum nitric oxide (NO) levels to the pathophysiology of CAP, and to compare the levels between children with CAP and controls.

Methods

Study populations

A total of 65 children with CAP (mean age 2.8±2.2 years, 29 males and 36 females) evaluated at pediatrics departments were recruited into this study. Moreover, 65 age- and sex-matched healthy control subjects (mean age 3.0±2.1years, 31 males and 34 females) selected from the hospital's outpatient clinic, were also enrolled, for comparison. Children with hospital-acquired pneumonia, active tuberculosis, diabetes mellitus, malignancy, and those with underlying immunological or pulmonary disease were excluded from the study. Individuals suffering from renal, gastrointestinal, endocrine or cardiac diseases and hypertension, and those taking medications such as antioxidants or antibiotics were excluded from the study as well. Diagnosis of CAP was based on the presence of clinical lower respiratory tract signs and symptoms, systemic manifestations and a new pulmonary infiltrate in chest radiography. Clinical diagnosis of CAP was supported by at least two of the following signs and symptoms: cough with

purulent or mucopurulent sputum, dyspnea, chest pain, auscultatory findings (rales and/or sign of consolidation), fever >38^{o}C, or leukocytosis (white blood cells >10x10^{3}/mm^{3}). Bacterial and viral pathogens detected by conventional methods. Microbiological tests such as blood culture, Gram stain, microscopy and culture of expectorated sputum or nasopharyngeal aspirates were performed to define the bacterial etiology of pneumonia, while serological tests were directed to atypical pathogens (*Mycoplasma pneumoniae, Chlamydia pneumoniae,* or *Legionella pneumophila*) or viruses. Local clinical ethics committee assessed and approved the study, and parental informed consent was obtained in each case. The present research was conducted according to the principles described in the Declaration of Helsinki.

Blood samples

In patients, blood sampling was done on admission. Biochemical analyses were done within 30 minutes after blood collection. For thiol/disulfide and NO analyses, serum was separated by centrifugation at 1500 *g* for 10 minutes, placed into plain tubes and stored at -80^{o}C until analysis.

Thiol/disulfide measurements

The contents of the native thiol (–SH) and total thiol (–SH + –S–S–) in the serum samples were measured using the reagent kits from Rel Assay Diagnostics (Gaziantep, Turkey). These spectrophotometric methods, developed by Erel and Neselioglu,[14] assayed in an auto-analyzer. The reducible disulfide bonds were first reduced to free-form functional thiol groups. The unused reductant, sodium borohydride, was consumed and removed with formaldehyde, and all of the thiol groups, including the reduced and

native ones, were measured after reacting with 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB). Half of the difference between the total and native thiols yielded the dynamic disulfide (–S–S) content.

NO analysis

Nitrate and nitrite are the primary oxidation products of NO, and the stable nitrate/nitrite levels in plasma are generally used as an indicator of NO formation. The technique (NO/ozone chemiluminescence) used in our study converts nitrate/nitrite back to NO and measures the NO level in a gaseous form. Serum NO levels were detected as described previously.[16] The serum samples were deproteinized with absolute ethanol at 0^{o}C in a 1:2 v/v mix, incubated 30 min at 0^{o}C followed by centrifugation at 14,000 rpm for 5 min. The pellets were discarded and the supernatant was used to measure NO levels by the NO/ozone chemiluminescence technique (Model 280i NOA, Sievers Instruments, Boulder, CO, USA). Samples and standards were injected into the purge vessel to react with the reducing agent (vanadium III chloride dissolved in 1 M HCl at 95^{o}C), which converted nitrite, nitrate, and S-nitroso compounds to NO. A continuous stream of pure nitrogen purged the resultant NO from the reaction vessel to the chemiluminescence chamber. NO concentration of the sample was determined by comparison with the standard curve (obtained with sodium nitrate). The NOAnalysis^{TM} software (version 3.21, Sievers, Boulder, CO, USA) was used for data collection and analysis.

Statistical analyses

All values were shown as the mean ± SD or percentage. The distribution of all variables was checked with the Kolmogorov–Smirnov test. Mann–Whitney U test was used for data with abnormal distribution. Otherwise, the differences between mean values of two

groups were analyzed using the unpaired Student's t test. Gender of the two groups was analyzed using the chi-square test. The Pearson's test was used to assess the correlations, but Spearman correlation analysis was performed in correlation analysis of data with abnormal distribution. GraphPad Instat (version 3.05, GraphPad Software Inc., San Diego, CA, USA) statistical software was employed. The statistical significance was accepted at the level of 0.05.

Results

Demographic and laboratory data of the children with CAP and in healthy controls are presented in Table 1. Compared with the controls, the average age, gender, weight, hemoglobin, platelet count, platelet distribution width, mean platelet volume, and neutrophils/lymphocytes ratio in the CAP group were similar. White blood cells, neutrophils, lymphocytes, NO, disulfide/native thiol ratio, and disulfide/total thiol ratio were all greater among CAP subjects, and native thiol, total thiol, disulfide, and native thiol/total thiol ratio were lower among CAP patients (Table 1).

Microbiological and serological test results revealed bacterial pneumonia in 73.8% (48/65), atypical pneumonia in 10.8% (7/65), and viral pneumonia in 15.4% (10/65) of study patients. Multiple pathogens were found in 27 patients (41.5%). The most common viruses were respiratory syncytial virus and influenza, and the most frequently detected bacteria were *Streptococcus pneumoniae* and *Haemophilus influenzae.*

Figure 1 shows the distribution of the native thiol, total thiol, and disulfide levels between the study groups. These values were markedly low in the CAP group ($P<0.0001$ for native thiol and total thiol levels, $P=0.0126$ for disulfide levels). Although the disulfide/native thiol

(*P*=0.0002), and disulfide/total thiol ratios (*P*=0.0004) were high, native thiol/total thiol ratio (*P*=0.0004) was low in the CAP group (Figure 2). Elevated NO levels were detected in the CAP patients (*P*=0.0003, Figure 3).

Correlation analysis showed that there were marked positive correlations between native thiol and total thiol, total thiol and disulfide, and disulfide and WBC (Table 2). Negative correlations were observed between native thiol and neutrophils, total thiol and neutrophils, and total thiol and C-reactive protein (CRP). No significant correlations were noted between thiol levels and NO or CRP levels.

Discussion

The present study is the first study to assess dynamic thiol/disulfide homeostasis in the serum of children with CAP by a novel automated spectrophotometric method. We observed marked reductions in native thiol and total thiol levels in CAP patients. Consequently, disulfide levels were also low in the CAP group. However, increased WBC, neutrophils, lymphocytes counts, augmented disulfide/native thiol and disulfide/total thiol ratios, and elevated CRP and NO levels were detected. Thus, our findings suggest that endogenous thiols were depleted, and dynamic thiol/disulfide homeostasis is affected by CAP.

In principle, the airway epithelium carries an effective antioxidant defense system, including enzymatic antioxidants (eg, catalase and superoxide dismutase), glutathione, and enzymes associated with glutathione.[17] Despite these protective mechanisms, oxidative stress in the airway epithelium is known to cause events such as DNA methylation, cell membrane damage, and lipid peroxidation, which result in widespread cellular and tissue damage contributing significantly to the worsening of lung infections.[18] Glutathione is well known as

the most efficient antioxidant in the lung, and its depletion is associated with oxidative stress in many other diseases.[19] Oxidative stress is also important in the pathogenesis of CAP. Accumulating evidence suggests that serum total antioxidant status is decreased, and oxidative stress parameters were increased in patients with CAP.[5,10,15,20] Blood samples from patients with pneumonia demonstrated low levels and activities of antioxidants such as glutathione peroxide, superoxide dismutase, and glutathione, compared with healthy controls, suggesting a high burden of oxidants.[10] Chen et al.[9] also demonstrated that severe CAP exhibited significant increase of oxidative stress and proinflammatory mediators (TNF-α and IL-6) in peripheral blood and lung. Recently, Parlak et al.[15] found that the total thiol and native thiol levels were significantly lower, and the disulfide/total thiol levels were significantly higher in the CAP group compared with the controls, but the disulfide levels were similar between the two groups. Our results were in part consistent with this report, but we found that disulfide levels were significantly lower in patients with CAP. Additionally, increased disulfide/native thiol ratio and diminished native thiol/total thiol ratio were recorded in our study. These ratios were not studied by Parlak et al.[15] Increased disulfide/total thiol and disulfide/native thiol ratios may indicate that the thiol/disulfide ratio had shifted towards disulfide bond formation, and that oxidative stress was augmented in CAP.

We found negative correlations between the neutrophils and thiol levels (both native and total thiols). This data may suggest that the ROS released from the high number of neutrophils in CAP patients decrease the thiol levels by oxidizing the thiols. This finding is also in agreement with the study published by Parlak et al.[15] who showed similar correlations. We also observed marked positive correlation between disulfide levels and WBC counts. Thus, it can be proposed that elevated ROS release from increased numbers of leukocytes generates high levels of disulfide.

NO is part of the innate inflammatory response and its levels are expected to rise in acute lung infection.[3] However, conflicting data about NO levels in pneumonia patients have been reported. In exhaled breath analysis, the concentration of nitrate, a stable oxidative end product of NO metabolism, in patients with CAP was found to be higher than that in controls.[21] In another study, no difference in exhaled NO levels was found between children with CAP and healthy controls.[22] We observed marked increased in serum NO levels in children with CAP. In agreement with our results, augmented NO production has been shown in the broncoalveolar lavage of children with infective pneumonia.[23] This increase probably derives from an elevated lung NO production. NO is synthesized by the epithelium of the respiratory tract together with inflammatory cells.[3] During pneumonia, a extensive influx of activated phagocytes into the lower airways is observed.[24] We have detected increased leukocytes and monocytes. Thus, these cells may contribute to the increased NO levels seen in our study. NO rapidly interacts with superoxide to form peroxynitrite which can oxidize a wide variety of biomolecules including thiols.[25] Therefore, NO can contribute to thiol/disulfide homeostasis in CAP.

In conclusion, our results showed that elevated oxidative stress and impaired thiol/disulfide homeostasis have a substantial role in the etiology of CAP in children. Increased ROS generation in CAP can lead to the depletion of endogenous thiol levels.

Disclosure

The authors declare no conflict of interest.

Author contributions

M.T.T. and A.T.D. designed the study; M.T.T., L.T. and M.E.C. enrolled patients and collected data; S.D., A.S., N.E., E.B. and A.M. performed experiments; S.D. and A.T.D. analyzed the data and wrote the manuscript; M.T.T., S.D. and A.T.D. interpreted the data and reviewed the manuscript; A.T.D. supervised the whole study process. All authors read and approved the final manuscript.

References

1. Harris M, Clark J, Coote N *et al.* British Thoracic Society guidelines for the management of community acquired pneumonia in children: update 2011. *Thorax.* 2011; **66 Suppl 2**: ii1-ii23.

2. World Health Organization. Pneumonia. Fact sheet, 7 November 2016. https://www.who.int/news-room/fact-sheets/detail/pneumonia (accessed on 17 June 2018).

3. Delclaux C, Azoulay E. Inflammatory response to infectious pulmonary injury. *Eur. Respir. J. Suppl.* 2003; **42**: 10s-14s.

4. Fialkow L, Wang Y, Downey GP. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. *Free Radic. Biol. Med.* 2007; **42**: 153-64.

5. Katsoulis K, Kontakiotis T, Baltopoulos G, Kotsovili A, Legakis IN. Total antioxidant status and severity of community-acquired pneumonia: are they correlated? *Respiration.* 2005; **72**: 381-7.

6. Laskaj R, Dodig S, Cepelak I, Kuzman I. Gamma-glutamyltransferase activity and total antioxidant status in serum and platelets of patients with community-acquired pneumonia. *Arch. Med. Res.* 2007; **38**: 424-31.

7. Manda-Handzlik A, Demkow U. Neutrophils: The role of oxidative and nitrosative stress in health and disease. *Adv. Exp. Med. Biol.* 2015; **857**: 51-60.

8. Pruchniak MP, Aražna M, Demkow U. Biochemistry of oxidative stress. *Adv. Exp. Med. Biol.* 2016; **878**: 9-19.

9. Chen Y, Luo G, Yuan J *et al.* Vitamin C mitigates oxidative stress and tumor necrosis factor-alpha in severe community-acquired pneumonia and LPS-induced macrophages. *Mediators Inflamm.* 2014; **2014**: 426740.

10. Cemek M, Caksen H, Bayiroğlu F, Cemek F, Dede S. Oxidative stress and enzymic-non-enzymic antioxidant responses in children with acute pneumonia. *Cell Biochem. Funct.* 2006; **24**: 269-73.

11. Lai KY, Ng WY, Osburga Chan PK, Wong KF, Cheng F. High-dose N-acetylcysteine therapy for novel H_1N_1 influenza pneumonia. *Ann. Intern. Med.* 2010; **152**: 687-8.

12. Turell L, Radi R, Alvarez B. The thiol pool in human plasma: the central contribution of albumin to redox processes. *Free Radic. Biol. Med.* 2013; **65**: 244-53.

13. Jones DP, Liang Y. Measuring the poise of thiol/disulfide couples *in vivo*. *Free Radic. Biol. Med.* 2009; **47**: 1329-38.

14. Erel O, Neselioglu S. A novel and automated assay for thiol/disulphide homeostasis. *Clin. Biochem.* 2014; **47**: 326-32.

15. Parlak ES, Alisik M, Hezer H, Karalezli A, Hasanoglu HC, Erel O. Evaluation of dynamic thiol/disulfide redox state in community-acquired pneumonia. *Saudi Med. J.* 2018; **39**: 495-9.

16. Alasehirli B, Demiryürek S, Arica E, Gürsoy S, Demiryürek AT. No evidence for an association between the Glu298Asp polymorphism of the endothelial nitric oxide synthase gene and fibromyalgia syndrome. *Rheumatol. Int.* 2007; **27**: 275-80.

17. Comhair SA, Erzurum SC. Antioxidant responses to oxidant-mediated lung diseases. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2002; **283**: L246-55.

18. Park HS, Kim SR, Lee YC. Impact of oxidative stress on lung diseases. *Respirology.* 2009; **14**: 27-38.

19. Ghezzi P. Role of glutathione in immunity and inflammation in the lung. *Int. J. Gen. Med.* 2011; **4**: 105-13.

20. Nowak D, Zieba M, Zawiasa D, Rozniecki J, Król M. Changes of serum concentration of lipid peroxidation products in patients with pneumonia. *Monaldi Arch. Chest Dis.* 1996; **51**: 188-93.

21. Corradi M, Pesci A, Casana R *et al*. Nitrate in exhaled breath condensate of patients with different airway diseases. *Nitric Oxide.* 2003; **8**: 26-30.

22. Carraro S, Andreola B, Alinovi R *et al*. Exhaled leukotriene B4 in children with community acquired pneumonia. *Pediatr. Pulmonol.* 2008; **43**: 982-6.

23. Grasemann H, Ioannidis I, de Groot H, Ratjen F. Metabolites of nitric oxide in the lower respiratory tract of children. *Eur. J. Pediatr.* 1997; **156**: 575-8.

24. Moldoveanu B, Otmishi P, Jani P *et al*. Inflammatory mechanisms in the lung. *J. Inflamm. Res.* 2009; **2**: 1-11.

25. Demiryürek AT, Cakici I, Kanzik I. Peroxynitrite: a putative cytotoxin. *Pharmacol. Toxicol.* 1998; **82**: 113-7.

Table 1 Demographic and laboratory data of the children with CAP and in healthy controls

	Control (n=65)	CAP (n=65)	*P*-values
Age (years)	3.0±2.1	2.8±2.2	0.5969
Gender			0.8603
Male (n %)	31 (47.7)	29 (44.6)	
Female (n %)	34 (52.3)	36 (55.4)	
Weight (kg)	14.3±1.5	13.9±1.8	0.1711
Hemoglobin (g/dL)	13.5±2.4	13.1±1.6	0.2656
White blood cells (x 10^3/mm^3)	6.9±3.0	12.1±6.5	<0.0001
Platelet count (x 10^3/mm^3)	261.8±73.1	257.4±77.3	0.7394
MPV (fL)	9.3±0.7	9.1±0.8	0.1318
PDW (%)	10.6±1.9	10.1±1.5	0.0983
Monocytes (x 10^3/mm^3)	0.8±0.3	1.2±0.7	<0.0001
Neutrophils (x 10^3/mm^3)	5.8±2.6	6.7±2.5	0.0464
Lymphocytes (x 10^3/mm^3)	3.1±1.7	3.7±1.5	0.0348
CRP (mg/L)	0.3±0.1	11.9±10.9	<0.0001
NO (μmol/L)	68.2±42.5	106.1±64.6	0.0003
Native thiol (μmol/L)	400.5±105.8	227.7±53.8	<0.0001
Total thiol (μmol/L)	448.4±111.7	266.3±58.9	<0.0001
Disulfide (μmol/L)	24.0±11.5	19.3±9.6	0.0126
Disulfide/Native thiol (%)	6.3±3.1	8.9±4.6	0.0002
Disulfide/Total thiol (%)	5.5±2.4	7.3±3.3	0.0004
Native thiol/Total thiol (%)	89.1±4.8	85.4±6.6	0.0004

Data show mean ± SD values. CRP, C-reactive protein; MPV, mean platelet volume; NO, nitric oxide; PDW, platelet distribution width

Table 2 Significant correlations between thiol and disulfide levels in children with CAP

Parameters	Correlation coefficient (r)	Coefficient of determination (r^2)	*P*-values
Native thiol ↔ Neutrophils	-0.3558	0.1266	0.0104
Total thiol ↔ Neutrophils	-0.2851	0.0813	0.0425
Disulfide ↔ WBC	0.2960	0.0876	0.0349
Total thiol ↔ CRP	-0.2786	0.0776	0.0477

WBC, White blood cells

Figure Legends

Fig. 1 Box-plot of native thiol, total thiol (A) and disulfide levels (B) between the study groups. Lower and upper margin of each box represents 25th and 75th percentiles, horizontal lines in the middle of the boxes represents median value, and whiskers represent lowest and highest values.

Fig. 2 Box-plot of disulfide/native thiol, disulfide/total thiol ratios (A) and native thiol/total thiol ratio (B) between the study groups. Lower and upper margin of each box represents 25th and 75th percentiles, horizontal lines in the middle of the boxes represents median value, and whiskers represent lowest and highest values.

Fig. 3 Box-plot of NO levels between the study groups. Lower and upper margin of each box represents 25th and 75th percentiles, horizontal lines in the middle of the boxes represents median value, and whiskers represent lowest and highest values.