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# Evaluation of nitric oxide metabolism and malondialdehyde levels as an indicator of oxidant stress in malign and parapneumonic pleural effusion

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**Ethics Committee Approval**
This study was approved by the Ethics Committee of Ankara University Medical Faculty (25 - 487, 28 February 2011).
All procedures in this study involving human participants were performed in accordance with the 1964 Helsinki Declaration and its later amendments.

**Conflict of Interest**
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## Abstract

**Background/Aim:** Pleural effusion is an important pathology which usually develops comorbid to varying diseases and negatively affects the quality of life. Studies to understand the etiopathogenesis of the disease are important. Although there are some studies in the literature about the arginine-NO metabolism in pleural diseases, there is not another study including all patient groups and the parameters examined in this study. Pleural fluid arginase and NOS (nitric oxide synthase) activities as well as NO (nitric oxide) and MDA (malondialdehyde) levels of patients were determined. The aim of our study was to investigate the possible relationship between these parameters and the mechanism of pleural fluid accumulation.

**Methods:** In this study, pleural fluid arginase and NOS (nitric oxide synthase) activities as well as NO (nitric oxide) and MDA (malondialdehyde) levels of patients with malignancy, pneumonia and CHF (congestive heart failure) were determined. Our study was a cross-sectional descriptive research and our study groups consisted of patients with pneumonia (n=28), malignancy (n=28) and CHF (n=24). NO and MDA level with arginase and NOS activity were determined spectrophotometrically. Results were expressed as mean (standard deviation).

**Results:** Pleural fluid arginase activity in CHF patients was significantly lower than in the malignancy and pneumonia groups ($P$=0.003). The pleural NO level and NOS activity were higher in the malignancy group than in the other groups ($P$<0.001). Compared to the other groups, MDA level was significantly increased in the pneumonia group. ($P$<0.001).

**Conclusion:** In the light of these results, it may be concluded that the arginase- NO metabolism and MDA formation are involved in the pathogenesis of pleural effusions.

**Keywords:** Arginase, Malondialdehyde, Nitric oxide, Nitric oxide synthase, Pleural effusion

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## Introduction

Arginase is an important hydrolytic enzyme involved in arginine degradation. Arginine is also used by NOS to produce NO, and NOS and arginase may compete to utilize that common substrate. The main task of arginase in tissues is to regulate the intracellular arginine concentration to diminish NO production by NOS [1].

NO is a short- lived polyfunctional signaling molecule and a well-known free radical [2]. There are three isoforms of NOS in the human body, and all are identified in the respiratory system. One of the three isoforms of NOS is iNOS (inducible NOS) which is expressed in response to bacteria and proinflammatory stimuli and produces large amounts of NO for a longer period [3]. All three isoforms (endothelial, inducible and neuronal NOS) participate in the regulation of respiratory physiology by cooperative production of NO. Any change in the activities of these enzymes may affect the NO level and play a role in pathogenesis of many respiratory diseases [4].

It is known that free radicals play a role in the development of cancer, inflammation damage and chemical toxicity and elevated levels of NO are associated with free radical production. NO may act both as an inhibitor and activator of lipid peroxidation under different conditions [5]. That is why we aimed to investigate the possible complex relationship between arginase, NOS/NO and MDA as a product of lipid peroxidation.

Pleural effusion is a common clinical problem in routine practice and is often seen as a complication of a systemic pathology or other organ diseases. The most common causes of pleural effusion include CHF, malignancy, pneumonia, and tuberculosis.

Various markers of oxidative stress can be detected in different biological specimens such as blood, sputum, bronchoalveolar lavage (BAL) fluid, pleural fluid, and exhalation air in lung diseases. These samples demonstrate both local and systemic effects of oxidative stress. However, the role of free radicals in pleural diseases is not yet fully known. Free radicals may occur as a byproduct of normal metabolism, as well as by the effects of infections, inflammation, carcinogenesis, drugs, and other harmful chemical substances. Therefore, in this study, the levels of NO, MDA and the activity of NOS and arginase in pleural fluid were examined in three groups of patients with heart failure, lung cancer and pneumonia, and the possible relationship between these parameters and etiopathogenesis of the diseases were investigated.

## Materials and methods

### Study population and protocol

This study was approved by the Ethics Committee of Ankara University Medical Faculty (25 - 487, 28 February 2011). Informed consents were obtained from all patients. The samples of pleural fluid were collected by thoracentesis from the patients in Ankara Atatürk Chest Diseases and Chest Surgery Training and Research Hospital. Patients with pleural effusions whose pleural collections were sampled for diagnostic and /or therapeutic purposes were included in the study. Patients were divided into three groups based on their final diagnoses. Similar numbers of patients to those in the literature were included in each group. We tried to reach the maximum number of patients within the planned research period. The first group of 28 patients had pneumonia with parapneumonic effusion (PPE). This group consisted of 21 males and 7 females. The mean age of the patients was 56.4 (18.0) years. Diagnosis of PPE was established based on the measurement of the pH, glucose and LDH (lactate dehydrogenase) levels of the pleural fluid using the Light criteria [6]. Also, inflammatory cells were seen in the cytopathological examination of the effusion, which was called "parapneumonic fluid". The second group consisted of 24 patients, 19 males and 5 females who had CHF related transudative effusion that was diagnosed by the presence of clinical and radiologic findings suggestive of cardiac dysfunction. Ejection fraction was <40% based on echoes performed on these patients and a diagnosis of heart failure was made. Patients in this group also had massive fluid accumulation in their lungs. Examination of these fluids showed that it is a transudate due to CHF. The mean age of the patients in this group was 70.8 (10.8) years.

The third group comprised 28 patients, 20 males and 8 females with malignancy. Their mean age was 60.9 (11.9). Malignant effusion was diagnosed by the presence of cytologic and /or histopathologic evidence of malignancy in pleural fluid or bronchoscopic specimens.

Pleural fluid specimens were stored at -80ºC until the day of biochemical analysis. All analyses were performed in the research laboratory of Department of Medical Biochemistry, Ankara University Faculty of Medicine.

Measurement of NO, NOS, MDA, and arginase: The pleural fluid supernatant was obtained after the centrifugation of primary tube at 3000 rpm for 15 minutes at room temperature and NO, NOS, MDA, and arginase values were determined by spectrophotometric methods. Arginase activity was measured using the method described by Chinard [7]. One unit of arginase activity was defined as 1 µmole liberated ornithine per minute at 37 ° C. Arginase activity was expressed as IU/L.

Measurement of the NO pool (consisting of $NO\cdot + NO\neg^{2}$) is based on a chemical reaction in which NO ($NO\cdot$) to a greater extent, and nitrite anion ($NO\neg^{2}$) to a lesser extent, gives a diazotization reaction with sulfanilic acid. The absorbance of complexone formed with N-(1-naphthyl-ethylene diamine) reflects the sum of $NO\cdot$ and $NO\neg^{2}$ levels in the reaction medium, termed the NO pool. NO pool values were given as mM. In this method, sodium nitroprusside was used as the chemical standard and the reaction scheme described by Durak et al. [8] was followed. NOS activity which is known to produce NO by catalyzing a five-electron oxidation of guanidino nitrogen of L-arginine was measured at the same time and expressed as IU/mL [9].

MDA, an end product of fatty acid peroxidation, reacts with thiobarbituric acid to form a colored complex that has maximum absorbance at 532 nm [10]. Results were expressed as nmol/mL. Absorbances were read using a Unicam HeλIOS-α UV-VIS spectrophotometer (Unicam, Cambridge, UK). All chemicals were purchased from Sigma and Merck Chemical Companies (St. Louis, MO).

### Statistical analysis

Data analysis was performed by using SPSS for Windows, version 15. Values in the groups did not show normal

distribution. Therefore, nonparametric Kruskal Wallis test was used for comparison of multiple groups. All data were expressed as mean (SD) and median. For all statistical calculations, $P<0.05$ was considered statistically significant. When there was a significant difference between the three groups, post hoc multiple comparison test was used to determine the groups from which the difference originated. In addition, Spearman correlation analysis was performed separately for the three groups participating in the study. Possible relationships between the parameters examined in the groups were investigated.

## Results

As seen in Table 1, there were significant differences between all three groups in terms of all parameters. Pleural fluid arginase activity in CHF patients was significantly lower than in the malignancy and pneumonia groups ($P=0.003$), and similar between the pneumonia and malignant groups ($P=0,983$). Pleural NO level and NOS activity were higher in the malignancy group than in the other groups ($P<0.001$), and similar between the pneumonia and CHF groups ($P=0.987$, $P=0.921$). MDA level was significantly increased in the pneumonia group compared to the other groups ($P<0.001$), and similar between the CHF and malignant groups ($P= 0.865$).

Table 1: NO and MDA levels and NOS and arginase activities of groups

| | Pneumonia (n=28) Mean (SD) Median (min-max) | Malignancy (n=28) Mean (SD) Median (min-max) | CHF (n=24) Mean (SD) Median (min-max) | *P*-value All groups Pneumonia vs. Malignancy | *P*-value Pneumonia vs. CHF Malignancy vs. CHF |
|---|---|---|---|---|---|
| Arginase (IU/L) | 198.6 (65.8) 53.8 (16.5-1643.0) | 78.4 (2.0) 78.7 (57.9 – 95.0) | 90.7 (36.9) 16.6 (2.0 - 848.8) | 0.003 0.983 | 0.027 0.003 |
| NO (mM) | 15.8 (1.6) 14.7 (8.7 - 56.7) | 45.5 (2.2) 48.2 (23.7 - 61.7) | 14.5 (1.2) 13.0 (8.7 - 39.7) | <0.001 <0.001 | 0.897 <0.001 |
| NOS (IU/ml) | 6.9 (0.2) 6.3 (5.4 - 9.1) | 10.9 (0.3) 10.9 (5.5 - 14.1) | 6.0 (0.4) 6.5 (2.9 - 9.6) | <0.001 <0.001 | 0.921 <0.001 |
| MDA (nmol/ml) | 2.5 (0.3) 1.9 (1.2 - 8.1) | 1.5 (0.2) 1.1 (0.5 – 5.0) | 1.4 (0.2) 1.3 (0.4 - 5.4) | <0.001 <0.001 | <0.001 0.865 |

Min: Minimum, max: maximum, SD: Standard Deviation

According to correlation analysis, only arginase and MDA levels in the pneumonia group were positively correlated ($P=0.002$).

## Discussion

The accumulation of fluid in the pleural space, usually due to an underlying disease, is called pleural effusion [11]. We have very little knowledge about the immunological and molecular mechanisms that play a role in pleural diseases and effusions. A large number of mediators and proteins released by the mesothelial cells play role in inflammation processes. One of these mediators is NO [12]. Experimental studies have shown that during an inflammatory process, pleural mesothelial cells increase NO synthesis by inducing cytokine and lipopolysaccharide. These proinflammatory cytokines increase nitrite/nitrate production [13].

In animal experiments, nitrite/nitrate levels were high in exudative pleural effusion which is generated by carrageenan injection to normal mice. In the same study, it was observed that in mice with iNOS gene loss, the inflammatory response was decreased compared to the pleurisy occurring in normal mice. Nitrite / nitrate levels were also lower in the pleural fluids of mice with iNOS gene loss [14]. The inflammatory reaction was reduced in the exudate formed by the administration of a NOS inhibitor. Regnault et al. [15] reported that NO production was higher in exudate pleural effusions which were induced by an inflammatory reaction. In our study, we found a significant difference between all groups for NO and mean NO levels were higher in exudate groups compared to transudate CHF group. Similar results were found for NOS activity. According to these findings, we may suggest that NO is a possible mediator playing a role in the development of exudates.

Arginase is a potent immune system inhibitor and has an inhibitory effect on lymphocyte proliferation [16]. In many different studies, serum arginase activity was investigated especially in patient groups with different malignancies. Two isoforms of arginase are present in the respiratory system [4]. Thus, in our study, we also aimed to evaluate the changes in arginase activity in pleural diseases and its relation to NO and NOS. Arginase median values of patients with CHF were lower than those of patients in the pneumonia and malignant groups, and similar between the latter two. Furthermore, as to the mean levels of arginase in the study groups, the malignancy group with the lowest mean activity also had the highest NO and NOS averages. This result may further support the supposed relationship between NOS and arginase sharing the common substrate [17]. High NOS activity may be the cause of decreased arginase activity in this group.

Free radicals and peroxides are involved in the pathogenesis of various inflammatory and malignant diseases. Most lipid-containing biological systems such as plasma membranes, organs, and cell membranes utilize the measurement of malondialdehyde in assessing lipid damage because of oxidative stress. Diseases related to the pleural cavity such as malignancy, tuberculosis or pneumonia may cause exudate and theoretically contribute to oxidative stress production. Conversely, transudative pleural effusions are not associated with local pleural pathology and are due to imbalance between hydrostatic and oncotic pressure. So, transudate is not expected to lead to the formation of reactive oxygen derivatives.

Various studies have been carried out to reveal the importance of free radicals in pleural diseases. In a study by Hammouda and colleagues [18] who measured pleural fluid MDA levels, MDA levels were significantly elevated in exudative fluids relative to transudative fluids. Kostikas and colleagues [19] found that oxidative stress was high in the exudative pleural effusion. According to the obtained values, MDA level measurement was a useful method that could be used to differentiate between transudative-exudative fluids.

We compared the pleural fluid MDA levels of three groups of patients as an oxidative stress indicator. We observed a significant difference between the three groups. In bilateral comparisons, the pneumonia group had significantly higher levels compared to malignancy and CHF groups. There was no significant difference between the malignancy and CHF groups. High MDA values in the pneumonia group were supportive of other studies [20], however, MDA levels in the malignancy group were not significantly different from the transudate CHF group.

### Limitations and strengths

The pleural effusion sample used in our study is difficult to obtain and requires an invasive procedure. Therefore, the small number of samples constitutes a limitation in our study. However, we examined more than one parameter in three different disease groups, which was the strength of our work.

### Conclusion

Considering our results, we think that arginine-NO metabolism may play a role in the etiopathogenesis of pleural effusions caused by different lung diseases. MDA seems to be involved in the pathophysiology of pneumonia as an indicator of lipid peroxidation.

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