ABSTRACT

The aim of this study is to evaluate the application of phospholipid liposomes in HCl - induced acute respiratory distress syndrome (ARDS) in rabbits. ARDS was induced by administration of 0.2 N HCl via intratracheal instillation for 45 min. After inducing of ARDS, the rabbits under artificial lung ventilation were retreated with liposomes for 60 min. Arterial blood gas analysis was performed at 15, 30, 45 and 60 min after liposome application. Untreated animals were ventilated for the same time. The rabbits were killed with thiopental and then the bronchoalveolar lavage (BAL) obtained was investigated for lipid and specific surfactant protein content (biochemical parameters). The equilibrium surface tension and dynamic surface tension characteristics of monolayers formed from the lavage fluid was determined in Langmuir through by Wilhelmy plate method (bio-physical parameters). HCl- lung injury caused decrease of arterial oxygen pressure/ fraction of inspired oxygen ratio more than 50% compared to the control. We obtained high respiratory acidosis - increase of arterial pressure of CO2 and decrease of blood pH. An increase of oxygen gradient was also detected. The instillation of liposomes led to reversion of gas exchange at 60 min after application almost to the control value. As well the biochemical and biophysical parameters of the bronchoalveolar lavage fluid were recovered to the control level after instillation of liposomes.

Introduction

The main characteristics of the ARDS are hypoxemia, decreased lung compliance, pulmonary edema (1, 2). The basis of these lung dysfunctions is the changes in the endogenous surfactant system, resulting in very high mortality of patients with ARDS (3, 4). A lot of direct and indirect pulmonary insults such as trauma, aspiration and sepsis also cause ARDS. The gastric acid aspiration is a common etiology of ARDS associated with changes in pulmonary epithelium and influx of edema fluid into the alveolar space. As a result the alveolar surfactant is inactivated and atelectasis appears (5, 6).

Application of ES has been tested as a therapeutic modality in patients with ARDS but the clinical trails reported to date have been inconsistent (7,8) since a lot of factors influence the administration of ES. They include the specific composition of surfactant preparations, the host’s response, the timing of the treatment, the

mode of mechanical ventilation, etc (9, 10). The efficacy of ES in vivo depends on its interaction with some components of the host’s alveolar environment (11, 12) and the nature of lung injury. Thus the surfactant therapy is very complicated and hence it is very important to evaluate different treatments in animal models of lung injury as first step for clinical setting.

The aim of this study is to evaluate the effects of administration of phospholipid liposomes in rabbit model of gastric acid aspiration for improvement of lung functions. In our previous investigations it was proved that the phospholipids liposomes obtained by us possessed specific lung and alveolar surfactant accumulation after i.v. application (13). The effects of change in the biochemical composition and the surface behavior of monolayers of the alveolar surfactant, before and after administration of liposomes, is also a goal of the study. The model of lipid monolayers has been utilized to study in vitro the properties of the alveolar surfactant at the air-water interface (the interface lining the alveoli), using rabbit BAL samples.

Materials and Methods
Animal preparation and treatment
Adult Chinchila rabbits, weighing 2.5 ± 0.3 kg, were premedicated intramuscularly with ketamine (50mg/kg) and acepromazine (0.05 mg/kg). Animals were placed in the supine position and 1% lidocaine was injected subcutaneously over the region of the trachea. The trachea was exposed and tracheotomy was performed by insertion of endotracheal tube (3.0 - 3.5 mm, with side-port adaptor) into the lower trachea trough a transverse incision between the cartilaginous rings below the thyroid cartilage. The animals received also pancuronium bromide (0.05 mg/kg) to inhibit spontaneous breathing. The heart rate was detected throughout the duration of the experiments. The rabbits were mechanically ventilated with the following parameters : FiO₂ of 1.0; the frequency of respiratory breathing was 30 breathings/min, the tidal volume - 10ml/kg, the PEEP - 5 cm H₂O and the inspiratory:expiratory ratio - 1:1. The ear blood rout was catheterized for ABG and for pH measurements. Catheter potency was maintained by using of 0.5 - 1.0 ml of heparinized (10 units/ml) 0.9% saline in order to clear the catheter after obtaining the blood samples. The blood measurements were performed with AVL OMNI (AVL, Austria) after 10 min stabilization period in order to establish the baseline value and to ensure the animal viability and stability, and to standardize the physiological and ventilatory parameters.

The rabbits were randomly assigned to three treatment groups:

Control group. The animals were prepared as describe above and then were subsequently ventilated for 110 min.

HCl- injured rabbits. After stabilization, 0.2 N HCl was instilled trough a sideport adaptor of the endotracheal tube (volume 4ml/kg) during the inspiratory phase of respiration in order to induce an acute lung injury. The acid was applied in two aliquots. The animal viability and stability were reliably predicted if arterial PO₂ (PaO₂) to FiO₂ was 50% of the pretreatment values at 45min after HCl administration (11). This value of oxygenation was used as an inclusion criterion for the animals included in this group.

Group of liposome administrated rabbits. Phospholipid-cholesterol liposomes (4ml/kg, concentration = 25mg/ml) were administered in small boluses during the inspiratory phase of ventilation through a side-port adaptor. The animals were ventilated 60 min after administration. ABG analysis was performed at 30, 45, 60 min after liposome application. The heart rate was measured continuously throughout the protocol. The animals were killed with a thiopental sodium overdose at the end of the experiments.

After euthanasia the lungs were lavaged.
as it is described (14) in order to recover BAL for further analysis.

**Liposome preparation**
Multilamellar liposomes were prepared by the reversed phases method (15) using soybean phosphatidylcholine (Sigma), containing 40% phosphatidylcholine, 12% phosphatidylinositol, 27% phosphatidylethanolamine, 20% phosphaticid acid, 1% fatty acids plus glicolipids and cholesterol (Sigma) in 2:1 molar ratio. The liposome concentration was 25mg/ml saline. Liposome suspension was stable at this concentration for one month at 4 ºC (13). We used freshly prepared liposomes, incubated at 37 ºC for 5 minutes before the experiments.

**Analytical methods**
Rabbit lung surfactant phospholipids were extracted by the method of Bligh and Dyer (16). Phospholipid content in BAL was determined by the method of Kahovkova and Odavic (17). The individual phospholipids were determined by TLC using silicagel G plates (Merk) and solvent system containing chloroform: methanol: isopropanol: 0.25% KCl: trietylamine in ratio 30:9:25:6:18 (v/v) (18). Cholesterol and protein content were determined by the method of Sperry and Webb (19) and Lowry (20) respectively.

**Lipid monolayers of bronhoalveolar lavage at the air-water interface**
Adsorbed monolayers from BAL were formed in a Langmuir trough (Biegler-Electronic, Austria) with area of 706 mm² (volume of BAL = 7 ml) and the surface tension \( \gamma \) (mN/m) was followed with time by the Wilhelmy plate method (21, 22). Kinetics of adsorption and the equilibrium surface tension \( \gamma_{eq} \) were monitored. Platinum float with size 1 x 1.6 cm was used. The trough temperature was controlled with a precision of ±0.5 ºC. Experiments were carried out at 37 ºC. The surface tension was measured with an accuracy of 0.5 % and each experiment was repeated at least three times with different sample preparations.

Dynamic surface tension characteristics of monolayers of BAL were measured during compression/decompression of the monolayers between 100% (\( \gamma_{max} \)) and 20% (\( \gamma_{min} \)) of the initial monolayer area (1710 mm²). The hysteresis area of the loop obtained after compression/decompression was also monitored. The compression/decompression rate was 3 minutes per cycle. The samples were continuously stirred by a Teflon-coated stirring bar. The monolayer surface tension dynamics was monitored up to the 3rd cycle in our studies by the Wilhelmy method. Experiments were carried out at 37 ºC. Accuracy of the measurements was 0.5 % and each experiment was repeated three times.

**Statistical analysis**
Five animals are used for each group. Values are means ± SE. Unpaired Student t-test was used to compare different experimental groups. P < 0.05 was considered as significant.

**Results and Discussion**
The improvement of gas exchange in patients with ARDS is one of the main problems in the therapy of pulmonary diseases. Application of exogenous surfactants in order to restore lung function shows different outcome. Investigations with various surfactants of different origin, natural (11, 14) or artificial (9,12,23,24) still did not show good response in all cases. In some cases, due to the permeability abnormalities resulted in protein leakage in the alveolar space of patients with ARDS a lung lavage before the surfactant application (11) is necessary to be performed in order to remove the inhibited surfactant. Nevertheless the response was not very effective after this procedure.

In our previous investigations we obtained and characterized phospholipid liposomes with specific lung and alveolar surfactant accumulation after i.v. application (13). The investigations on the mecha-
TABLE 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, kg</td>
<td>2.5 ± 0.30</td>
</tr>
<tr>
<td>(\text{Pa } \text{O}_2/\text{FiO}_2), mmHg</td>
<td>222.18 ± 5.80</td>
</tr>
<tr>
<td>(\text{PaCO}_2), mmHg</td>
<td>25.14 ± 1.20</td>
</tr>
<tr>
<td>pH</td>
<td>7.36 ± 0.01</td>
</tr>
<tr>
<td>(\text{SaO}_2), %</td>
<td>98.82 ± 0.20</td>
</tr>
<tr>
<td>([\text{HCO}_3]), mmol/l</td>
<td>17.25 ± 0.60</td>
</tr>
<tr>
<td>BB, mmol/l</td>
<td>39.45 ±0.70</td>
</tr>
<tr>
<td>A-a (\text{PaO}_2), mm Hg</td>
<td>436.70 ± 7.50</td>
</tr>
</tbody>
</table>

Values represent mean ± SE. \(\text{PaO}_2\) – arterial oxygen pressure; \(\text{PCO}_2\) – arterial carbon dioxide pressure; A-a \(\text{PaO}_2\) – oxygen gradient; pH – blood pH; \([\text{HCO}_3]\) – blood concentration; BB – blood concentration of all basis, \(\text{SaO}_2\) – arterial oxygen saturation. #This parameter was calculated by the method of Hinkov (25).

The baseline characteristics of the experimental animals obtained 10 minutes after the beginning of mechanically ventilation are presented in Table 1.

Half of the \(\text{HCL}\) - treated group was instilled with phospholipid – cholesterol liposomes and the animals were ventilated for 60 min. The ABG and pH analysis was performed at 30, 45 and 60 min after administration. Fig. 1 illustrates the temporal changes in mean \(\text{PaO}_2/\text{FiO}_2\) ratio after \(\text{HCl}\) and liposome administration. The results showed that the \(\text{PaO}_2/\text{FiO}_2\) ratio of the control group remained unchanged during the period studied. The \(\text{HCl}\)-treated group showed continuous decrease of this value over the period of ventilation. A progressive increase in oxygenation in the liposome-treated group was observed after the 45th minute and at the 60 minute after administration the ratio \(\text{PaO}_2/\text{FiO}_2\) reached about 95% of the baseline value. The parameters of oxygen transport reached almost the values of the untreated animals. The mean of blood pH, saturation, \([\text{HCO}_3]\) returned to the normal level at 45 min after implication of liposomes.

In order to investigate the changes in the alveolar surfactant due to the \(\text{HCL}\) lung injury we obtained BAL samples from the three animal groups. In Table 2 are presented the protein recovery, cholesterol and phospholipid content in BAL samples of control and \(\text{HCl}\)-treated group. The protein and cholesterol content increases and the total phospholipid content decreases in BAL of \(\text{HCl}\) – injured group compared to the control one. The increased cholesterol level led to increase of the surface tension which caused alveolar flooding and an influx of protein in the alveolar space occurred (26). Investigation on the nature of these proteins showed that they consisted in high respiratory and metabolic acidosis. All these parameters of ABG showed a common picture of ARDS. The control group was mechanically ventilated for the same time under the same conditions. The results obtained showed that the detected blood parameters indicated severe impairment of lung function, compared to the untreated group.

In order to investigate the changes in the alveolar surfactant due to the HCL lung injury we obtained BAL samples from the three animal groups. In Table 2 are presented the protein recovery, cholesterol and phospholipid content in BAL samples of control and HCl-treated group. The protein and cholesterol content increases and the total phospholipid content decreases in BAL of HCl-injured group compared to the control one. The increased cholesterol level led to increase of the surface tension which caused alveolar flooding and an influx of protein in the alveolar space occurred (26). Investigation on the nature of these proteins showed that they consisted...
Fig. 1. Temporal changes in ratio of arterial oxygen pressure (PaO$_2$) to inspiratory fraction of O$_2$ (FiO$_2$) in control (■), HCl - treated (●) and liposome treated (▲). Arterial blood gas analysis was obtained at specific intervals during the course of ventilation. No changes in oxygenation were observed in control group. HCl treatment caused rapid deterioration of PaO$_2$/FiO$_2$ ratio, while liposome administration led to progressive increase of oxygenation. All animals were ventilated on a FiO$_2$ of 1.0.

TABLE 2
Total protein recovery, cholesterol and phospholipid content of BAL in control and HCl - treated animal groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>HCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein recovery,</td>
<td>34.40 ± 3.80</td>
<td>284.80± 42.80 ***</td>
</tr>
<tr>
<td>mg/kg body weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol, μg/mg Pr BAL</td>
<td>2.87 ± 0.30</td>
<td>11.60 ± 1,20 ***</td>
</tr>
<tr>
<td>Phospholipid,</td>
<td>71.8 ± 4.48</td>
<td>29.80 ± 1.90 **</td>
</tr>
<tr>
<td>μg/mg Pr BAL</td>
<td></td>
<td></td>
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</table>

Values are means ± SE; **P<0.01; *** P<0.001 as compared to the control group.

of serum proteins (11). It is well known that serum proteins inhibited surfactant functions (2) which resulted in lung dysfunctions.

There were changes in phospholipid composition as well. The results on the percent participation of the individual phospholipids in total phospholipid extract of BAL in untreated and HCl - treated groups revealed that the BAL samples of the control group consisted mainly of PC-about 80% of the total PL. The HCl lung injury caused significant decrease of the percent participation of PC to 36% from the total amount of PL. An increase of the amount of SM from 2% to 20%; for PG from 4.3% to 9.6% and for PE from 3% to 14.5% of HCL-treated animals was detected. The decreased amount of phosphatidylethanolamine might be due to several reasons such as: impaired synthesis, activation of lung phospholipase A$_2$ or diminished in lung lipid transfer proteins activity, which transfer the newly synthesized PC to the alveolar surfactant. One of the routes for SM synthesis is via sphingomyelin synthetase which substrate is the PC. So it seems quite probable that there might be an activation of that enzyme due the increased cholesterol level which also led to a decrease of percent participation of PC. The cholesterol molecule was shown to be

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Fig. 2. Surface pressure-area curves of monolayers obtained from BAL during compression/ decompression. The figures present results from the first cycle. A - BAL from control group, B – BAL from HCl-treated rabbits, C – BAL from liposome treated animals.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HCl</th>
<th>Liposomes</th>
</tr>
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<tbody>
<tr>
<td>$\gamma_\text{eq}$ (mN/m)</td>
<td>41.5±0.80</td>
<td>54.37±1.30*</td>
<td>44.43±0.60*</td>
</tr>
<tr>
<td>Cycle 1</td>
<td>$\gamma_{\text{max}}$</td>
<td>45.6</td>
<td>59.3</td>
</tr>
<tr>
<td></td>
<td>$\gamma_{\text{min}}$</td>
<td>25.9</td>
<td>45.3</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>1.441</td>
<td>0.998</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>$\gamma_{\text{max}}$</td>
<td>51.2</td>
<td>65.0</td>
</tr>
<tr>
<td></td>
<td>$\gamma_{\text{min}}$</td>
<td>25.3</td>
<td>33.4</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>1.186</td>
<td>0.812</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>$\gamma_{\text{max}}$</td>
<td>44.6</td>
<td>65.0</td>
</tr>
<tr>
<td></td>
<td>$\gamma_{\text{min}}$</td>
<td>27.1</td>
<td>31.5</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>1.154</td>
<td>0.937</td>
</tr>
</tbody>
</table>

Values for surface tension ($\gamma_\text{eq}$) are means ± SE, * P<0.05 vs. control, # P<0.05 vs. HCl – treated group. Dynamic surface tension characteristics of monolayers of BAL were measured during compression/decompression of the monolayers between 100% ($\gamma_{\text{max}}$ (mN/m)) and 20% ($\gamma_{\text{min}}$ (mN/m)). A (mN) - hysteresis area of the loop obtained after compression/decompression. The compression/decompression rate was 3 minutes per cycle. The monolayer surface tension dynamics was monitored up to the 3rd cycle. Accuracy of the measurements was 0.5% and each experiment was repeated three times.

Since the lung physiological activity depends on the lung surfactant dynamic properties at the air-water interface (the interface lining the alveoli) we investigated the change of the surface tension during compression/decompression of BAL monolayers between 100% ($\gamma_{\text{max}}$) and 20%($\gamma_{\text{min}}$) of the initial monolayer area. The hysteresis area (A) of the loops obtained during 3 compression/decompression cycles was monitored. The results are presented in Fig 2 for the 1st cycle and in Table 3 for the 3 consecutive cycles.

One can see that the values of $\gamma_{\text{max}}$ increased during cycling for the tree groups...
(due probably to the lost of surfactant components from the surface), being always higher for the HCl-treated group and lower for the liposome-treated group in comparison with the control group. The hysteresis area was lower for the HCl-treated, comparable to the other two, in each cycle. We detected that the surface tension (\(\gamma\)) values increased after HCl treatment from 45.6 mN/m to 56.8 mN/m compared to the control group and followed by sharp decrease after liposome treatment to value of 41.8 mN/m (8 \(\gamma_{eq} = 10\) mN/m). Regarding the surface behavior of surfactant components measured under dynamic conditions, the recovery of the increased \(\gamma_{min}\) and \(\gamma_{max}\) values in the HCl treated animals to the levels of control group was clearly detected (Table 3 and Fig. 2) These results demonstrated the recovery potential of the phospholipid–cholesterol liposomes and their ability to increase surface activity of BAL under in vivo conditions. In Table 3 are also shown the changes of the hysteresis area of the compression/decompression loop. The latter decreased after HCl treatment and the shape of the loop was changed which was connected with the decline in the lung function (Fig. 2). After liposome instillation the hysteresis area increased in parallel with the recovery of gas exchange. The investigations on the surface tension and on the dynamic characteristics of monolayers of BAL as well revealed that the surface activity of BAL in vitro is recovered to the normal values after liposome treatment. Significant differences in the shape of the curves were also monitored which it was, however, difficult to interpret without additional experiments (Fig. 2).

In conclusion, our results demonstrated an improvement in vivo of gas exchange after liposome administration. The intratraheal instillation of liposomes was performed in small doses because of some literature data which demonstrated that the application of surfactant in small doses led to significant smaller lung injury score (28). As it was shown in this work (Fig. 1) the PaO2/FiO2 values of liposome- treated group was recovered very closely to the control values- 210 ± 4.20 mm Hg. All parameters of ABG analysis returned to the normal level after liposome treatment as well. The blood pH, base concentration and the saturation values revealed also almost the same values as these of the baseline after liposome treatment. The PaCO2 value decreased to 28.90 ± 1.30 mm Hg. These parameters correlate well with the in vitro data for the biophysical parameters of BAL, which indicated significant recovery of surfactant properties. On the basis of these results we may speculate that it is likely that the liposomes assessed in this study might be used for in vivo improvement of oxygenation in acid aspiration induced ARDS. More investigations are needed to establish the optimal conditions for such kind of therapy.

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REFERENCES