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IN VITRO ANALYSIS OF BRONCHO-ALVEOLAR LAVAGE FROM A PATIENT WITH PULMONARY ALVEOLAR PROTEINOSIS

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Abstract

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Pulmonary alveolar proteinosis (PAP) is a heterogenous disorder of genetic or acquired etiologies characterized by intraalveolar accumulation of lipoproteinaceous material. The clinical course of the disease is variable, ranging from spontaneous remission to respiratory failure. The aim of the present study was to compare the biochemical and biophysical characteristics of broncho-alveolar lavage (BAL) from a patient with PAP, during the whole lung lavage (WLL) taken after each stage of the procedure. For this purpose biochemical and biophysical analysis of the clinical samples were made. The phospholipids (PLs) and the proteins concentrations of the samples were measured. For determination of protein content in broncho-alveolar lavage samples Lowry protein assay (Peterson's modification) was used. The PL's concentration was determined via extraction by the method of Blight and Dyer. Thin-layer chromatography was used for determining the phospholipid profile of the separate phospholipid components. In addition, by using the method of Axisymmetric Drop Shape Analysis, the surface characteristics: equilibrium, maximal and minimal surface tension during 10 cycles of compression-decompression in the dynamic conditions, were determined. Our results showed consecutive proteins and phospholipids content decrease during the procedure. Logically, the equilibrium surface tension was increased as a result of the decreased Phospholipids/Proteins ratio. After WLL the physiological condition of the patient was improved. The present study will be of great interest for effective implementation of the procedure of whole lung lavage in the clinical practice.

Key words: axisymmetric drop shape analysis, lipid concentration, protein concentration, surface tension, thin-layer chromatography, whole lung lavage

Abbreviations: ADSA – Axisymmetric Drop Shape Analysis, AS – Alveolar Surfactant, BAL – broncho-alveolar lavage, PAP – Pulmonary Alveolar Proteinosis, PLs – Phospholipids, SP – surfactant protein, TLC – Thin-Layer Chromatography, WLL – Whole Lung Lavage

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Introduction

Pulmonary alveolar proteinosis (PAP) is a diffuse pulmonary disease characterized by the accumulation of periodic acid-Schiff-positive lipoproteinaceous material, primarily phospholipid surfactant and specific surfactant apoproteins in the distal air spaces, which results in impaired gas transfer (Campo et al., 2012). The alveolar surfactant (AS) at the air-water interface has an average half-life of about 36 hours. It is metabolized by the macrophages and adsorbed back in the aqueous subphase by pneumocytes type II. Up to 90% of the main surfactant phospholipid dipalmitoylphosphatidylcholine is being recycled from the alveoli to pneumocytes type II by receptor mediated signaling, activated by the specific alveolar protein A (SP-A) and a clathrin dependent endocytosis while the other 10% are being degraded by the alveolar macrophages (Crowther et al., 2006).

Pulmonary alveolar proteinosis is an extremely rare disorder, occurring worldwide with an estimated prevalence of 0.1 per 100,000 individuals. The onset of clinical disease is atypical, with a subacute indolent course that often delays the diagnosis by months to years (Campo et al., 2012). PAP occurs in three clinically distinct forms: congenital, secondary, and acquired. The congenital form comprises a heterogeneous group of disorders caused by mutations in the genes encoding specific surfactant protein B (SP-B), which leads to its deficiency. Together with the surfactant lipids, SP-B is responsible for keeping low surface tension values in during inhalation/exhalation. It is known that the two hydrophobic surfactant proteins SP-B and SP-C stimulate the metabolism, as well as the intake of the surfactant lipids by the pneumocytes type II (Rice et al., 1989). The important role of SP-B for normal lung function is confirmed by the fact that newborns with SP-B deficiency caused by genetic disorder develop lethal respiratory distress syndrome (Nogee et al., 1993). The secondary PAP is caused by underlying conditions that reduce the number of or functionally impair alveolar macrophages. Such conditions include some hematologic cancers, pharmacologic immunosuppression, inhalation of inorganic dust (e.g. silica) or toxic fumes, and certain infections (Trapnell et al., 2003; Mazzone et al., 2013). The genesis of the acquired PAP is autoimmune: anti-granulocyte-macrophage colony stimulating factor antibodies are formed, which in 90% of the cases leads to dysfunction of the alveolar macrophages, defect in the surfactant homeostasis and difficult degradation of the surfactant (Seymour et al., 2001).

Whole lung lavage (WLL), introduced by Jose Ramirez-Rivera in the late 1960s, is still the gold-standard therapy (Ramirez, 1966). Indeed, this technique has been much im-

proved over the years, thus enhancing effective removal of material from the alveoli. WLL involves the induction of general anesthesia followed by isolation of the lungs with a double-lumen endotracheal tube and performance of single-lung ventilation while large-volume lavages are performed on the non-ventilated lung. Warmed normal saline solution in 1-liter aliquots (total volume up to 20 liters) is instilled into the lung, chest physiotherapy is performed and the proteinaceous effluent is drained with the aid of postural positioning. The sequence of events is repeated until the effluent, which is initially milky and opaque, becomes clear (Beccaria et al., 2004; Indira et al., 2007; Webb et al., 2008; Michaud et al., 2009; Steneva et al., 2011).

The aim of the present study was to analyze the broncho-alveolar lavage (BAL) from a patient with PAP, by comparing the biochemical and biophysical characteristics of samples taken during the different stages of WLL. For this purpose the phospholipids (PLs) and the proteins concentrations of the samples were measured. Thin-layer chromatography was used for determining the profile of the separate phospholipid components. In addition, by using the method of Axisymmetric Drop Shape Analysis (ADSA), the surface characteristics: equilibrium surface tension, as well as maximal and minimal surface tension during 10 cycles of compression-decompression, was determined.

Materials and Methods

Broncho-alveolar lavage samples. The whole lung lavage samples were collected from a male patient during 15 WLL cycles for both lungs. Every cycle was made with 1 liter 0.9% NaCl saline (10 ml/kg). The procedure was performed first on the right lung and seven days later on the left lung. The analyzed samples were gathered as follows:

Sample 1: mean sample after lavage with 1 liter 0.9 % NaCl saline (10 ml/kg); final lavage volume after the first cycle of the procedures: 0.8 liter BAL for the right and 1 liter BAL for the left lung.

Sample 2: taken and analyzed after 4 x 1l saline was added to sample 1; final volume after the cycle: 5l.

Sample 3: new lavage cycle with 4 x 1 liter saline for treated lung; final volume: 4l.

Sample 4: new lavage cycle with 4 x 1 liter saline for lung; final volume: 4l.

Sample 5: new lavage cycle with 3 x 1 liter saline for the left lung and 4 x 1 liter saline for the right lung; final volumes: 2l and 3l, respectively.

Biochemical analysis. For determination of protein content in BAL samples Lowry protein assay (Peterson's modification) was used (Peterson, 1983), taking 0.1 ml of each

sample. The phospholipid concentration was determined after extraction (initial volume of 1 ml) by the method of Blight and Dyer (Bligh et al., 1959). The total PLs content was measured by quantity determination of inorganic phosphorus (Kahovkova et al., 1969). The separate phospholipid components were analyzed by thin-layer chromatography (TLC) with triethylamine (Sigma-Aldrich). Silica-gel plate (DC-Alufolien Kieselgel 60, Merck) was activated for 1 h at 105°C. After the PLs extraction the equal volume from each sample, dissolved in chloroform, as well as a standard with individual PLs were applied.

Biophysical analysis. By the method of ADSA the surface characteristics of small amounts (only 50 μl) of the tested samples was determined. A tensiometer KSV CAM 101 (KSV Instruments Ltd., Finland) was used. The setup was computer controlled by a Windows-integrated program, including the ADSA surface tension calculation algorithm. Fifteen minutes were necessary after the formation of a symmetric pending drop for adsorption of surface-active molecules at the air-water interface and reaching the equilibrium value of the surface tension, γ equilibrium (γ_{eq} , $\text{mN}\cdot\text{m}^{-1}$). After recording the equilibrium value, the pending drop was subjected to 10-fold compression and decompression, thus its surface area changed 5 times during the time of 90 s. In these dynamic conditions the following surface parameters: maximal value of the surface tension (γ_{max} , $\text{mN}\cdot\text{m}^{-1}$) at 100 % drop surface area and minimal value of surface tension (γ_{min} , $\text{mN}\cdot\text{m}^{-1}$) at 20 % drop surface area were detected.

Results and Discussion

The present study analyzed BAL samples from a patient with PAP. For this purpose the biochemical and biophysical characteristics of samples taken during the different stages of the applied whole lung lavage procedure were compared.

The impaired respiratory function due to PAP is caused by inactivation of the AS as a result of the increased amount of the main serum proteins (plasma albumin, fibrinogen, etc.). It is known that in case of respiratory distress syndrome the albumin concentration of the alveolar liquid reaches up to 25–100 $\text{mg}\cdot\text{ml}^{-1}$. It is considered that as a consequence of strong adsorption and high concentration the serum proteins cover the alveoli, forming dense film that makes alveolar surface unreachable for the native AS (Zasadzinski et al., 2005).

In the present study the biochemical analysis of the total proteins concentration in BAL samples showed that the main protein quantity, which impaired the alveolar function was washed away with the first three samples (Figures 1 and 2). The total protein concentration decreased during whole lung lavage procedure: from 0.91 $\text{mg}\cdot\text{ml}^{-1}$ in the first sample from

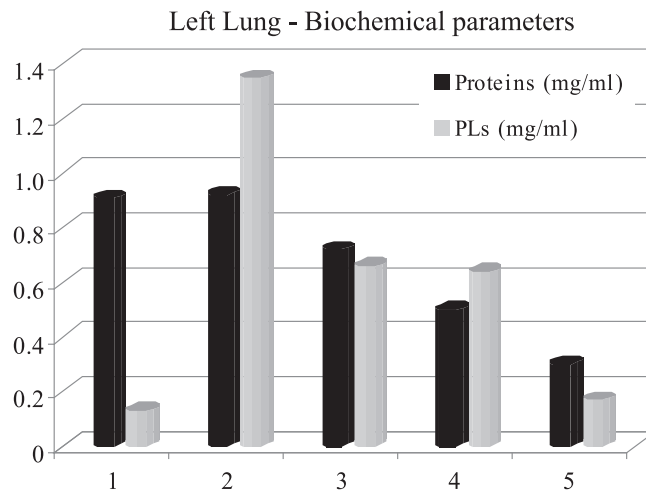


Fig. 1. Total concentration of proteins and phospholipids in broncho-alveolar lavage liquid samples from the patient's left lung

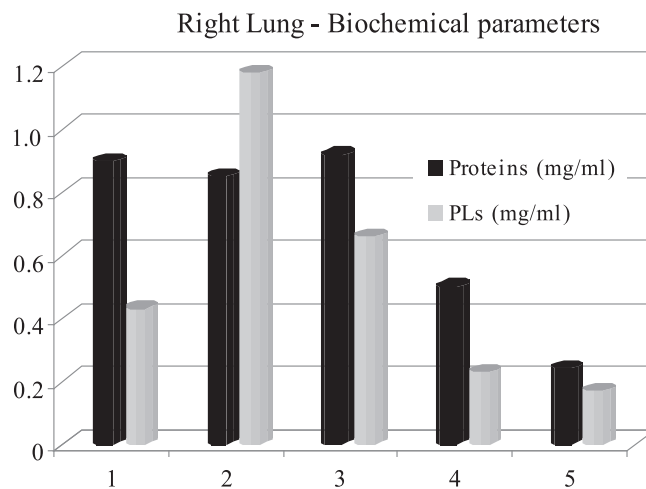


Fig. 2. Total concentration of proteins and phospholipids in broncho-alveolar lavage liquid samples from the patient's right lung

left lung to 0.30 $\text{mg}\cdot\text{ml}^{-1}$ in the final sample, and from 0.90 $\text{mg}\cdot\text{ml}^{-1}$ in the first sample from right lung to 0.24 $\text{mg}\cdot\text{ml}^{-1}$ in the final sample, respectively (Figures 1 and 2; Tables 1 and 2). It was seen that after final WLL cycles from each lung more than 10 g proteins were removed.

Consequently, the PLs content determined in each sample showed that the highest quantity was found in sample 2 (Figures 1 and 2; Tables 1 and 2). Most probably this was due to the vibrating massage applied on the chest of the pa-

Table 1
Biochemical parameters of left lung

Sample	Proteins (mg/ml)	PLs (mg/ml)	Proteins (g/total volume)	PLs (g/total volume)	PLs/Proteins
1	0.91	0.13	0.91	0.13	0.14
2	0.92	1.35	3.70	5.40	1.46
3	0.72	0.66	2.87	2.65	0.92
4	0.50	0.64	2.00	2.56	1.28
5	0.30	0.17	0.69	0.34	0.56
Total, g			10.17	11.08	

Table 2
Biochemical parameters of right lung

Sample	Proteins (mg/ml)	PLs mg/ml	Proteins (g/total volume)	PLs (g/total volume)	PLs/Proteins
1	0.90	0.43	0.72	0.35	0.48
2	0.85	1.18	3.39	4.70	1.39
3	0.92	0.66	3.51	2.52	0.72
4	0.50	0.23	2.27	0.89	0.46
5	0.24	0.17	0.73	0.50	0.71
Total, g			10.62	8.96	

tient after the first WLL cycle that was supposed to improve the removal of the undesired protein excess in the alveoli. However, after the whole procedure a significant PLs content was washed out along with the proteins: about 9 g from right lung, and about 11 g from the left lung. Nevertheless, according to literature data PLs/Proteins ratio in the alveolar surfactant is about 9:1 (Schürch et al., 1992). The results obtained from the biochemical analysis showed a significant difference according to this parameter in the lavage taken: PLs/Proteins ratio is much lower reaching values under 1, as a result of the increased protein concentration in the lung (Tables 1 and 2). This observation led to the conclusion that irrespectively from the high PLs content in the lavage, after the applied procedure, normal alveolar function was restored. In addition, the comparison between the samples taken from the patient's right and left lung showed higher protein amount and respectively lower PLs/Proteins ratio for the right lung. These results were also confirmed by image diagnostics showing deterioration of the condition of the right lung compared to the left (data not shown).

In addition to total PLs determination, the individual PLs profile in the different samples of the analyzed BAL thin-lay-

er chromatography was performed. The TLC results showed that the total amount of individual PLs in the samples from the left lung is higher than samples, taken from right lung (Figure 3), confirming the previous biochemical analysis about total PLs content. Moreover, compared to the standard, a gradual decrease in the individual PLs, sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and free fatty acids (FA) after each stage of the WLL in both lungs was observed. The most significant PLs reduction was found after the third WLL stage, where the individual PLs are not identified clearly, more pronounced in the case of right lung (Figure 3).

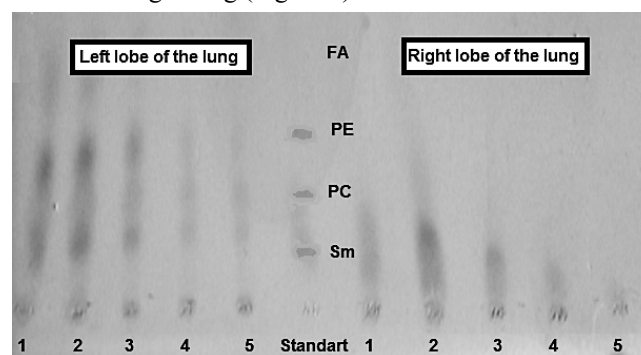


Fig. 3. Thin-layer chromatography for determination of individual phospholipids in BAL samples

In addition to the biochemical analysis, the surface tension characteristics of the broncho-alveolar lavage samples were determined by ADSA. We measured the following surface parameters: equilibrium, minimal and maximal surface tension. The results obtained are shown in Figures 4, 5 and 6, respectively.

It is known that the main property of AS is to reduce surface tension at the air-liquid interface in the alveoli thus preventing the lung collapse. The mono- and multilayer films of AS, existing *in vivo*, are characterized with a rapid adsorption from the subphase to the air-water interface, an equilibrium value of surface tension about 25 mN.m⁻¹ with a volume concentration of PLs higher than 50 mg.ml⁻¹, and high stability at surface compression (Lalchev, 1997). Our data showed that the values of γ_{eq} in both broncho-alveolar samples (from left and right lung were approximately 50-51 mN.m⁻¹ (Figure 4). The surface tension results showed a tendency of increased γ_{eq} values after the initial saline infusion in the left lung after the applied vibrating massage: the surface tension values changed from 49.4 mN.m⁻¹ with sample 2 to 54.5 mN.m⁻¹ with the last sample after final WLL. These results corresponded to the lower PLs content in the samples (Table 1, Figure 1). The increase in γ_{eq} was not so obvious in

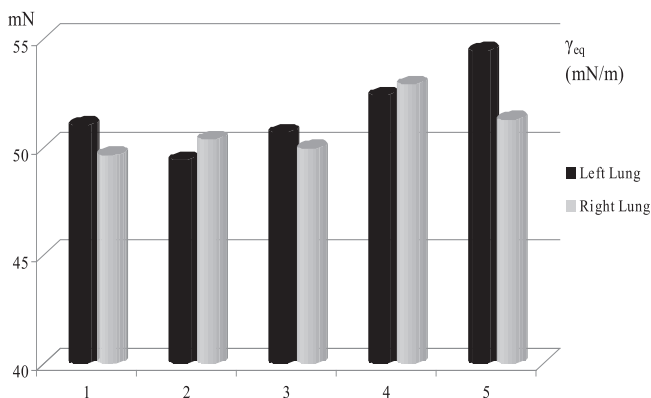


Fig. 4. Equilibrium surface tension values γ_{eq} (mN/m) in broncho-alveolar lavage samples from left and right lung

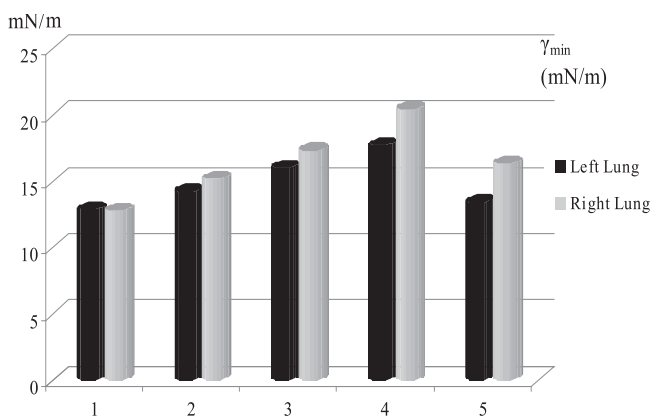


Fig. 5. Minimal surface tension values γ_{min} (mN/m) in broncho-alveolar lavage samples from left and right lung

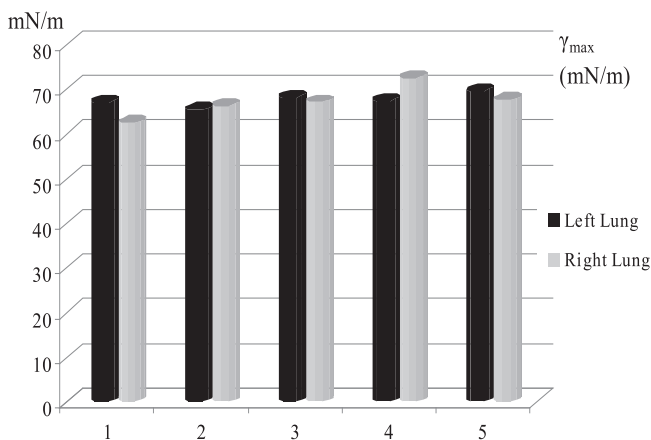


Fig. 6. Maximal surface tension values γ_{max} (mN/m) in broncho-alveolar lavage samples from left and right lung

the deteriorated right lung (from 50.4 mN.m⁻¹ with sample 2 to 51.3 mN.m⁻¹ at the end of the procedure). It is known that normal function of AS depends on optimal PLs/Proteins values and lower surface tension. In the studied case of PAP this ratio is abnormal, especially for the right lung (Tables 1 and 2). However, the equilibrium surface tension, which was registered in static conditions, is not significant for AS' behavior *in vivo*, as the physiological function of alveoli is associated with a permanent change of the surfactant monolayer surface.

The analyzed values of the dynamic surface characteristic γ_{min} also confirm the deterioration of the right lung. The minimal γ in all of the BAL samples after the applied vibrating massage showed higher values for the right lung as compared to the left one (Figure 5). Moreover, the increase in γ_{min} noticed in both lungs suggested that with each saline solution applied the inhibiting normal AS function agents were removed from the lung and the final sample was characterized with restored surface function. The reason for the increase in γ_{min} noticed in both lungs in the final sample was the lung edema after the introduction of significant amount of fluid. The results showed that the lavage was effective to the application of ten liters of saline.

The results for the maximal surface tension (γ_{max}) in broncho-alveolar lavage samples from the left lung showed that these values did not differ significantly from γ_{max} values of the right lung samples (Figure 6). Unlike the minimal surface tension, we did not observe a similar tendency between γ_{max} and the function of the lung.

Conclusion

The results obtained from the biochemical analysis showed differences in the proteins and PLs content between the left and the right patient's lung. It was observed higher protein concentration and respectively lower PLs/Proteins values in the samples from the right lung. This result was confirmed by image diagnostics showing deterioration of the right lung, as compared to the left. In addition, the TLC results showed that the total amount of individual PLs in the samples from the left lung is higher than samples, taken from right lung, confirming the biochemical analysis about total PLs content.

The analyzed surface parameters (γ_{eq} and γ_{min}) also confirmed results from image diagnostics. Our results showed a tendency of increased γ_{eq} values after the initial saline infusion in the left lung after the applied vibrating massage which was expected due to the lower PLs content in the samples. This increase was not so obvious in the deteriorated right lung. It is known that normal function of AS equals optimal

PLs/Proteins values and lower surface tension. Obviously, in the studied case of PAP this ratio is abnormal, especially for the right lung.

The results from the biochemical and biophysical analysis of the WLL samples confirmed the efficiency of the applied PAP treatment, especially during the first three cycles of the procedure. A following study of the analyzed parameters 72 hours after the procedure and the possibility of recovery of the optimal AS content will be of great interest for effective implementation of the WLL procedure in clinical practice.

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